Microbial analysis of MAP pot-stewed duck wings under different conditions during 15 °C storage

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Abstract This study was to investigate the changes of microbial community and counts of MAP pot-stewed duck wing (PSDW) under different packaging films and spices ratio during 15 °C storage, using the traditional bacterial cultivation and PCR-DGGE. Results of microbial counting showed that the shelf-life of PSDW during 15 °C storage for recommendation was within six days, and the packaging films and spices ratio didn't affect the change of microbial numbers in PSDW during storage. PCR-DGGE analysis revealed that *Staphylococcus equorum*, *Weissella* sp., *Leuconostoc mesenteroides* became the dominating bacteria of PSDW at the end of storage, and high barrier cover film, general barrier base film and spice ratio 1:1, had a better inhibition effect on bacteria in PSDW products, which could be used as the condition for PSDW storage. This study will help PSDW processing enterprises visualize the biodiversity of PSDW during storage, and choose the best condition for the subsequent processing.

Keywords Pot-stewed duck wings · Modified atmosphere packaging (MAP) · Microbial community · Storage · PCR-DGGE

Introduction

Modified atmosphere packaging (MAP) is considered as an effective method for packaging various meat and meat products (Patsias et al. 2008; Koutsoumanis et al. 2008; Al-Nehlawi et al. 2013; Zhang et al. 2015; Lloret et al. 2016). It involves placement of product in good sealing material under mixtures of non-toxic gases, in proportions different than air, so as to reduce microbial growth and enzymatic spoilage (Young et al. 1988). Recently, commercial MAP meat products have been emerged in China market, but not a lot in China market, so as to maintain the quality of meat products and extend the shelf-life, in which pot-stewed duck wing (PSDW) is a representative of meat products with the use of MAP in China. PSDW product is a traditional meat product, and more and more popular because of its delicate piquancy flavor in China. Compared with the vacuum packaging type, not only could show the shape of the product, but the application of MAP in PSDW product will change the microbial community of bacteria in products.

Generally, meat products could be spoiled by different species of bacteria, which is related with several factors, the main ones being represented by the meat characteristics, the use of different spices and packaging types. The microbial community of bacteria in meat products under different storage conditions has been traditionally studied on basis of cultivation, isolation and biochemical identification (Borch et al. 1996; Nychas and Skandamis 2005; Jiang et al. 2010; Osés et al. 2013). However, recently, the diversity of microorganisms in a wide variety of meat products during storage has been extensively studied by the culture-independent approaches, mainly PCR combined with denaturing gel gradient electrophoresis (PCR-DGGE) (Cocolin et al. 2013). The use of PCR-DGGE technique

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could visualize the microbial diversity of meat products during storage in a single image and assess at the species level without growing each bacterium in synthetic growth culture.

In literature, the microbial diversity of many traditional meat products under different conditions has been evaluated (Marui et al. 2014; Polka et al. 2015; Pisacane et al. 2015; Greppi et al. 2015). In other country, there were no researches associated with PSDW product for references, where PSDW has a special characteristic in RTE meat products. Thus in China, researches associated with pot-stewed duck product in previous studies were seldom, most of which focused on the process improvement, the quality change during storage and its flavor analysis (Yin 2008; Xie 2012). Otherwise, the MAP type can also change the bacterial community. Thus, researches on the microbial population of PSDW product, especially under different packaging and spices ratio conditions were limited. Therefore, the aim of this study was to analyze the microbial populations in PSDW product at 15 °C storage under different conditions (the factors including packaging films and spices ratio), which combined PCR-DGGE analysis with the monitor of total number of colonies along the shelf-life, and the gas proportion was measured at the same time. The final aim was to understand the microbial community of PSDW products under different conditions, which was concerned by the processing enterprises, thereby giving the basis for the shelf-life extension.

Materials and methods

Meat samples

PSDW products were produced according to different procedures, where the packaging films and spices ratio were chosen as the main factors according to the pre experiments. The samples were divided into four groups, and every group was designed by orthogonal test, shown in Table 1.

Storage conditions and sampling

All the samples in four groups were stored at 15 °C, and samples in each group were sampled every day.

Monitor the total number of colonies in PSDW products

For culture-based microbiological analyses, 25 g of duck meat samples were aseptically removed and homogenized in 225 mL of sterile saline for 2 min using a Stomacher (Bag Mixer 400VW, Interscience Co.). Further decimal dilutions with the same diluent were made, and the total viable counts on Plate Count Agar (PCA, LuQiao Company, Beijing, China) were carried out on duplicate agar plates, which were then incubated at 37 °C for 48 h. Each experiment was performed in five repetitions.

Bacterial DNA extraction and PCR-DGGE analysis

30 mL of sample homogenate from “Monitor the total number of colonies in PSDW products” was centrifuged at 800 rpm for 2 min, and the supernatant was transferred to another sterile centrifuge tube, then a second centrifugation was performed at 12,000 rpm for 10 min. The sedimentation was immediately used for bacterial DNA extraction using the TIANamp Bacterial DNA Kit (Tiangen Biotech Beijing Co., Ltd., China) with some modifications. Finally, the concentration and purity of extracted DNA were confirmed by a UV–Vis Spectrophotometer (NANODROP 2000C, Thermo, America).

The V3-region of bacterial 16S rRNA genes for DGGE analysis was amplified by nested PCR as described by Jiang et al. (2011). The PCR products were visualized in a 1.2% agarose gel. DGGE analysis was performed on Dcode universal mutation detection system (Bio-Rad, USA) as previously described (Muyzer et al. 1993; Jiang et al. 2010) with the following modifications. The PCR products were loaded onto 8% polyacrylamide gels in 0.5× TAE, and the optimal electrophoresis experiments were performed at 60 °C by using gels containing a linear 32–47% denaturant gradient (100% corresponding to 7 mol/L urea and 40% acrylamide).

Table 1 Different parameters of four groups' samples

Group	Cover film ^a	Base film ^b	Natural spices ratio
1	General barrier film	General barrier film	1:2
2	General barrier film	High barrier film	1:1
3	High barrier film	High barrier film	1:2
4	High barrier film	General barrier film	1:1

^a High barrier cover film—smart packaging, active barrier for oxygen, PE/active barrier/PE/adhesives/OPET; general barrier cover film—PE/EVOH/PE/adhesives/OPP

^b General barrier base film—APET/adhesives/PE; high barrier base film—APET sheet

^c Natural spices ratio—weight ratio of pungent spices and fragrant spices

Electrophoresis was performed at a constant voltage of 200 V for 10 min, and then at 85 V for 14 h. After that, gels were dyed with Gelred dye for 10 min, and rinsed in ddH₂O, and photographed under UV light using the GelDoc 2000 system (Bio-Rad, USA) and QUANTITY ONE soft to view the image. The selected bands were excised from DGGE gel and eluted in DEPC water, and incubated overnight at 4 °C. Then the eluted DNA was re-amplified with the primer without the GC clamp. After the verification of agarose gel electrophoresis, the PCR products were sent to Invitrogen Company for sequencing. The sequences were compared with the GenBank database using the BLAST algorithm (www.ncbi.nlm.nih.gov/blast/).

Gas measure

Five packages in each group were chosen every day for the analysis of gas proportion. Oxygen and carbon dioxide concentrations were measured using a digital O₂ and CO₂ analyzer (OXYBABY 6 O₂/CO₂, WITT, Germany). A

syringe needle was inserted into each packaging box, and the proportions of O₂ and CO₂ were shown.

Results and discussion

Changes of gas proportion in pot-stewed duck wings during storage

As observed in Figs. 1 and 2, compared with two control groups, gas proportions in PSDW products have been changed, where O₂ percentage decreased to 0.1%, and CO₂ percentage increased to about 2.5% in four treatment groups. Yet the O₂ and CO₂ percentage in the control groups kept in the initial percentage, which were around 0.8 and 0.1%, respectively. It indicated that the aerobic microorganism in PSDW products were active at the beginning of storage, which depleted O₂ and produced CO₂ through bacterial metabolism. At the end of storage, the

Fig. 1 Change of O₂ percentage in PSDW packaging boxes during storage

Fig. 2 Change of CO₂ percentage in PSDW packaging boxes during storage

Fig. 3 Change of total bacterial counting in PSDW products during storage

increase of CO₂ percentage may be due to the metabolism of anaerobic microorganism. During the different treatment groups, the change trend of gas proportions was consistent, which could prove that these packaging materials with different cover films and base films had the same effect on the gas proportions in N₂-fulfilled PSDW packaging boxes. The gas-barrier properties of the packaging materials should be sufficient to maintain the desired gas composition (Al-Bachir 2005; Pastoriza and Bernardez 2011). However, the changes of gas proportions in PSDW packaging boxes in this study were mainly due to the activity of microorganism. Many studies also showed the consistent change of gas in food packaging (Tucker 2008; Gunes and Kirkin 2012; Chaix et al. 2015).

Change of bacterial numbers in pot-stewed duck wings during storage

The total bacterial numbers in different PSDW products were counted. As shown in Fig. 3, the number of the initial contaminated bacteria in PSDW products was about 2 log CFU/g, which began to grow in fourth days of storage. With the extension of storage time, the total bacterial count with approximate 5 log CFU/g was detected in the sixth days, which was up to the limitation of total numbers of colony in cooked meat products in the China National Standard GB 2726—2005 (80,000 CFU/g). Therefore, the shelf-life of these PSDW products was lower than 6 days during the 15 °C storage. Through the

Fig. 4 DGGE profiles of V3 regions of 16S rRNA bacterial gene from pot-stewed duck wings in four groups. 1–1 to 1–9 represented the sample at each day in Group 1; 2–1 to 2–9 represented the sample

at each day in Group 2; 3–1 to 3–9 represented the sample at each day in Group 3; 4–1 to 4–9 represented the sample at each day in Group 4

Table 2 DGGE bands identified in this study by means of 16S rRNA gene sequencing

Bands no.	Closest relative (s)	Length (bp)	Identity %	Accession no.
1	<i>Weissella confusa</i>	169	94	NR 113258.1
2	<i>Leuconostoc mesenteroides</i>	169	92	NR 118557.1
3	<i>Weissella diestrammenae</i>	195	100	NR 118386.1
4	<i>Weissella diestrammenae</i>	170	93	NR 118386.1
5	<i>Leuconostoc mesenteroides</i>	201	100	NR 118557.1
6	<i>Staphylococcus equorum</i>	196	100	NR 027520.1
7	<i>Phyllobacterium loti</i>	171	94	NR 133818.1
8	<i>Streptococcus parauberis</i>	170	98	NR 102798.1
9	<i>Leuconostoc pseudomesenteroides</i>	170	96	NR 109004.1
10	<i>Weissella thailandensis</i>	173	94	NR 040822.1
11	<i>Moraxella osloensis</i>	172	94	NR 113392.1
12	<i>Acinetobacter kyonggiensis</i>	170	98	NR 116714.1
13	<i>Lactobacillus graminis</i>	173	97	NR 042438.1
14	<i>Macrococcus caseolyticus</i>	190	100	NR 074941.1
15	<i>Streptococcus parauberis</i>	190	100	NR 102798.1
16	<i>Psychrobacter pulmonis</i>	181	100	NR 118026.1
17	<i>Pseudomonas monteilii</i>	184	100	NR 121767.1
18	<i>Staphylococcus saprophyticus</i>	171	91	NR 041324.1
19	<i>Psychrobacter cryohalolentis</i>	193	100	NR 075055.1
20	<i>Psychrobacter cryohalolentis</i>	184	100	NR 075055.1
21	<i>Streptococcus pasteurianus</i>	191	100	NR 102811.1
22	<i>Acinetobacter bereziniae</i>	180	100	NR 117625.1
23	<i>Weissella confusa</i>	172	99	NR 113258.1
24	<i>Weissella diestrammenae</i>	168	98	NR 118386.1
25	<i>Weissella confusa</i>	170	99	NR 113258.1
26	<i>Weissella diestrammenae</i>	173	99	NR 118386.1
27	<i>Leuconostoc mesenteroides</i>	169	94	NR 118557.1
28	<i>Staphylococcus equorum</i>	167	98	NR 027520.1
29	<i>Staphylococcus equorum</i>	168	98	NR 027520.1
30	<i>Propionivibrio dicarboxylicus</i>	169	92	NR 026477.1
31	<i>Propionivibrio dicarboxylicus</i>	172	100	NR 026477.1
32	<i>Acinetobacter kyonggiensis</i>	171	98	NR 116714.1
33	<i>Macrococcus caseolyticus</i>	172	98	NR 074941.1
34	<i>Streptococcus parauberis</i>	174	99	NR 043001.1
35	<i>Psychrobacter ciconiae</i>	171	99	NR 134796.1
36	<i>Psychrobacter cryohalolentis</i>	171	97	NR 075055.1
37	<i>Streptococcus lutetiensis</i>	182	100	NR 121743.1

comparison with the results of different treatment groups, there were no obvious differences in the total counting numbers. Recently, due to the need for gas- and water-barrier properties, high barrier cover film and base film have emerged (Pettersen et al. 2004), but its effect on microorganism in different food products was not confirmed. Simultaneously, many kinds of spices, which was classified as pungent spices and fragrant spices were used in PSDW products. For the pungent spices, it included pepper, ginger, Chinese prickly ash and onion. For the

fragrant spices, it included lilac, Chinese cinnamon, fennel, orange peel, anise and et al. (Wang et al. 2010). Both spices were made as the mixture, which may be the main factor for bacterial growth. This result showed that the packaging materials and natural spice ratio had little effect on the numbers of total bacterial counts, which may be indicated that the effect of other storage condition (such as 15 °C storage temperature) was far greater than that of packaging materials and natural spice ratio.

Shift of microbial community of pot-stewed duck wings in four groups during storage

Figure 4 and Table 2 shows the dynamics of the bacterial population of PSDW product found by PCR-DGGE profiles under different conditions during storage. A biodiversity was found in the initial stages of the storage, where *Streptococcus parauberis* (Band 15, 8, 21, 34) was present in all groups, and *Moraxella osloensis* (Band 11) and *Phyllobacterium loti* (Band 7) were shown in DGGE profile of Group 1 and Group 2, thus *Acinetobacter kyonggiensis* (Band 32) and *Propionivibrio dicarboxylicus* (Band 30 and 31) were shown in Group 3 and Group 4. After day 3 or day 4, however, changes of microbial community began to occur in all groups. Bands, including *Staphylococcus equorum* (Band 6, 28 and 29), *Weissella diestrammenae* (Band 26, 3, 4 and 24), *Weissella confusa* (Band 1, 23 and 25), *Leuconostoc mesenteroides* (Band 27, 5 and 2) and *Psychrobacter cryohalolentis* (Band 19, 20 and 36) were migrated to upper positions in DGGE profiles, and became dominant bacteria at the end of the monitoring in each group. Thus some bands (Band 15, 11, 30, 31, 7 and 34), disappeared or weakened at the end of storage. In previous related study, Yin (2008) analyzed the microbial community of vacuum-package pot-stewed duck neck using culture-dependent method and found that the dominate bacteria of this product were *Brochothrix thermosphacta*, *Enterobacteria*, *Pseudomonas* sp. and et al. However, it was not suitable for comparison. Duck neck may be contaminated by microorganism during the bloodletting in slaughter, and its bacterial community was different from that of duck wings, and Yin (2008) used traditional culture-dependent methods, which had certain limitation. Otherwise, the MAP type can also change the bacterial community. Besides, it was different among the bands of four groups, where bands in Group 3 and Group 4 after day 3 were relatively less than that of Group 1 and Group 2, and bacteria in Group 4 had the best inhibition effect (Table 2).

Conclusion

In conclusion, the shelf-life of PSDW products during 15 °C storage was recommended up to six days. Factors, such as the packaging films and spices ratio, didn't effectively control the lag phase and extend the shelf-life during 15 °C Storage. However, according to the analysis of microbial flora, *S. equorum*, *Weissella* sp., *L. mesenteroides* became the dominate bacteria of PSDW products at the end of storage, and high barrier cover film, general barrier base film and spice ratio 1:1, had a better inhibition effect on bacteria in PSDW products, which could be used

as the condition for PSDW storage. These results could monitor bacterial population dynamics in PSDW products, and provide theoretical basis for further extending the shelf life of this product.

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