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Microbial Diversity Analysis of Chilled Beef with MAP during Storage

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The microbial diversity and dynamic changes of predominant bacteria in beef stored under low temperature (-2°C, 0°C, 5°C and 10°C) with modified atmosphere packaging (MAP1: 65% O_2 and 35% CO_2, MAP2: 80% O_2 and 20% CO_2 and air packaging) were respectively analyzed by denaturing gradient gel electrophoresis (PCR-DGGE). The Shannon-Wiener index (*H*), richness (*S*), evenness (E_H) and similarity indices were used to represent changes of bacterial community. Thirteen kinds of spoilage bacteria including *Bacillus* sp., *Lactococcus* sp., *Carnobacterium* sp., etc. were identified after sequencing of DGGE fragments. *Bacillus* and *Carnobacterium* were the initial spoilage organisms and they play an important role during the storage of -2°C - 5°C. Under the condition of 10°C, less species of spoilage bacteria were detected and *Lactococcus* and *Lactobacillus* turned into predominant bacteria gradually. In conclusion, study of the response and adaptability of the species under different conditions (chill temperature, MAP and storage time) will be meaningful for improving storage system and prolonging the shelf life of meat products.

Key words: Microbial diversity; Chilled beef; PCR-DGGE; MAP.

As a popular food, chilled beef is recognized as one of the most perishable foods for its high water content and abundance of essential nutrients. The shelf-life of meat depends on many factors including the species and quantities of microbes initially and their subsequent growth, in addition, the storage temperatures can play an important role in the handling of the raw meat products (Danilo *et al.*,2010; Borch *et al*., 1996; Nychas *et al*., 2008). In order to improve the quality, chilled meat is recommended to be stored under refrigeration but not frozen (0 °C - 4 °C). In addition, modified atmosphere packaging (MAP) using gas mixtures containing variable O_2 and CO_2 concentrations is one of the innovative food packaging concepts that has been introduced as a response to demands of consumers for high quality, safety and shelf-life extension of red meat (beef, mutton, pork). Substantial fractions of CO_2 (20% - 35%) are normally used to retard the growth of organisms produced by aerobic spoilage, and a certain concentration of O_2 (65% - 80%) is employed to preserve meat color.

Microbial growth and reproduction has been considered as a major reason for meat spoilage during storage. During refrigerated storage, most microbes are retarded while some psychrophilic bacteria can even survive which may damage the quality and safety of chilled meat. So it's essential to study the diversity and changes of microbe in chilled beef. Up to now, there have been several bacterial diversity studies on meat (Blixt & Borch,

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2002; Nápravníková *et al*., 2002). These research were traditionally using cultivation-based method which is time-consuming and cannot reflect the bacterial community fully because only 1% of the total number of actively resiring bacterial cells can be cultured on laboratory media (Luo *et al*., 2004; Ward *et al*., 1992; Amann *et al*., 1995). As an alternative molecular method, denaturing gradient gel electrophoresis (DGGE) has become a very important tool in the study of microbial communities, as it is believed to overcome problems associated with selective cultivation and isolation of bacteria from natural samples (Jiang *et al*., 2010). PCR-DGGE has been only in some cases optimized to monitor changes of spoilage microbial in meat (Danilo *et al*., 2010; Ercolini *et al*., 2006; Fontana *et al*., 2006; Diez *et al*., 2008) while it is widely exploited for the characterization of fermented foods (Ercolini *et al*., 2004).

The aim of this work was using PCR-DGGE to analysize the dynamic changes of microbial community in chilled beef under three different packaging conditions and to investigate the effects of MAP during refrigerated storage.

MATERIALS AND METHODS

Beef samples

Four boneless beef cuts (purchased in a muslim beef mutton company) were transported to the laboratory at 4 °C within 30 minute. Each cut was divided into small steaks (about 25 g) and steaks originating from the same cut were rubbed together in order to spread the naturally acquired contamination evenly. Then four steaks (about 100 g) from the same cut were placed in a sterile PET bag and modified-atmosphere packaged by using a packaging machine. The beef samples were packed using MAP1(65% O_2 and 35% CO_2), MAP2 (80% O_2 and 20% CO_2) and air packaging, and they were stored at four refrigerated temperatures (-2 °C, 0 °C, 5 °C and 10 °C). These samples were taken for analysis after several days of storage, respectively (Table 1).

DNA extraction

Beef sample were diluted in 100 mL sterile saline water after shaking at 300 r min^{-1} for 5 min. The dilution was centrifuged for 3 min at 2000 r min^{-1} and the supernate was centrifuged for 10 min at 5000 ×*g* to precipitate bacteria, then the sediment was washed two times by 25 mL and 1 mL sterile saline water, respectively. After centrifugation of 5 min at 5000 ×*g*, the sediment was used to extract bacterial DNA by using Biospin bacteria genomic DNA extraction kit (BioFlux) according to the manufacturer's instructions, and then the bacterial DNA was suspended in 100 µL TE before stored at -20°C.

PCR amplification

The primers V3-2 (10µM) and V3-3 (10µM) were used to amplify the variable V3 region of 16S rRNA gene, which giving PCR products of about 200 bp. Amplifications were performed in a PCR amplifier (Eppendorf AG 22331 Hamburg). Each mixture(final volume, 20 µL) contained 10 µL of Premix Ex Taq , 8 µL of ddH_2O, 0.5 µL of each primers and 1 µL of template DNA. Template DNA was denatured for 5 min at 94 °C, then a touchdown PCR was performed. The initial annealing temperature was 65 °C and this was decreased 0.8 °C every cycle for 18 cycles, finally 12 cycles were performed at 55 °C. The extension for each cycle was carried out at 72 °C for 45 s while the final extension was at 72 °C for 10 min. Aliquots (4 µL) of PCR products were routinely checked on 1% agarose gels.

DGGE analysis

PCR products were analyzed by DGGE by using a Bio Rad Dcode apparatus. Samples were applied to 7% (wt/vol) polylamide gels (acrylamide/ bisacrylamide=37.5:1) in 1×TAE buffer. Parallel electrophoresis experiments were performed at 60 °C by using gels containing a 45%-55% urea-formamide denaturing gradient (100% corresponded to 7M urea and 40% (wt/vol) formamide). The gels were run for 16 h at 60 V and stained with SYB Green I for 15 min, then rinsed and phographed by Gel Doc™ XR+ system. For each lane in DGGE gels, band patterns and average intensities of individual bands were analyzed using Quantity One image analysis software 4.0. To measure changes in the bacterial community, the Shannon-Wiener index (*H*), richness(*S*) and evenness(E_H) were calculated according the following equation:

$$H = -\sum_{i=1}^{S} P_i \ln P_i \qquad E_H = H / \ln S$$

where Pi is the ratio between the specific band intensity and the total intensity of all bands,

S is the total number of bands in each samples (Luo *et al*., 2004). Then similarity of all samples were analyzed by calculating similarity indices with interval data in software of NTSYS-pc 2.10e and cluster analysis were made by SHAN and UPGMA.

Sequencing of DGGE fragments

The DGGE bands to be sequenced were suspended in 50×TE. One µL of the DNA of each DGGE band was re-amplified by using V3-1 (10µM) and V3-2 (10µM) under the conditions described above. The PCR products were purified by Universal DNA purification kit (TIANGEN, Germany) according to the manufacturer's instructions. Then the DNA were cloned by pGM-T cloning kit and sequenced by ShengGong biological engineering company (Shanghai, China). Sequences were aligned with those in GenBank with Blast. Construct phylogenetic tree with Bootstrap-NJ in software of MEGA 3.1.

RESULTS

The PCR-DGGE profiles of PCR products obtained from DNA directly extracted from beef cuts under different conditions are presented in Fig. 1 and a fluctuation in the population was observed. The bands on DGGE gels represented microbial species (Ercolini, 2004).

Under the condition of -2 °C, a high microbial diversity was noticed but only band A1and A2 were with high intensity. After 15 days of storage under MAP1 condition, bands A7, A8 and A9 were found to be the dominant ones, and then A12 and A13 increased. Bands A1 and A8 occurred during the whole period of three packaging conditions and they played a high degree of intensity during the storage of air packaging. In the latter spoilage phase of MAP2, bands A13 and A12 were also detected. Band A13 was found during the whole storage of air packaging while band A12 was not found at all the other samples.

Under the condition of 0 °C, the beef showed the presence of B1, B2 and B3 at day 0. Under the MAP1 condition, the intensity of some other bands became more intense and bands B5, B7, B8 and B9 became the dominant ones after 25 days of storage. The similar tendency occurred in MAP2 and air packaging conditions. They were not the dominant until the end of the monitoring under MAP1 and MAP2 conditions, bands B5 and B9 were with high intensity since the beginning of the storage under air packaging condition. Band 10 was only existed in the latter period of storage under MAP1 and MAP2 conditions.

Under the condition of 5 °C, the initial bacteria contained C1, C2, C3 and C4. At day 5 under MAP1 condition, bands C4, C5 , C7, C8,C9 were shown with similar intensity, and bands C9 and C10 increased from day 5 to day10. At the

Table 1. Sample analysis time under different temperatures. The time were chosen according growth curve of precipitate bacteria

Temperture (°C)	Time(d)			
-2	0	15	30	
0	0	6	15	25
5	0	5	10	15
10	0	2	5	10

Table 2. Shannon-Wiener index (H), evenness (E_H) and richness (S) of each beef samples stored under different packaging conditions and temperatures. The lanes were indicated on the DGGE gels shown in Fig.1

Lanes	S	H	E_H	Lanes	S	H	E_H	Lanes	S	H	E_H	Lanes	S	H	E_H
a1	26	3.05	0.94	b1	10	1.78	0.77	c1	14	2.18	0.83	d1	12	1.94	0.78
a2	16	2.36	0.85	b2	18	2.64	0.91	c2	21	2.76	0.91	d2	13	1.87	0.73
a3	17	2.54	0.90	b3	15	2.48	0.92	c3	14	2.21	0.84	d3	15	2.47	0.91
a4	22	2.88	0.93	b4	13	2.22	0.87	c4	13	2.41	0.94	d4	18	2.58	0.89
a5	17	2.48	0.86	b5	9	2.13	0.97	c5	15	2.28	0.84	d5	20	2.67	0.89
a6	24	2.91	0.92	b6	10	2.00	0.87	c6	16	2.37	0.85	d6	22	2.83	0.92
a7	19	2.63	0.89	b7	14	2.32	0.88	c7	22	2.64	0.85	d7	18	2.62	0.91
				b8	13	2.41	0.94	c8	20	2.7	0.90	d8	21	2.78	0.91
				b9	16	2.47	0.89	c9	20	2.58	0.86	d9	18	2.6	0.90
				b10	19	2.59	0.88	c10	8	1.97	0.95	d10	19	2.64	0.90

beginning of the storage under MAP2 condition, bands C8, C10,and C12 was with high intensity, then bands C12 and C8 decreased and bands C13 and C10 increased rapidly to be dominant. Band C13 turned out to be with highest intensity. Under air packaging condition, band C14 was also with high intensity, but this species was not detected in any other samples.

Under the condition of 10 °C, bands D1, D2 and D3 were identified the dominant. The sample d2 which was stored under MAP1 for 2 days was exactly the same as the initial sample. The intensity of bands D4, D5 and D6 increased to be the dominant at the end of storage under MAP1 condition. Band D5 was detected during the whole storage of three packaging conditions. Band D4 increased to dominant at day 5 in MAP2 and air packaging which was earlier than in MAP1, and then it turned to faineter. Band D6 increased later in MAP1 and air packaging than MAP2. Band D1 decreased a lot during the storage of three packaging conditions. Bands D8 and D9 showed a high intensity at the prior period in MAP2 and air packaging. Under air packaging condition, band D5 became to be dominant at the end of the storage

Table 3. DGGE bands identified in this study by means of partial 16S rDNA gene sequencing. Bands are lettered as indicated on the DGGE gels shown in Fig.1

Closest relative	Bands	Accession no.
Bacillus sp.	A7,A8,A9,B1,B2,B3,B15,C1,C2,C4,C5,C6,C7, D1,D2,D3,D9,D11	KC433413-KC433430
Lactococcus sp.	A5,A10,A18,B6,B11,B14,C12,D5,D7,D12,D13	KC433431-KC433441
Carnobacterium sp.	A1,A15,B5,B7,B10,C3,C10,C11,C15,D8	KC433442-KC433451
Acinetobacter sp.	A2,A17, B4,C8	KC433452-KC433455
Brochothrix sp.	A13,B8,B9,C16	KC433456-KC433459
Enterobacter sp.	A3,A4,A6,D6,D10	KC433460-KC433464
Psychrobacter sp.	A14,B13,C14	KC433465-KC433467
Staphylococcus sp.	A12,B12	KC433468, KC433469
Pseudomonas sp.	B16,B17	KC433470, KC433471
Arthrobacter nitroguajacolicus	A11	KC433472
Lactobacillus sakei	C9,C13, D4	KC433473-KC433475
Aeromonas sp.	A19	KC433476
Micrococcus sp.	A16	KC433477

Table 2 shows that the values of richness(E_H) and Shannon-Wiener index(H) of each beef sample were 1.87-3.04 and 0.73-0.97, respectively. The three initial sample(b1,c1 and d1) but a1 performed close values of $E_H$and H. $E_H$and H of samples during storage represented a small increase compared to the initial ones.

The similarity indices of the DGGE profiles of all the samples are reported in Fig.2 and similarity level can be evaluated by Jaccard's q. Under the condition of -2 °C, the similarity index between a1 and other samples was low (0.45) which being a moderate dissimilar level. During the storage under MAP1 (MAP2, air packaging), the similarity index increased from 45% to 57% (67%, 63%) which indicated the microbial diversity decreased. In the former period of storage, the similarity index of samples stored in MAP2 and air packaging (a4 and a6) was higher (79%), while in the subsequent storage higher similarity (73%) existed among a3 and a5 which were stored in MAP1 and MAP2. This indicated the influence of MAP during storage. Under the condition of 0 °C and 5 °C, the similarity index of most samples were in medium similar level. It was found that the similarity index of beef samples with different packaging was not high even if stored for the same time. Furthermore, the similarity index among the samples during same MAP storage was also not high. This indicated that the influence of the storage time and modified atmosphere packaging on beef were rather obvious, and this was in agreement with previous studies that both storage time and packaging had a strong effect on the microbial communities in chilled pork (Li *et al.,* 2006). Under 10 °C, the high similarity index was

revealed between d1 and d2 (0.92), which indicated that the changes of microbial species was not obvious in the first two days of storage under MAP1. The similarity index of all the other samples were in similar level which revealed that the influence of storage time and packaging were not significant under the condition of 10 °C.

The results of highlighted band by DNA sequencing are reported in Table 2. Fragments migrating with the same distance in DGGE gels were repeatedly sequenced giving the same results in terms of closest relative species and percent of identity. Results of DNA sequencing were submitted to GenBank database to obtain accession number (Table 2, KC433413- KC433477). Thirteen kinds of spoilage bacteria were found in this study and *Carnobacterium* and *Bacillus* were always identified as the initial spoilage organisms. They have been shown to play an important role during the storage of -2 °C-5 °C. Under the condition of 0 °C, *Brochothrix* was the predominant bacteria during the whole storage of

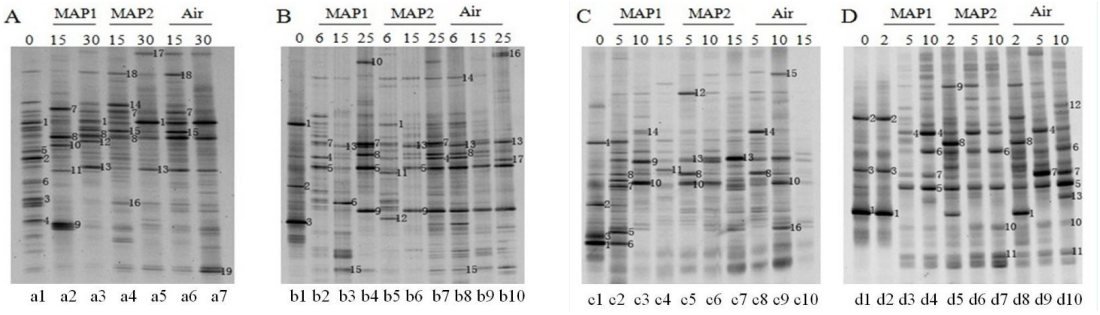

Fig.1. PCR-DGGE profiles of PCR products of chilled beef stored at (A)-2°C, (B)0°C, (C)5°C and (D)10°C after several days of storage (Table 1).The sampling times (days) are indicated at the top of each lane and the number of these lanes are marked at the bottom. The packaging conditions are MAP1 (65%O_2 and 35%CO_2), MAP2 (80%O_2 and 20%CO_2) and air packaging. The numbers on the right of the bands indicate the sequenced bands reported in Table 2, and fragments labeled with the same number showed identical sequences

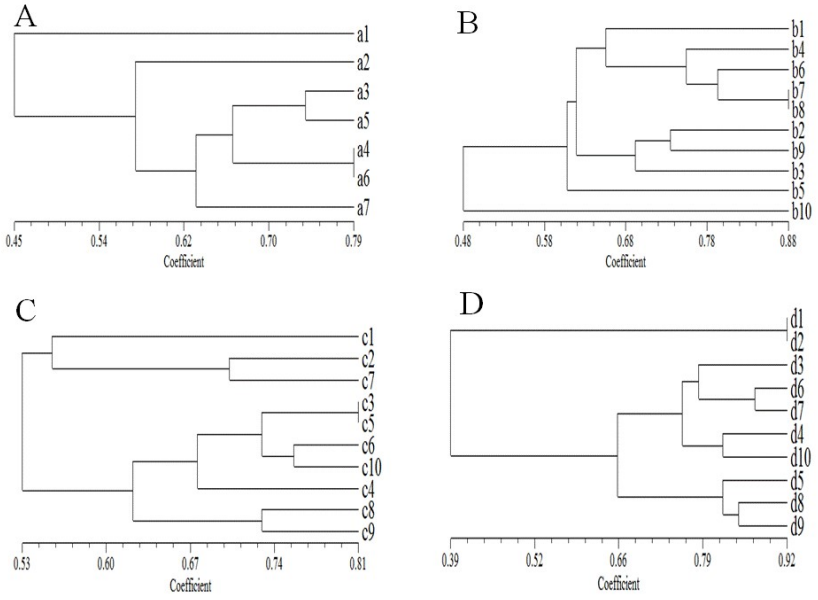

Fig. 2. Cluster analysis of molecular banding patterns generated by PCR-DGGE of Fig. 1. The dendrogram was obtained with the UPGMA clustering algorithm. Percentage similarity between different samples is shown on the scale under the dendrogram

air packaging with higher oxygen content, while it became dominant at later storage of MAP1 and MAP2. *Pseudomonas* were only detected at the end of the storage of air packaging. These indicated the inhibition of CO_2 on *Brochothrix* and *Pseudomonas*. In most cases, the bacteriostatic effect is obtained by a combination of decreased O_2 and increased CO_2 concentrations (Anne-Marie *et al*., 2004). Under the condition of 10°C, five kinds of spoilage bacteria were detected during storage which was less than those stored under other storage temperatures and *Lactococcus* and *Lactobacillus* turned into predominant bacteria gradually.

The phylogenetic tree was constructed based on sequencing of DGGE fragments (Fig.3). They were grouped into two clusters. Some of the bacterium which was identified in the same family were grouped in the same cluster, such as A8, A9, B2, C5 *et al.*, but some like A7, B1, B15 and C4 which belong to this genera as well were grouped into the other cluster. This may be due to their different species with different nucleotides. And the homology of A3 and A4, B8 and B9, C10 and C15, and D10 and D6 were rather close, but there was a difference between their mobility of the corresponding bands in DGGE gels, which showed a structure difference on them. But on the other hand, it's noteworthy that several bands for a species in a DGGE profile do not necessarily represent different strains of that species due to the presence of multiple copies of the ribosomal genes and the fact that the gene copies have evolved differently (Jiang *et al*., 2010; Ueda *et al.,* 1999). There were high homology between A18, B14, C12 and D12, and they were all in the top of the DGGE gels, so it's possible that they were the same bacterium and the different mobility may be caused by the little difference of the four gels.

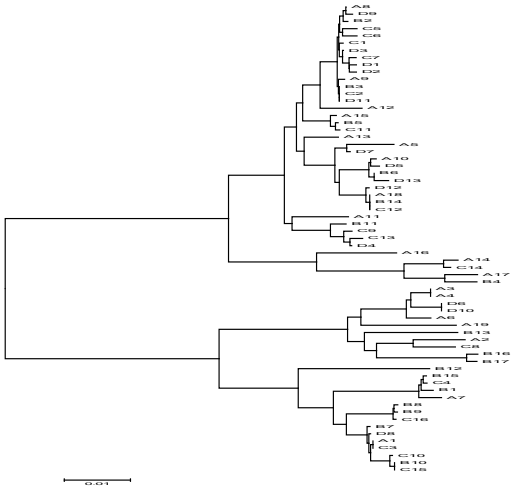

Fig. 3. The phylogenetic tree of DGGE bands identified in this study. Bands are lettered as indicated on the DGGE gels shown in Fig.1

DISCUSSION

The changes of these bands in DGGE lanes showed the complexity and variability of bacterial communities in chilled beef. It is easy to find that the bacteria species changed a lot irregularly with packaging conditions and time of storage. In addition, under the condition of -2 °Cÿthe increase of the similarity index implied the decrease of the bacterial diversity which was in accordance with previous studies that found a micro-biological diversity reduce after storage (Li *et al*., 2006).

The values of richness(E_H) and Shannon-Wiener index(H) of the initial beef samples (b1, c1 and d1) except a1 were similar, the difference may be caused by the different initial microbial contaminating level of the four blocks. It is generally accepted that the surface of the meat is easier to be contaminated during the period of animal slaughter and darcass segmentation processing. It is interesting that so many different microbial species can cohabitate in beefs. Some spoilage organism belongs to *Bacillus* sp., *Acinetobacter* sp., *Psychrobacter* sp. *Lactococcus* sp. *Enterobacter* sp. *Carnobacterium* sp. and *Brochothrix* sp. were detected in the initial samples in this study and the samples stored under -2°C were contaminated even more severe. In addition, *Staphylococcus sp*., *Aeromonas* sp., *Micrococcus* sp., and *Pseudomona*s sp. were also found in beef samples during chilled storage. *Bacillus, Lactococcus* and *Carnobacterium* existed under conditions of all the four temperatures. *Arthrobacter nitroguajacolicus, Aeromonas* and *Micrococcus* were detected under the condition of -2 °C, this may be due to adaptability of low temperature. All of these spoilage organisms had been detected in beef by previous research. And a wider microbial diversity was observed previously (Li *et al*., 2009). This probably due to the different

sources of these samples. *Enterobacter* sp. is members of the *Enterobacteriaceae* that are seldom isolated from meat, but it is commonly seen in animal wastes, sewage, soil and water, so it may be associated with human infection. *Arthrobacter nitroguajacolicus* was not be perceived as predominant bacteria for the band was not obvious all the time and this was in agreement with the previous research (Li *et al.*, 2008). Up to now, little research about *Arthrobacter* have been carried out. Under the condition of 10°C, *Lactococcus* sp. was reported as one of the predominant species in chilled beef at the last phase of storage, while other spoilage bacteria decreased, which confirmed that *Lactococcus* sp. can compete against other bacteria (Russo *et al*., 2006). During storage, the species of the spoilage bacteria changed a lot and the changes varied with different packaging, so the influence of storage time and packaging were clear. What's more, it was obvious that specific spoilage bacteria became predominant with temperature which indicated the influence of storage temperature. So it's essential to choose corresponding dominant bacteria as indicators of meat corruption.

In this study, the DGGE profile of c10 was not clear, which may be due to the lower PCR product yield of DNA extracted directly from beef. In this study, PCR-DGGE was used to monitor the changes of bacterial communities of packaged beef during chilled storage and the PCR-DGGE technique is applicable to monitoring bacterial population dynamics in chilled beef and the identification of the spoilage-related microbial can help in the effective establishment of storage condition. But identification of these bacteria at species levels could not be achieved in this study and it was not possible to obtain univocal identifications for most of the DGGE fragments (Danilo *et al*.). So it will be better to synthesize cultivation-based method and PCR-DGGE method to analysis microbial diversity.

CONCLUSION

In this study, the changes in the bacterial communities of packaged beef during chilled storage were analyzed by PCR-DGGE and thirteen kinds of spoilage bacteria were identified. The influence of chill temperature, packaging and storage time have been proved and the study of the response and adaptability of the species of different conditions will be fundamental for improving and implementing storage system aimed at preventing the spoilage and prolonging the shelf life of meat products.

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