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The response of bacterial communities to carbon dioxide in high-oxygen modified atmosphere packaged beef steaks during chilled storage

Jun Yang [a,b], Xiaoyin Yang [a,b,*], Rongrong Liang [a,b], Lixian Zhu [a,b], Yanwei Mao [a,b], Pengcheng Dong [a,b], David L. Hopkins [a,c], Xin Luo [a,b], Yimin Zhang [a,b,*]

[a] Lab of Beef Processing and Quality Control, College of Food Science and Engineering, Shandong Agricultural University, Tai'an, Shandong 271018, PR China
[b] National R&D Center for Beef Processing Technology, Tai'an, Shandong 271018, PR China
[c] Centre for Red Meat and Sheep Development, NSW Department of Primary Industries, Cowra, NSW 2794, Australia

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ABSTRACT

The objective of this study was to establish the effect of CO_2 on the bacterial community in beef steaks held under both high-oxygen modified atmosphere packaging (HiOx-MAP) types (CO_2 treated MAP: 50% O_2/40% CO_2/10% N_2; control MAP: 50% O_2/50% N_2). Steaks were stored at 2 °C for 20 days. Gas composition, meat color, pH values, total volatile basic nitrogen values, total viable counts (TVC) and microbial community dynamics were monitored. Compared to the control MAP, the high level of CO_2 in the contrast MAP significantly delayed bacterial growth, resulting in a bright red color as well as extending the shelf-life to over 20 days. The microbial diversity decreased with prolonged storage in both MAP types, but it was more complex in high-CO_2 treated MAP steaks. When TVC values approached the shelf-life threshold for the control MAP, *Pseudomonas* and *Brochothrix* were the predominant bacteria, while *Pseudomonas* and *Serratia* under the CO_2 containing MAP were at a lower abundance than under the control MAP. The dominant *Pseudomonas* species causing spoilage in the control MAP steaks was *P. fragi*, and this species was inhibited significantly by CO_2, followed by *P. weihenstephanensis*. Inversely, *P. versuta* instead of *P. fragi* became the dominant *Pseudomonas* species under the CO_2 treated MAP. Overall, the application of CO_2 in HiOx-MAP influenced microbiota succession, which played an important role in retaining beef quality.

1. Introduction

Meat spoilage caused by the growth and metabolic activities of specific spoilage organisms (SSOs) has become a major concern in meat industry (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). High oxygen modified atmosphere packaging (HiOx-MAP) has been widely applied to ensure chilled fresh beef has an attractive appearance. However, high oxygen environments facilitate the growth of aerobes, increase lipid oxidation accompanied by oxidative or rancid off-flavors, and all these are detrimental to extending meat shelf-life. Therefore, decreasing O_2 and increasing CO_2 concentration moderately based on conventional HiOx-MAP (80% O_2/20% CO_2) is becoming a feasible way to improve beef quality and limit the growth of spoilage bacteria (Gill & Tan, 1980; Zakrys, O'sullivan, Allen, & Kerry, 2009). However, once the CO_2 concentration is above 40%, it may cause packaging film collapse (McMillin, Huang, Ho, & Smith, 1999). Based on these facts, a novel gas composition containing 50% O_2/40% CO_2/10% N_2 has been shown to be an alternative to prolong the microbial shelf-life of beef steaks (Yang et al., 2016).

The type and rate of microbial spoilage in HiOx-MAP beef has a direct relationship with microbiota development, which will be affected even if there is a slight change in MAP gas composition. Yang, Zhu, Zhang, Liang, and Luo (2018) noted that *Brochothrix* and *Pseudomonas* were dominant in 80% O_2/20% CO_2 packaged beef steaks, accompanied by high microbial amino acid and lipid metabolism levels at the end of storage. However, *Leuconostoc* were found to be the most prevalent bacteria and the potential producers of undesirable volatile compounds in the same packaged beef (Jääskeläinen, Hultman, Parshintsev, Riekkola, & Björkroth, 2016). Furthermore, HiOx-MAP (70% O_2/30% CO_2) beef was reported to be dominated by *Carnobacterium* and *Brochothrix* (Säde, Penttinen, Björkroth, & Hultman, 2017). On the other hand, the interactions between microorganisms also play an inhibitor or stimulator role on meat spoilage (Doulgeraki, Ercolini, Villani, & Nychas, 2012). Considering that the gas combination with 50% O_2, 40% CO_2 and

* Corresponding authors at: 61, Daizong Str., Taian, Shandong 271018, PR China.
E-mail addresses: yangxiaoyin@sdau.edu.cn (X. Yang), ymzhang@sdau.edu.cn (Y. Zhang).

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10% N_2 was an effective gas mixture for retarding bacterial spoilage, it is necessary to investigate the microbial structure and interactions in beef steaks held under this packaging type to provide a new insight into the antibacterial effect of CO_2.

As the most important bacteriostatic gas in MAP, CO_2 plays a fundamental role in the shelf-life extension of steaks held in the HiOx-MAP system with 40% CO_2. The classic antibacterial theories of CO_2 are summarized as follows: (1) Penetration of bacterial membranes, leading to intracellular pH changes; (2) Inhibition of enzymes or decreases in the rate of enzyme reactions; (3) Direct influence on molecular CO_2 and HCO_3^- on metabolism; (4) Changes in the physico-chemical properties of proteins (Daniels, Krishnamurthi, & Rizvi, 1985; Farber, 1991). In fact, the effect of CO_2 on bacterial growth is fairly complicated, and its alteration in the bacterial community of HiOx-MAP beef steaks during chilled storage has been poorly explored until now. It has been suggested that CO_2 is more effective against Gram-negative spoilage organisms like *Pseudomonas* and *Enterobacteriaceae*, whereas it has very limited effects on Gram-positive organisms, such as lactic acid bacteria (LAB) and *B. thermosphacta* (Gill, 1996). However, it is noteworthy that a mixture of 30% O_2/70% CO_2-MAP suppressed the growth of LAB and *Enterobacteriaceae*, and completely inhibited *Pseudomonas* and *B. thermosphacta* in minced beef, whereas MAP with 70% O_2/30% CO_2 favored the growth of LAB and *Enterobacteriaceae*, as well as restricted the growth of *Pseudomonas* and *B. thermosphacta* (Esmer, Irkin, Degirmencioglu, & Degirmencioglu, 2011). In this regard, CO_2 has an influence on microbial communities, which may be determined by the combined gas components in MAP. However, to our knowledge, the microbial succession selected by CO_2 in HiOx-MAP beef is not clear.

Moreover, the selective inhibition by CO_2 also extends to the bacterial species level. *Lactobacillus sakei* and *Lactobacillus oligofermentans* predominated under high concentration CO_2 packaging (100% CO_2), but the low level of CO_2 atmospheres (20% CO_2/80% N_2) enhanced the growth of *Lactococcus Piscium* (Nieminen, Nummela, & Björkroth, 2015). Although researchers have indicated that *Pseudomonas* was considered as one of the most sensitive genera to CO_2 inhibition (Gill, 1996), there are still different conclusions on *Pseudomonas* species. Jones and Greenfield (1982) summed up the tolerance to CO_2 inhibition is higher for *P. fragi* than for *P. fluorescens*. In contrast, Stanbridge and Davies (1998) found *P. fragi* were suppressed more effectively by CO_2 than *P. fluorescens*. It is known that the diversity of *Pseudomonas* species depends not only on food matrices (Caldera et al., 2016), but also on gas compositions (Ercolini, Russo, Torrieri, Masi, & Villani, 2006). In addition to *P. fragi* and *P. fluorescens*, *P. lundensis*, *P. putida*, *P. weihenstephanensis* and *P. psychrophila* were also found to be the dominant species in modified atmosphere packaged beef (Doulgeraki & Nychas, 2013; Hilgarth, Lehner, Behr, & Vogel, 2019). The differentiated physiological activities of these *Pseudomonas* species will directly determine meat spoilage characteristics (Papadopoulou et al., 2020). However, limited information is available regarding the effect of CO_2 on *Pseudomonas* diversity in HiOx-MAP beef.

Taken together, the overall goal of this work was to explore prolongation of shelf-life through examining the bacterial components of beef steaks packaged under HiOx-MAP with different CO_2 levels, and to determine the influence of these microbiota dynamics on meat physicochemical changes related to spoilage. This study may be helpful for enriching the antibacterial theory of CO_2 from the point of bacterial community.

2. Materials and methods

2.1. Meat materials

The M. *longissimus lumborum* muscles were collected from four crossbred Simmental bulls (18–24 months old, 350–370 kg carcass weights) in a commercial abattoir. Carcasses were conventionally chilled at 2–4 °C for 48 h. After trimming excessive fat and connective tissues, both left and right loins were cut into 2.54-cm thick steaks in the abattoir. Afterwards, the steaks were vacuum packaged and transported to the laboratory on ice within 2 h for subsequent storage under 2 different MAP conditions.

2.2. Packaging and storage

Two steaks from both loins of each carcass were randomly assigned to the CO_2 treated MAP (TMAP: 50% O_2/40% CO_2/10% N_2), or the control MAP (CMAP: 50% O_2/50% N_2) for respective storage times (5, 10, 15, and 20 days), and two steaks from each carcass were analyzed prior to packaging as initial samples at day 0. Thereby, each packaging treatment at each storage time interval had eight steaks as replicates (n = 8). Steaks were individually placed on polypropylene trays (O_2 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., Shanghai, China) containing Dri-Loc® soak pads (DLS-25, Sealed Air Corp., Danbury, USA). Trays were then flushed with the desired gas mixture with a gas headspace to meat ratio of 3:1, and sealed with oxygen-barrier film (O_2 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., Shanghai, China) using a DT-6D packaging machine (Dajiang Machinery Equipment Co., Ltd., Wenzhou, China). All packages were stored in a dark chiller at 2 °C. Steaks were removed from packages on each sampling day for microbiological and physicochemical analysis.

2.3. Enumeration of microorganisms

The enumeration of total viable counts (TVC) was conducted according to Yang et al. (2020). Briefly, individual meat samples (10 g) from each steak were aseptically collected as a thin slice of tissue from the top surface of each steak after meat color were measured (the contact face of aperture was disinfected by alcohol wipes), and then transferred aseptically into stomacher bags (BagMixer® 400; Interscience, St Nom, France). Ninety mL of sterile peptone-saline water (0.1% peptone, 0.85% NaCl) was added to each stomacher bag and the mixture was homogenized in a stomacher for 2 min at room temperature. Subsequently, serial 10-fold dilutions were carried out and 1 mL of serial dilution was mixed with Plate Count Agar (PCA; LandBridge, Beijing, China) in dishes and incubated at 37 °C for 48 h. Each dilution was performed in triplicate, and the results were expressed as log of the number of colony - forming units/g (CFU/g) sample.

2.4. Microbial assessment using 16S rDNA sequencing

2.4.1. DNA extraction

Total bacterial genomic DNA was extracted from beef steaks under different packaging as described by Yang et al. (2018) with slight modifications. In more detail, the homogenized liquid of each sample (60 mL) obtained in Section 2.3 was collected and centrifuged at 800*g* (5 min, 4 °C). A sample of supernatant (15 mL) was collected and transferred into a new sterile tube and then centrifuged at 10,000*g* for 5 min. The supernatant was discarded and the pellet was washed with sterile saline solution (0.15 M NaCl), and then the microorganisms were gathered by centrifugation at 12,000*g* for 5 min. Total DNA was directly extracted using a Shannuo™ Bacterial DNA Kit (Tianjin Charme Co., Ltd., China). The V3 and V4 regions in 16S rRNA were amplified by the polymerase chain reaction (PCR) with the specific primers 341F: 5′ - CCTAYGGGRBGCASCAG - 3′ and 806R: 5′ - GGACTACNNGGGTATCTAAT - 3′ with a nucleotide barcode, respectively. The PCR thermal cycling was conducted using an initial denaturation step at 98 °C for 1 min; followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified PCR products were verified by 2% agarose gel electrophoresis and purified by Qiagen Gel Extraction Kit

(Qiagen, Germany). Thereafter, the sequencing libraries were performed using TruSeq® DNA PCR -Free Sample Preparation Kit (Illumina, USA) with unique indices following the manufacturer's instructions. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

2.4.2. Bioinformatical analysis

The raw sequencing data from different steaks were merged using Fast Length Adjustment of Short (FLASH) to obtain the raw tags (Magoč & Salzberg, 2011). Then raw tags were filtered by Quantitative Insights Into Microbial Ecology (QIIME) to obtain clean tags (Caporaso et al., 2010). The clean tags were compared to the SILVA database by the UCHIME algorithm to chimera sequences and obtain the effective tags finally. The effective tags were obtained and clustered into operational taxonomic units (OTUs) with a 97% similarity threshold by UPARSE software. The taxonomy assignment of OTUs was aligned based on the Mothur algorithm in the SILVA database (Quast et al., 2012).

Alpha diversity indices such as Chao1, ACE, Shannon and observed species were calculated with QIIME (Version 1.7.0) to evaluate the species richness and genetic diversity of microbial communities. Beta diversity on both weighted unifrac were calculated by QIIME software (Version 1.9.1). Linear discriminant analysis effect size (LEfSe) was applied to illustrate the differential abundance bacteria under both MAP with a log linear discriminant analysis (LDA) score >4. And functional genes of communities were predicted by microbiota phylogenetic investigations using PICRUSt and annotated to their biological function according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Langille et al., 2013). Venn diagrams, principal component analysis (PCA) and heatmap were generated with OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA).

2.5. Pseudomonas species isolation and identification

Pseudomonas species were additionally identified from both TMAP and CMAP beef steaks on day 20. Five individual colonies were randomly isolated on the Cephalothin-Sodium Fusidate-Cetrimide (CFC) Agar with CFC selective supplement (CM0559B with SR0103E, OXOID, UK) of each beef steak at the highest dilution after incubation at 25 °C for 48 h, and then purified on the *Pseudomonas* selective medium (Carrizosa et al., 2017). A total of forty isolates were obtained from each packaging type. The diversity assessment of *Pseudomonas* isolates was measured with 16S rRNA gene sequencing analysis. Briefly, DNA was extracted from enriched *Pseudomonas* using a Bacteria Genomic DNA Kit (Beijing Cowin Biotech Co., Ltd, China), and then was amplified by the universal primers 27F (5′ - AGAGTTTGATCCTGGCTCAG - 3′) and 1492R (5′ - GGTTACCTTGTTACGACTT - 3′). The PCR products were purified and sequenced, and the sequences were compared to the GenBank databases using the BLAST function. Multiple gene alignments were performed using the software program ClustalW, and phylogenetic trees were constructed by the neighbor-joining method using the software MEGA 7 (Hilgarth et al., 2019). Furthermore, in order to verify 16S rRNA results, a multiplex PCR assay targeting the *carA* gene for identificating *P. fragi*, *P. lundensis* and *P. putida* was performed, and a species-specific PCR for *P. fluorescence* was detected with the primer 16SPSEfluF and 16SPSER according to previous studies (Ercolini et al., 2007; Scarpellini, Franzetti, & Galli, 2004).

2.6. Gas composition

Concentrations of oxygen and carbon dioxide inside the packages were monitored on each sampling day (CheckPoint O_2/CO_2, PBI-Dansensor, Ringsted, Denmark). The instrument needle was inserted through a rubber septum attached to the covered material.

2.7. Total volatile basic nitrogen (TVB-N) and pH measurement

The TVB-N levels of beef steaks were measured based on a China National Food Safety Standard method (GB 5009.228-2016). Samples were taken from multiple locations of the beef steaks, and frozen at −80 °C until analysis. A 10 g of sample was placed into a triangle bottle with 75 mL of distilled water. The mixture was equilibrated for 30 min at room temperature (20–25 °C) and was transferred to a distillation tube. After 1 g of magnesium oxide was added into the distillation tube, the distillation tube was connected to an automatic Kjeldahl nitrogen determination apparatus (K - 355, Buchi, Switzerland). The distillation was absorbed by 30 mL of 20 g/L boric acid solution, and then titrated with 0.01 mol/L hydrochloric acid. The amount of TVB-N (mg/100 g) was calculated by the consumption of hydrochloric acid.

At each analysis day, pH values of beef steaks were measured directly by a pH meter (SenvenGo, Mettler-Toledo, Switzerland) after calibration in buffers solutions at pH 7.00 and 4.00, respectively. Each beef steak was measured in triplicate for further statistical analysis.

2.8. Color measurement

The surface color of beef steaks was measured on each sampling day (blooming for 30 min at 2 °C on day 0) using an X-Rite SP62 spectrophotometer (4 mm diameter aperture, Illuminant A, 10° observer, Grand Rapids, USA). The CIE lightness (L^*), redness (a^*) and yellowness (b^*), and reflectance values in the range of 400 nm to 700 nm at 10-nm intervals were recorded. The reflectance ratio R630/580 was calculated to estimate the color stability of beef steaks. The data given are means of 6 measurements for each beef steak after opening packaging immediately.

2.9. Statistical analysis

The experiment adopted a split-plot design. For the whole plot, carcasses served as blocks. Within the subplot, steaks from each carcass were randomly assigned to the initial treatment or the combinations of two packaging treatments and four storage times (d 5, 10, 15 & 20). The MIXED procedure of SAS Version 9.0 (SAS Institute Inc., Cary, NC) was used with packaging treatments, storage time and their interaction as fixed factors and carcass as a random factor. Least squares means were separated using the PDIFF option and were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Microbial counts

There was a significant packaging × storage time interaction for TVC ($P < 0.05$). The TVC were approximately 4.94 log CFU/g at day 0 as shown in Table 1, indicative of an unhygienic environment and operation in the source abattoir. The TVC in CMAP steaks increased significantly after day 5 and exceeded 7.4 log CFU/g at the end of storage exceeding the recommended microbial spoilage threshold of 7 log CFU/g (ICMSF, 1986). It is worth noting that the presence of CO_2 completely inhibited the bacterial growth in TMAP steaks, in which the TVC only increased by 0.25 log CFU/g throughout a 20-day storage period and was far below the spoilage limit. In accordance with our results, İrkin, Esmer, Degirmencioğlu, and Degirmencioğlu (2011) also showed that a gas composition of 50% CO_2 and 50% O_2 could maintain the TVC of minced beef at approximately 4 log CFU/g until two-weeks of storage. Although the specific action of CO_2 against microorganisms is still not well known, the overall bacteriostatic effects are considered as an extension of the lag phase of growth and a decrease in growth rate during the logarithmic phase (Farber, 1991).

Table 1
Effects of packaging conditions and storage time on the colony counts, pH and TVB-N values of beef steaks during chilled storage.

Traits	Packaging	Storage time (days)					SE[f]	*P*-value		
		0	5	10	15	20		PT[e]	Day	PT × Day
TVC (log CFU/g)	TMAP	4.94^{ab}	4.88^{ab}	4.84^{bz}	4.94^{abz}	5.19^{az}	0.14	<0.001	<0.001	<0.001
	CMAP	4.94 ^{cd}	4.92^{d}	5.27^{cy}	6.38^{by}	7.44^{ay}				
pH	TMAP	5.57	5.57	5.57	5.57	5.58^{z}	0.02	0.065	0.002	0.054
	CMAP	5.57^{b}	5.57^{b}	5.57^{b}	5.57^{b}	5.63^{ay}				
TVB-N (mg/100 g)	TMAP	11.04^{b}	11.64^{ab}	11.13^{b}	11.09^{bz}	12.24^{az}	0.35	<0.001	<0.001	<0.001
	CMAP	11.04^{c}	12.10^{b}	12.09^{b}	13.05^{by}	15.83^{ay}				

[a-d] Means within the rows with different letters differ at $P < 0.05$.
[y-z] Means within the columns with different letters differ at $P < 0.05$.
[e] PT = packaging types: TMAP = 50%O_2 /40% CO_2/10% N_2; CMAP = 50% O_2/50% N_2.
[f] Standard error.

3.2. Microbial community by using high-throughput sequencing

3.2.1. Sequencing data analysis

Amplicon sequencing of the 16S rRNA gene provided a clear view of the diversity and abundance of microbial communities under both MAP types. A total of 2,318,058 high quality effective tags (an average of 64,391 tags per sample) were gathered by merging and filtering raw sequences from each sample with an average length of 426.03 bp. Good's coverage was higher than 99% for all steaks, indicating that most bacteria in both MAP steaks were detected. The number of observed OTUs in both MAP were significantly decreased at the last stage of storage, and the number of OTUs in TMAP were higher than that in CMAP (Table 2). As observed in the Venn diagrams (Supplementary Fig. S1), the number of common OTUs across both packaging types decreased gradually during storage, and the unique OTUs under TMAP were higher than CMAP. ACE and Chao1 indexes can reflect the species richness of communities, and Shannon index reflects the species diversity of communities. In this study, ACE, Chao1 and Shannon indexes all decreased from day 5 under both MAP, which suggested that the microbial diversity decreased with prolonged storage. TMAP steaks had the higher values of these three indexes than CMAP steaks, indicative of a higher microbial diversity in TMAP steaks during storage. Generally, the microbial diversity in meat continues to decline over time with only a few species as survivors in the end (Mansur et al., 2019). These differences in the alpha diversity of both MAP may be attributed to the selective antibacterial actions of CO_2 on SSOs. As a result, the growth of other bacteria was enhanced, because nutrient bioavailability was provided and toxicity was decreased through inhibiting dominant SSOs (Cauchie et al., 2020).

The beta diversity for both MAP steaks at different storage times was analyzed by PCA, in which PC1 and PC2 accounted for 75.7% and 21.8% of the total variance, respectively (Fig. 1). The samples were clearly separated throughout storage regardless of the packaging types, but the samples for both HiOx-MAP at respective sampling point were located at similar sites within 20 days, except for day 10. This indicated that both MAP types resulted in pronounced changes in the microbiota structure over time, but the shift in microbial communities was rather similar for both gas conditions.

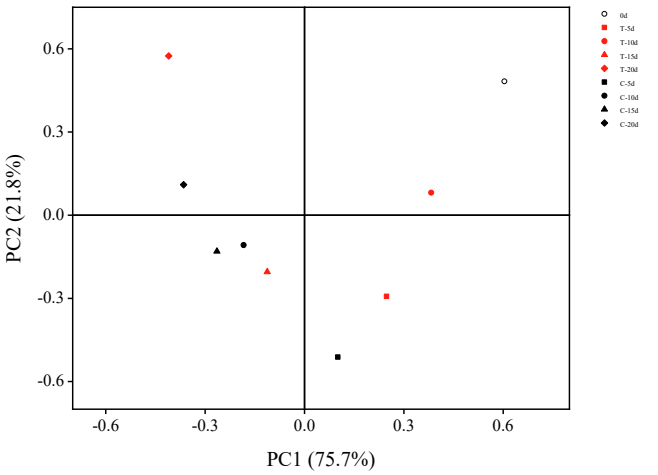

Fig. 1. Principal Component Analysis (PCA) of the bacterial community during the chilled storage of beef steaks packaged under both MAP types. Sample numbers represent storage days. T and C = TMAP (50% O_2/40% CO_2/10% N_2) and CMAP (50% O_2/50% N_2), respectively.

Table 2
Bacterial diversity estimators of beef steaks under both MAP types during chilled storage.

Traits	Packaging[a]	Storage time (days)				
		0	5	10	15	20
Read number	TMAP	65,249	62,420	64,597	65,730	62,697
	CMAP	65,249	64,355	63,871	64,922	65,831
OTUs	TMAP	767	904	1257	484	724
	CMAP	767	566	529	678	545
Chao1	TMAP	509	413	616	260	376
	CMAP	509	333	301	265	192
ACE	TMAP	512.90	416.15	631.30	271.37	391.40
	CMAP	512.90	344.10	309.58	274.61	230.77
Shannon	TMAP	4.56	4.19	5.00	2.97	2.48
	CMAP	4.56	3.52	2.91	2.58	2.22
Coverage	TMAP	99.88%	99.90%	99.83%	99.90%	99.83%
	CMAP	99.88%	99.90%	99.90%	99.90%	99.93%

[a] TMAP = 50% O_2/40% CO_2/10% N_2; CMAP = 50% O_2/50% N_2.

3.2.2. Composition of bacterial community under MAP

The shift in beef microbiota compositions at different packaging and storage conditions was shown at the phylum and genus level (Fig. 2). Based on high-throughput sequencing results, more than five phyla were observed, in which *Proteobacteria*, *Firmicutes*, *Actinobacteriota* and *Bacteroidota* were the most abundant phyla, accounting for > 95% of the total bacterial community (Fig. 2a). *Proteobacteria* were the most predominant microorganisms during storage in both MAP types, but then they were all replaced by *Firmicutes* until the end of storage. This may have accounted for the subsequent outgrowth of the genus *Brochothrix*.

A total of twenty-one genera of bacteria appeared in beef steaks for both TMAP and CMAP (Fig. 2b). The initial bacterial composition is dominated by *Acinetobacter* (29.5%), *Psychrobacter* (21.3%), and *Kocuria* (15.0%) in this study. They were recognized as undesirable bacteria in food processing environment generally. For example, *Kocuria rhizophila*, one of the most initial bacteria at the species level (15.0%), have been detected on the knives used in cattle abattoirs and the small intestinal gut in beef cattle (Moretro, Langsrud, & Heir, 2013; Whon, Kim, & Bae, 2018). In addition, *Intestinibacter*, *Faecalibacterium*, and *Blautia* were detected at relatively low proportions in all samples during storage.

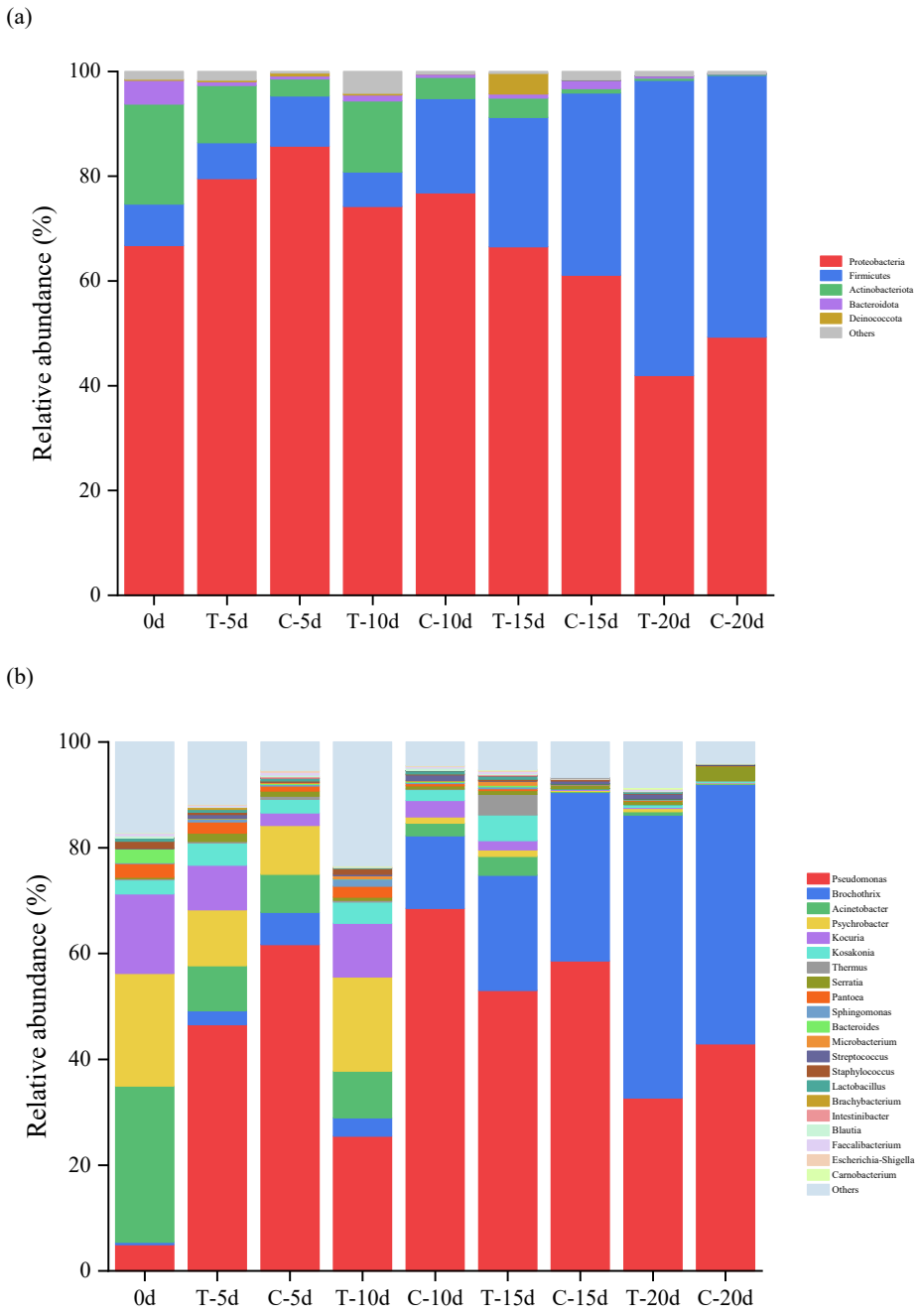

Fig. 2. Relative abundance (%) of the bacterial community during the chilled storage of beef steaks packaged under both MAP types. (a) phylum level; (b) genus level. Sample numbers represent storage days. T and C = TMAP (50% O_2/40% CO_2/10% N_2) and CMAP (50% O_2/50% N_2), respectively.

These genera have previously been identified as fecal bacteria of forage-fed horses and sheep (Shepherd, Swecker, Jensen, & Ponder, 2012; Xulu et al., 2020). Unlike other microbiota studies that examined beef cut in a laboratory environment (Hanlon et al., 2021; Yang et al., 2018), this work represents the actual microbial contamination in a Chinese abattoir and simulated a more realistic storage of steaks. Based on the results, additional decontamination procedures are needed when removing cattle hides and intestinal contents, along with clean tool surfaces, to avoid these autochthonous contaminants.

The gas composition affected the bacterial development during storage intensely. In more detail, several initial genera, such as *Acinetobacter*, *Psychrobacter* and *Kocuria*, decreased gradually (Fig. 2b). Instead, *Pseudomonas* grew rapidly during the first 5 days, and were identified as a dominant genus in both gas conditions over time. Compared to others, *Pseudomonas* with a stronger proteolytic activity can gain an ecological advantage through penetration into the meat because the bacteria then have access to new host material for exploitation (Nychas et al., 2008). Additionally, the dominance of *Brochothrix* were observed in both HiOx-MAP at the end of storage. Given that the TVC approached the spoilage threshold in the CMAP on day 20, *Pseudomonas* and *Brochothrix* were the main contributors to the spoilage of steaks. The relative abundance of *Pseudomonas* dropped rapidly in TMAP steaks over the last 5 days. This decline was mainly due to the outgrowth of *Brochothrix* and the inhibition of *Pseudomonas* growth by CO_2. TMAP steaks showed a lower percentage of *Pseudomonas* and higher percentage of *Brochothrix* at the end of storage, indicative of *Brochothrix* being more resistant to CO_2 than *Pseudomonas* under TMAP, which were in consistent with Gill (1996). We also found the abundance of *Serratia* under

TMAP was lower than under CMAP. In support, Carrizosa et al. (2017) reported that the MAP with a higher CO_2 concentration (composed of 20% O_2/45% CO_2/35% N_2) significantly restricted the growth of *Serratia* in goat meat compared to the MAP with 55% O_2, 20% CO_2 and 25% N_2. In general, high levels of CO_2 will select LAB as the main flora (Ercolini et al., 2006; Gill, 1996; Nieminen et al., 2015). However, only limited abundance of *Lactobacillus* and *Carnobacterium* were detected in the current study, which may be due to the specific initial microbial loads and high O_2 atmospheres.

The abundant microbial taxa were assigned by LEfSe analysis to exhibit detailed comparative information regarding bacterial communities in both MAP beef steaks (Fig. 3). In detail, the microbiome of TMAP steaks was more diverse than CMAP steaks during chilled storage, because it was characterized by three indicators distributed in different lineages, including *Firmicutes*, *Proteobacteria* and *Deinococcota*; in contrast, the indicator OTUs of CMAP steaks throughout 20-days were

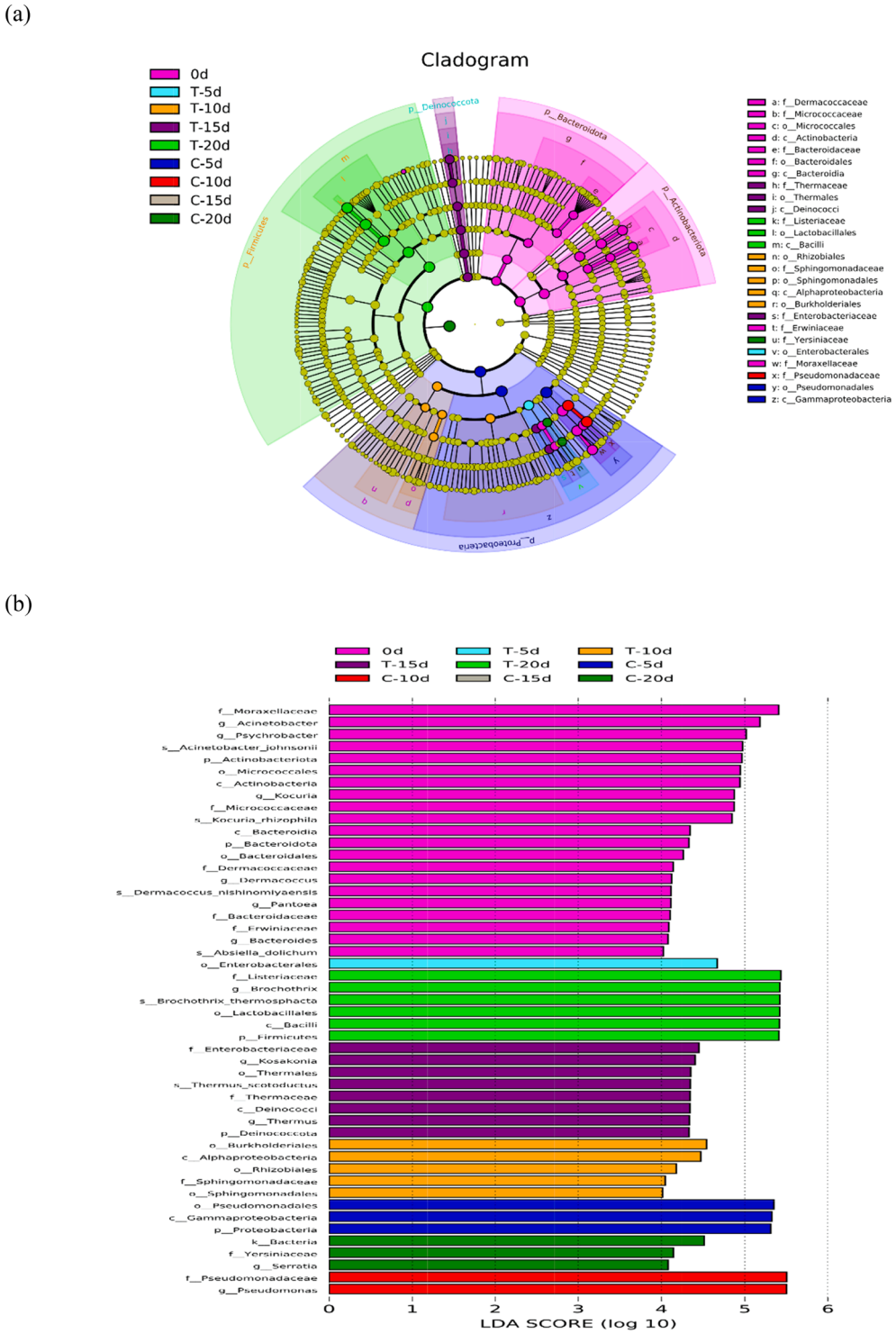

Fig. 3. LEfSe analysis of microbiota during the chilled storage of beef steaks packaged under both MAP types. (a) The taxonomic structure and the relative abundance of the identified taxa of beef steaks are represented in cladogram; (b) The most differentially abundant taxa in each group identified by LDA showed in bar gragh. Sample numbers represent storage days. T and C = TMAP (50% O_2/40% CO_2/10% N_2) and CMAP (50% O_2/50% N_2), respectively.

assigned to one indicator *Proteobacteria* (Fig. 3a). Fig. 3b revealed the CMAP indicator OTUs were assigned to *Pseudomonas* on day 10, suggesting that *Pseudomonas* proliferated overwhelmingly and accelerated the spoilage process of CMAP steaks. At end of storage, the CMAP indicator OTUs were assigned to *Serratia* (Fig. 3b), which are the common spoilage bacteria detected from HiOx-MAP meat and have been recognized as putrescine and cadaverine producers causing the spoilage of raw meat (Carrizosa et al., 2017; De Filippis et al., 2013; Yang et al., 2018). In addition, *Lactobacillales* and *B. thermosphacta* indicator OTUs were identified in TMAP steaks (Fig. 3b) and played an important role in the microbiota at day 20. They all belong to Gram-positive bacteria, and the thick peptidoglycan layer in the cell wall may contribute partially to protect Gram-positive bacteria against CO_2 (Gill & Tan, 1980; Reith & Mayer, 2011).

3.2.3. *Pseudomonas* compositions under MAP

Numerous studies have reported that psychrotrophic *P. fragi*, *P. lundensis*, *P. fluorescens*, and *P. putida* are often isolated from spoiled beef (Ercolini et al., 2007; Hilgarth et al., 2019). In this study, no *P. lundensis*, *P. fluorescens* or *P. putida* were detected in CMAP steaks. As expected, *P. fragi* were found as the most competitive species in CMAP steaks, accounting for 70% in total *Pseudomonas* species (28/40; Table 3), followed by *P. versuta*, *P. weihenstephanensis* and, to a lesser extent, other species such as *P. psychrophila*. Hilgarth et al. (2019) identified *P. fragi* and *P. weihenstephanensis* as the dominant species in high oxygen packaged minced beef. Some experiments evaluating the microbial growth rate in minced beef have observed that *P. fragi* strains have a shorter lag time than *P. fluoresces* and *P. putida* (Lebert, Begot, & Lebert, 1998; Papadopoulou et al., 2020). As a result, *P. fragi* could reach the multiplying exponential phase in advance and became the predominant flora. *P. fragi* played an important role in the development of beef spoilage such as off-odors, discoloration, and slime formation (Bala, Marshall, Stringer, & Naumann, 1977; Mansur et al., 2019; Nychas et al., 2008). Additionally, *P. versuta* have been detected in fresh minced beef and spoiled blunt snout bream (Hilgarth et al., 2019; Li et al., 2020), which was related to the hydrolyzing activity against sarcoplasmic proteins and green discoloration in fish (Li et al., 2020).

In terms of TMAP, these isolates were identified to have a close relationship with *P. versuta*, *P. fragi*, *P. fluorescens*, *P. lundensis*, *P. weihenstephanensis* and *P. taetrolens* in that order (Table 3). When compared the *Pseudomonas* abundance under both MAP types, we found that CO_2 exhibited an antibacterial influence on *P. fragi* and *P. weihenstephanensis*. Wang, Ma, Chen, et al. (2018), Wang, Ma, Zeng, et al. (2018) reported that 30% CO_2/70% N_2-MAP reduced the extracellular protease activity and cell metabolism (e.g., aerobic respiration, DNA replication and repair) of *P. fragi* NMC25 in anaerobically stored poultry meat. However, there are only a few reports on the inhibitory mechanism of high levels of CO_2 on *P. fragi* strains in beef during chilled storage, and further studies need to be implemented to elucidate the antibacterial action of CO_2 on *P. fragi* under HiOx-MAP. It is noteworthy that to the best of our knowledge, the spoilage role of *P. versuta* in beef is poorly understood, but *P. fragi* are regarded as the most dominant species on aerobically chilled meat with a high spoilage potential (Papadopoulou et al., 2020). As mentioned by Hofmann, Huptas, Doll, Scherer, and Wenning (2020), *P. fragi* are positive for tributyrin lipolysis, but *P. versuta* are not available for this at 4 °C. Therefore, *P. versuta* may have lower lipolytic activity than *P. fragi*. The comparation of spoilage potential between *P. fragi* and *P. versuta* during beef storage is hitherto underexplored yet, which is worthy of investigation.

3.2.4. Comparative functional analysis of microbiome

Meat shelf-life is not only dependent on bacterial counts, but also their metabolic properties. The microbial spoilage potential is determined by their ability to produce spoilage metabolic compounds (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015). Thus, it is necessary to further understand the bacterial metabolic pathway based on the predicted function of 16S rRNA sequencing. In the present study, carbohydrate, amino acid and lipid metabolism were three main metabolic pathways for microbial communities in the spoilage process, but the abundance of amino acid metabolism related genes in CMAP communities was higher than those in TMAP communities during the latter stage (Fig. 4). This probably results from the fact that more *Pseudomonas* in CMAP steaks exhausted the glucose and lactate present in the meat and began to metabolize nitrogenous amino acids compared to TMAP steaks (Nychas et al., 2008), while the range of amino acids that could be utilized by the dominant *B. thermosphacta* is very restricted (Pin, de Fernando, & Ordónez, 2002). The importance of amino acid metabolism in accelerating the meat spoilage process has been shown, for example, by the fact that leucine, isoleucine, and valine metabolic degradation were more abundant in CMAP at the end than in TMAP from day 5 (Fig. 4). Casaburi et al. (2015) summarized that these metabolism pathways were related to the accumulation of branched-chain fatty acids (e.g., 2 or 3-methylbutanoic acid), which together impart a sweety, fruity, waxy, and cheesy odor.

3.3. TVB-N and pH analysis

TVB-N originates from microorganisms and their enzyme activity, mainly consisting of ammonia, amine and trimethylamine (Bekhit, Holman, Giteru, & Hopkins, 2021). A significant interaction of packaging types × storage period was evident for TVB-N values (Table 1). The TVB-N values for the CMAP beef steaks increased significantly during storage and reached 15.83 mg/100 g at day 20, which has exceeded the spoilage threshold (≤15 mg/100 g) defined by National Food Safety Standard of China (GB 2707–2016). By contrast, TMAP steaks showed lower ($P < 0.05$) TVB-N values than CMAP steaks over the last 5 days, which were mainly attributed to the lower bacterial populations.

Only storage time had a significant effect on meat pH ($P < 0.05$). Similarly, there was a markedly increased pH in CMAP beef steaks after day 15 (Table 1), and this may have been caused by the bacterial production of NH_3, amines, and organic sulphides through protein breakdown to produce free amino acids (Stanbridge & Davies, 1998).

Table 3
Identification of *Pseudomonas* sp. by the 16S rRNA gene sequencing and species-specific PCR assay from beef steaks under both MAP types at the end of storage.

TMAP (%)	CMAP (%)	Identities (16S rRNA gene)	Identity (%)	Accession number	PCR amplification
10^{bx}	70^{aw}	*P. fragi*	99	NR 024946.1	*P. fragi*
7.5^{axy}	0^{by}	*P. fluorescens*	99	KX 186944.1	*P. fluorescens*
2.5^{yz}	0^{y}	*P. lundensis*	99	KX 186958.1	*P. lundensis*
2.5^{byz}	12.5^{ax}	*P. weihenstephanensis*	99	KP 738720.1	–
0^{z}	2.5^{y}	*P. psychrophila*	99	KX 186965.1	–
75^{aw}	15^{bx}	*P. versuta*	99	NR 149823.1	–
2.5^{yz}	0^{y}	*P. taetrolens*	99	KX 186991.1	–

[a-b] Means within the rows with different letters differ at $P < 0.05$.
[w-z] Means within the columns with different letters differ at $P < 0.05$.
TMAP = 50% O_2/40% CO_2/10% N_2; CMAP = 50% O_2/50% N_2.

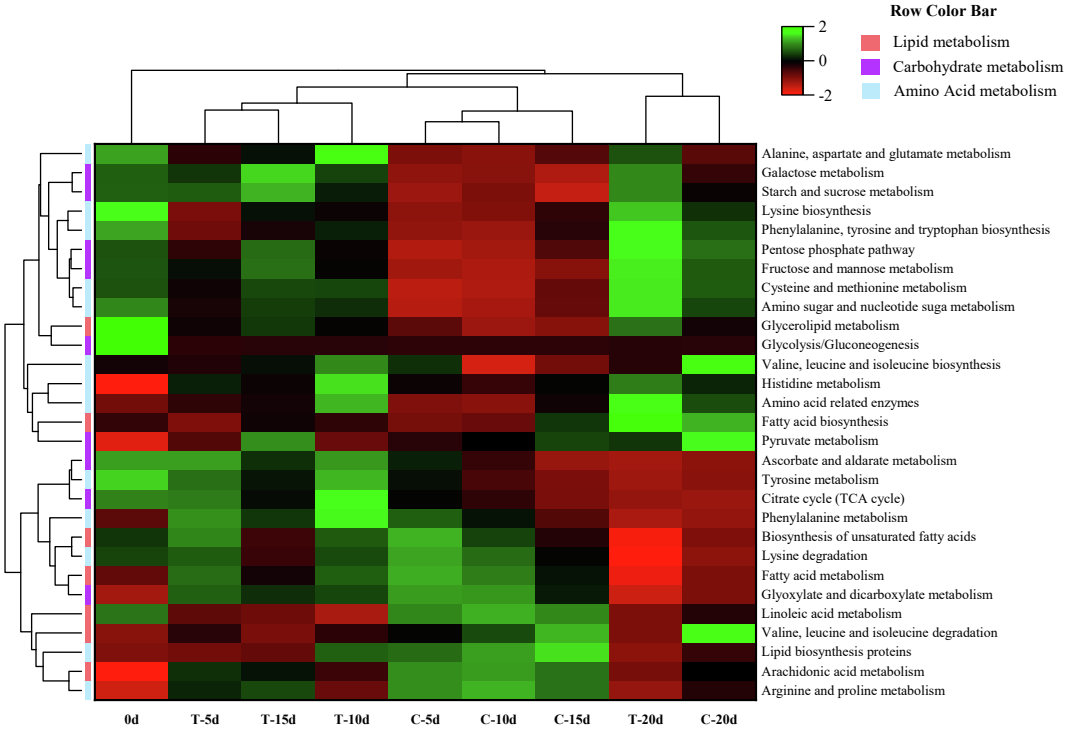

Fig. 4. Heatmap of potential bacterial metabolic pathways predicted by bacterial community in MAP beef steaks during storage. The level 3 pathways annotations were described on the right and colored according to level 2 categories. Sample numbers represent storage days. T and C = TMAP (50% O_2/40% CO_2/10% N_2) and CMAP (50% O_2/50% N_2), respectively.

3.4. Headspace gas analysis

In TMAP, the CO_2 concentration showed a downward trend within the first 10 days, and then increased significantly. By contrast, the O_2 concentration increased firstly and declined after day 10 (Table 4). The decreased CO_2 concentration in the early stage resulted from CO_2 dissolving in the meat aqueous and lipid phase until attaining saturation or equilibrium (Devlieghere, Debevere, & Van Impe, 1998). As for the CMAP, the O_2 concentration decreased and the CO_2 concentration increased constantly throughout the entire storage period, especially after day 10. This change was due to the increased bacterial respiratory metabolism accompanied by a microbial outgrowth from day 10. *Pseudomonas* as aerobic bacteria maybe an important inducement causing the rapid O_2 consumption (Cauchie et al., 2020; Gill, 1996). In practice, the gaseous composition within a MAP is not static, and its dissolution in the meat or permeation toward external atmospheres also continually alter the inner gaseous concentration. Albeit a significant increase in CO_2 levels was observed in CMAP from day 10, the antibacterial effect of CO_2 on the microbial growth has always been attributed to the initial CO_2 concentration in the gas-phase of packaging (Devlieghere et al., 1998; Zhao, Wells, & Mcmillin, 1994). Moreover, once meat products are contaminated by potent spoilage organisms, even the presence of CO_2 may play a minimal role in delaying the spoilage (Gill, 1996).

Table 4
Changes in the headspace compositions of both MAP types during chilled storage.

Storage time (days)	Headspace compositions under packaging of			
	TMAP[e]		CMAP[e]	
	%O_2	%CO_2	%O_2	%CO_2
0	51.45^{b}	39.80^{a}	51.00^{a}	0.92^{d}
5	51.52^{b}	35.47^{c}	45.38^{b}	5.55^{c}
10	53.23^{a}	35.21^{c}	43.85^{b}	7.00^{c}
15	50.93^{bc}	36.39^{b}	28.01^{c}	21.60^{b}
20	49.98^{c}	36.60^{b}	19.96^{d}	29.90^{a}
SE[f]	0.47	0.25	1.33	1.39
P-value	<0.001	<0.001	<0.001	<0.001

[a-d] Means within the columns with different letters differ at $P < 0.05$.
[e] TMAP = 50% O_2/40% CO_2/10% N_2; CMAP = 50% O_2/50% N_2.
[f] Standard error.

3.5. Color

Color is a critical aspect of meat quality that determines consumers' purchase decisions to a large degree (Esmer et al., 2011). In this study, neither the interaction of packaging × storage time nor packaging had an effect on L^* values (Table 5, $P > 0.05$), and only storage time resulted in an effect ($P < 0.05$). The L^* values for all packaged steaks gradually increased throughout the 15 d ($P < 0.05$), and then decreased significantly, irrespective of packaging types. Both a^* and b^* values were affected by the packaging × storage time interaction ($P < 0.05$). In the TMAP, both a^* and b^* values increased within the first 10 days and then with slight decline to different degrees. From day 10, CMAP steaks exhibited a significant decrease in a^* and b^* values, and the lowest a^* and b^* values ($P < 0.05$) were obtained at the end of storage. Considering beef color is considered acceptable when a^* values are equal to or above 14.5 (with 95% acceptance; Holman, van de Ven, Mao, Coombs, & Hopkins, 2017), the steaks under CMAP at day 20 would have been rejected by consumers, whereas the TMAP steaks still maintained an acceptable red color.

R630/580 ratio can be used as an indicator reflecting color stability, in which a higher ratio means lesser amount of metmyoglobin (MetMb), and thus a greater meat color stability (AMSA, 2012). Packaging and storage time interacted ($P < 0.05$) to affect the R630/580. As illustrated in Table 5, CMAP steaks demonstrated a significant decrease in R630/580 after day 5, and the lowest R630/580 was obtained at day 20 ($P < 0.05$), which was associated with greater MetMb accumulation and a noticeable brown discoloration. Yang et al. (2020) have explained that the outgrowth of *Pseudomonas* in HiOx-MAP beef was responsible for a quick discoloration by decreasing partial O_2 pressure. Therefore, the

Table 5
Effects of packaging conditions and storage time on the meat color of beef steaks under both MAP types during chilled storage.

Traits	Packaging	Storage time (days)					SE[f]	*P*-value		
		0	5	10	15	20		PT[e]	Day	PT × Day
*L**	TMAP	37.12^{d}	39.46^{c}	41.83^{b}	42.95^{a}	41.32^{b}	0.30	0.596	<0.001	0.528
	CMAP	37.12^{d}	39.26^{c}	41.73^{b}	43.05^{a}	41.93^{b}				
*a**	TMAP	16.52^{c}	19.18^{ab}	19.71^{a}	18.66^{aby}	18.14^{by}	0.56	<0.001	<0.001	<0.001
	CMAP	16.52^{b}	19.11^{a}	19.48^{a}	15.28^{bz}	11.92^{cz}				
*b**	TMAP	14.50^{b}	16.40^{a}	16.81^{a}	16.57^{ay}	16.45^{ay}	0.30	<0.001	<0.001	<0.001
	CMAP	14.50^{c}	16.29^{a}	16.89^{a}	15.24^{bz}	13.56^{dz}				
R630/580	TMAP	4.20^{a}	4.09^{a}	3.85^{ab}	3.54^{by}	3.50^{by}	0.14	<0.001	<0.001	<0.001
	CMAP	4.20^{a}	4.10^{ab}	3.76^{b}	2.70^{cz}	2.08^{dz}				

[a-d]Means within the rows with different letters differ at $P < 0.05$.
[y-z]Means within the columns with different letters differ at $P < 0.05$.
[e] PT = packaging types: TMAP = 50% O_2/40% CO_2/10% N_2; CMAP = 50% O_2/50% N_2.
[f] Standard error.

much higher ($P < 0.05$) R630/580 for TMAP steaks than for CMAP steaks from day 10 may be attributed to the inhibitory effect of CO_2 on microbial growth and oxygen consumption, thus resulting in a better meat color stability.

4. Conclusions

This study reveals the impact of CO_2 under HiOx-MAP on physicochemical properties and bacterial communities of beef steaks throughout a 20-day storage. In this study, the presence of CO_2 significantly inhibited bacterial growth and extended the shelf-life of HiOx-MAP steaks. High-CO_2 treated MAP steaks had higher microbial diversity indices than control MAP steaks during storage, and *Pseudomonas* as well as *B. thermosphacta* finally became the dominant spoilage bacteria under both MAP. However, the *Pseudomonas* abundance under high-CO_2 treated MAP was lower than that under control MAP without CO_2. Furthermore, the dominant *P. fragi* and *P. weihenstephanensis* in control MAP steaks were inhibited by CO_2 effectively, but *P. versuta* as dominant *Pseudomonas* species in high-CO_2 MAP steaks were more insensitive to the CO_2 inhibition than other bacteria. All these may be beneficial for keeping meat an acceptable color and stable pH, and retarding the production of TVB-N. This work provides new insights into microbial successions and their related spoilage phenomena occurring in CO_2 packaged steaks, and consequently enrich the antibacterial mechanism of CO_2 regarding microbial community dynamics. But additional studies into CO_2 bacteriostatic mechanisms on specific spoilers are needed to control their proliferation in meat during storage.

CRediT authorship contribution statement

Jun Yang: Investigation, Writing – original draft. **Xiaoyin Yang:** Conceptualization, Supervision, Project administration, Funding acquisition. **Rongrong Liang:** Methodology. **Lixian Zhu:** Visualization, Validation. **Yanwei Mao:** Formal analysis. **Pengcheng Dong:** Software, Data curation. **David L. Hopkins:** Supervision, Writing – review & editing. **Xin Luo:** Resources. **Yimin Zhang:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110872.

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