Analysis on Microbial Flora Changes During Processing and Storage of Spiced Goose Based on Pcr-Dgge Combined with Conventional Microbial Culture Methods

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Abstract. During the processing and storage process of spiced goose, samples were collected from raw goose meat (R0), spiced goose meat cooled for 2 hours (2h) and spiced goose meat stored for 1day (1d), 3 days (3d) and 5 days (5d). Microbial flora changes in spiced goose during the processing and storage process were analyzed by conventional microbial culture methods, polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE). The results indicated that more abundant microbial diversity was determined in goose meat through PCR-DGGE technology than conventional microbial culture methods. According to the PCR-DGGE results, the dominant bacteria in R0 and 2h were *Aeromonas* and *Acinetobacter*. On 1d, the dominant bacteria also included *Weissella* and *Enterobacter*, and on 3d, *Aeromonas* was no longer dominant. The dominant bacteria became *Weissella*, *Enterococcus* and *Staphylococcus* on 5d.

1 Introduction

Rongchang Spiced White Goose is a geographical indication product of Rongchang District in Chongqing. Based on statistics, the daily consumption of spiced goose in Rongchang District is 8000-10000. Due to its delicious flavor, spiced goose meat is popular, meanwhile the rich nutrients, high protein content and low fat content in spiced goose meat make it an ideal meat product[1]. Endogenous and exogenous microbial contamination occurs during slaughtering of geese, cleaning of raw goose meat and cooling process of spiced goose, resulting in the existence of certain microorganisms in the processed spiced goose products. The initial microorganisms will result in decay and deterioration of spiced goose during processing and storage and have the worry of food security. Studying changes in the microbial flora during the processing and storage of spiced goose is helpful to understand the pollution source of initial microorganisms and the microbial community of spiced goose meat, and provide a theoretical basis for prolonging the expiration date of spiced goose.

So far there has been little research on the microorganism of spiced goose. Lijun et al. studied the major pollution microorganisms and growth law in spiced goose during the production, transport, and sales process using conventional microbial culture methods[2]. At the same time studies on microbial flora changes in other domestic spiced meat products during processing and sale also were carried out using conventional microbial culture methods [3-4]. The results obtained from these methods

are reliable and can be used to directly understand the dominant spoilage microorganisms in meat products. However, the results showed that more than 99% of the microorganisms in the natural environment were not able to be cultured in the laboratory. Therefore, utilizing traditional culture methods to analyze the composition of microbial flora in meat products is not comprehensive and can cause the loss of some unculturable microorganisms[5-6]. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is a molecular fingerprinting technique for investigating microbial community composition and their genetic relationships and is a powerful tool for bacterial classification and identification[7-10].that is able to efficiently, directly, quickly and comprehensively analyze microbial composition and community structure. This technique [11] can directly detect total bacterial DNA in a sample, determine the microbial species and abundance, as well as avoid the limitations of conventional microbial culture method in studying microorganisms that are hard to culture or uncultivable. However, this method can't distinguish the distribution of living bacteria or nonliving bacteria, and can only qualitative analyses the microflora changes of meat products. Therefore some of studies investigate the microbial diversity of products based on PCR-DGGE combined with conventional microbial-culture methods [12-13]. In this study, the conventional microbial culture method and PCR-DGGE technology also were employed to investigate the microbial diversity of spiced goose during processing and storage, so as to understand the pollution source of initial microorganisms and the microbial community of spiced goose meat then

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provide a theoretical basis for the preservation and to prolong the expiration date of spiced goose.

2 Materials and methods

2.1 Materials and reagents

A spiced goose processing enterprise and its sales outlets were selected as the sample source of this study. The raw goose and spiced goose products involved in the experiment were collected from the enterprise and the sales outlets.

The culture media used to detect the major pollution microorganisms during processing and storage process of spiced goose which listed in table 1 were: Plate count agar (PCA) for total aerobic bacteria, violet red bile agar (VRBA) for the Coliform, Baird-Parker agar and freeze-dried rabbit plasma for the *Staphylococcus aureus*, MRS medium, MC medium and MRS medium modified with mupirocin lithium salt for the Lactic acid bacteria, violet red bile glucose agar (VRBGA) for the Enterobacteriaceae, Bengal red agar for the mold and yeast, Pseudomonas CFC selective agar for the *Pseudomonas* and STAA agar for the Brochothrix thermosphact were purchased from Qingdao Hope Bio-Technology Co., Ltd. Agarose was brought from Oxoid (UK) and 2×Taq PCR MasterMix and DNA extraction kits were purchased from TIANGEN Biotech (Beijing) Co., Ltd. Proteinase K and urea were bought from Sigma (USA). Ammonium sulfate (APS), acrylamide (Acr) / methyl bis acrylamide (Bis) were from Bio-rad (USA). PCR primers were synthesized by BGI Tech Co., Ltd.

2.2 Instruments and equipment

Sterilization pot, Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory; GZX- GF101- II type electric heating constant temperature blast drying box, Shanghai Yuejin Medical Instrument Co., Ltd.; SW-CJ-1FD type purification workbench, Suzhou Purification Equipment Co., Ltd.; BPC-150F biochemical incubator, Shanghai Yiheng Scientific Instrument Co., Ltd. MyCycler™ Thermal Cycler PCR Instrument, GS 800 Gel Imaging Analysis System, D-code system and Horizontal Electrophoresis System, American Bio-rad Company; Vertical Low Temperature Refrigerator and Vertical Refrigerator, Qingdao Haier Group; High Speed Refrigerated Centrifuge, UK Thermo.

2.3 Experimental methods

2.3.1 Sample processing

Processing flow chart of spiced goose

Clean raw goose meat, spiced goose meat cooled for 2h, as well as spiced goose meat vacuum-packed to store at 15 °C for 1d, 3d and 5d from sales were collected to perform traditional microbial culture and PCR-DGGE analysis. Three independent geese of each group were sampled and tested.

2.3.2 Determination of the major pollution microorganisms during processing and storage process of spiced goose by conventional microbial culture method

25g samples were weighed under sterile conditions and pulverised in 225ml sterile saline. After vigorous shaking, 1ml sample was added into 9ml sterile saline to prepare 10⁻² dilution. Based on the previous procedures, 10x serial diluted sample solutions were prepared. Three sample solutions of the appropriate dilution were selected to perform colony counting according to the requirements in the standard which listed in table 1. The results obtained were statistically analyzed using excel.

2.3.3 Determination of microbial flora changes during processing and storage of spiced goose by PCR-DGGE

(1) DNA extraction

After each 9 g sample was cut into pieces, it was added to 91 mL sterile saline and homogenized for 10 min. Subsequently, the microbial solution was centrifuged at 1000×g for 3 min at 4°C, and the supernatant was placed in another sterilized centrifuge tube to centrifuge at 8000×g for 10 min at 4°C. The pellet was collected and DNA was extracted using a DNA extraction kit (Beijing Tiangen) according to the manufacturer's instructions. After DNA was detected by 2.0% agarose and when the target strip appeared, it was stored in a refrigerator at -20 °C for further use.

Table 1 Classification and culture conditions of microbial culture

(2) PCR amplification of bacterial 16S rDNA fragments

Table 1 Classification and conditions of bacterial culture

Microbial species	Medium	Culture condition	Reference standard
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Total number of colonies	PCA	36°C±1°C 48h	GB 4789.2-2010
Coliform	VRBA	36°C±1°C 24h	GB 4789.3-2010 Rule No. 2
<i>Staphylococcus aureus</i>	Baird-Parker agar, Freeze-dried rabbit plasma	36°C±1°C 48h	GB 4789.10- 2010 Rule No. 2
Lactic acid bacteria	MRS medium, MC medium, MRS medium modified with mopiroxin lithium salt	36°C±1°C 48h	GB 4789.35- 2010
Enterobacteriaceae	VRBGA	36°C±1°C 24h	GB 4789.41- 2016 Rule No. 1
Mold, yeast	Bengal red agar	28°C±1°C 5d	GB 4789.15- 2016 Rule No. 1
<i>Pseudomonas</i>	CFC agar	25°C±1°C 48h	SN/T 4044-2014
<i>Brochothrix thermosphacta</i>	STAA agar	30°C±1°C 48h	ISO 13722-1996

HDA1-GC (5'-C G C C C G G G G C G C G C C C C G G G C G G G G G C A C G G G G G A C T C C T A C G G G A G G C A G C A G T - 3') and HDA2(5'-G T A T T A C C G C G G C T G C T G G C A C - 3') were used as the primers and the front end of the HDA1-GC primer had a GC cap structure. PCR reaction conditions (25 µL): 2 × Taq MasterMix (CW BIO, Beijing) 12.5 µL, 1 µL of upstream and 1 µL of downstream primers (10 µmol/L), 1 µL of template DNA, and adding ddH₂O to achieve the final volume of 25 µL. A negative control group was designed. Reaction conditions were: 94 °C for 4 min; 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, 30 cycles; 72 °C for 10 min. The length, concentration of the PCR product fragment and the negative control were observed by 2.0% agarose gel electrophoresis. The PCR products were analyzed via denaturing gradient gel electrophoresis in DCode™ System electrophoresis apparatus, where the denaturation concentration ranged from 35% to 65%. After electrophoresis for 5 minutes at 200V, electrophoresis was carried out at 60 °C and 100 V for 16 h. The direction of electrophoresis was consistent with that of denaturation. After electrophoresis, staining was performed with silver nitrate staining, followed by imaging in a GS 800 gel imaging system (Bio-Rad, USA).

(3) Band recovery and sequencing

After PCR-DGGE staining, specific and non-specific DNA bands in each group were selected for DNA recovery and the DNA was recovered by polyacrylamide gel DNA recovery kit (Solarbio). The recovered fragments were free of GC clip primer to carry out PCR amplification according to the conditions in (2). When the PCR was complete, the DNA fragments were sequenced by Shanghai MAJOR BIO Co. Ltd after visualizing the fragments on a 2.0% agarose gel.

(4) Data analysis

All data were sorted and imported into Excel for preliminary processing. Cluster analysis was performed using NTSYS-pc V2.10 (Exeter software, USA) and one-way ANOVA analysis was carried out by SPSS 19.0 (SPSS Inc., USA). A curve of obtained data was drawn using GraphPad Prism 6.0 (GraphPad Software Inc., USA). The resulting sequences were subjected to nucleic acid sequence homology analysis on the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3 Results

3.1 Microbial flora changes of spiced goose during processing and storage analyzed by conventional microbial culture method

As shown in Fig. 1, the total bacteria in the raw goose meat R0 was 4.66 log CFU/g. Since the temperature was kept at about 100 °C for 1 h during the stewing process and most of the microorganisms in the raw goose meat were killed, no colonies were detected in spiced goose meat after it was cooled for 2 h (2 h). After 3d of storage, the total number of colonies was 6.83 log CFU/g, which surpassed the maximum limitation 5 log CFU/g in GB 2726 -2016(2016). On the fifth day of storage, the total number of colonies reached 9.79 log CFU/g.

According to Fig. 1, the spoilage organisms in the raw goose meat (R0) mainly included *Enterobacteriaceae*, *Pseudomonas*, *Lactic acid bacteria*, molds and yeasts, and the numbers of these microorganisms had no significant difference. No colonies were detected in the spiced goose after it had been cooled for 2 h (2h). Only *Lactic acid bacteria* were detected in spiced goose meat stored for 1 d at 2.35 log CFU/g. On the third day of storage, the dominant organisms were *Enterobacteriaceae*, *Lactic acid bacteria*, *Pseudomonas*, *Brochothrix thermosphacta*,

molds and yeast, and coliforms in order from most prevalent to least prevalent. On the fifth day of storage, the dominant organisms from most abundant to the least abundant were *Enterobacteriaceae*, *Pseudomonas*, Lactic acid bacteria, *coliforms*, *Brochothrix thermosphacta*, mold and yeast. During the storage of spiced goose, Lactic acid bacteria have always been the dominant bacteria, and due to the faster growth rate of *Pseudomonas* and *Enterobacteriaceae*, they finally developed into the dominant spoilage bacteria. The pathogen *Staphylococcus aureus* was not detected.

Fig.1. Microbial flora changes of spiced goose during processing and storage as determined by conventional microbial culture method

3.2 Microbial flora changes of spiced goose during processing and storage process analyzed by PCR-DGGE technology

3.2.1 PCR-DGGE gels map sequencing results of spiced goose during processing and storage

The PCR-DGGE detection results of bacteria in spiced goose during processing and storage are shown in Fig. 2. It can be seen that the repeatability among parallel samples was better, and the brightness of some bands was different. 37 main bands labeled in the profiles were recovered, cloned, and sequenced. As shown in Table 2, the sequence results were submitted to NCBI database for homology analysis. The results are shown in Table 1. Bands 1, 15, 27, 30, 31, 35 and 37 were *Aeromonas* sp., and strips 20, 24 belonged to *Acinetobacter* sp.; bands 33, 34, 36 were *Enterobacter* sp., and band 32 was *Salmonella* sp.; bands 3, 23, 28 were *Streptococcus* sp.; bands 4, 5, 7, 9, 10 and 13 belonged to *Weissella* sp.; band

8 was *Lactococcus* sp.; band 11 was *Leuconostoc* sp., and bands 14 and 26 were *Enterococcus* sp.; band 17 was *Carnobacterium* sp.; band 2 was *Oceanobacillus* sp., and bands 18, 29 belonged to *Staphylococcus* sp. Bands 21, 22, 25 were of the genus *Macroccoccus*; bands 6, 12, 16, 19 were uncultured bacteria.

Fig. 2. PCR-DGGE Profiles of microbial flora in spiced goose during processing and storage

3.2.2 Microbial flora changes of spiced goose during processing and storage process

As can be seen from Fig. 2, the microbial flora in raw goose meat (R0) was composed of *Aeromonas*, *Streptococcus*, *Acinetobacter* and *Macroccoccus*. Due to their brighter bands, *Streptococcus*, *Acinetobacter* and *Aeromonas* became the dominant bacteria. *Aeromonas* belongs to the Gram-negative bacteria[14] and since it was widely distributed in aquatic systems and soils, drinking water was a potential source of contamination. People can be infected mainly through consumption of water or food contaminated by the bacteria[15-16]. After cooking at a high temperature for a long time, bands 3, 23, 28 in raw goose meat R0 disappeared and the streptococci were inactivated, as they were not detected during the subsequent storage of the spiced goose. The microbial flora in spiced goose meat after 2-hour cooling (2h) consisted of *Aeromonas*, *Weissella*, *Acinetobacter*, *Macroccoccus* and *Oceanobacillus*, among which *Aeromonas* and *Acinetobacter* were the dominant bacteria. The positions of the bands in spiced goose stored for 1 day exhibited higher similarity to that after 2h, but the brightness of each band in the 1 d sample was higher. The dominant bacteria of 1d included *Weissella*, *Acinetobacter*, *Aeromonas* and *Enterobacter*. When stored for 3 days, the *Aeromonas* bands disappeared. At this time, the dominant bacteria were *Weissella*,

Carnobacterium, *Enterococcus* and *Enterobacter*. The bacterial composition in spiced goose meat stored for 5d included *Aeromonas*, *Weissella*, *Enterococcus*,

Staphylococcus, *Salmonella*, *Enterobacter*, and the dominant bacteria were *Weissella*, *Enterococcus* and *Staphylococcus*.

Table 2. Gene sequence homology analysis results of microbial DGGE strips in spiced goose during processing and storage

No.	Similar strains	ID	Similarity (%)
1	Uncultured Aeromonadaceae bacterium	LT695071	84%
2	Oceanobacillus senegalensis strain Marseille-P3587	NR_147387	95%
3	Streptococcus dysgalactiae strain 14I	MF113285	97%
4	Weissella cibaria strain AA10	JN851746	100%
5	Weissella halotolerans	LC064886	97%
6	Uncultured bacterium isolate DGGE gel band bst_L05_01	KU579293	98%
7	Weissella cibaria strain AA10	JN851746	99%
8	Lactococcus garvieae strain CIV.46	MF628997	97%
9	Weissella thailandensis strain CAU3107	MF429421	99%
10	Weissella confusa strain RCM3	KT626390	100%
11	Uncultured Leuconostoc sp. clone K-67-13-36	KJ804056	93%
12	Uncultured bacterium clone 418_OIs2-E2	JN884204	97%
13	Weissella paramesenteroides strain RC2	JN863617	100%
14	Enterococcus faecium strain ZGP-Efa.8	KM921921	99%
15	Aeromonas veronii strain ASH07	KU725740	100%
16	Uncultured bacterium clone GXTJ5A301AC0K3	KF323312	95%
17	Carnobacterium sp. UST050418-652	FJ596440	100%
18	Staphylococcus kloosii strain A15_iii	KP728024	96%
19	Uncultured bacterium clone SDSF_8F_e06	KC713055	97%
20	Acinetobacter sp. 796	KU670099	96%
21	Macroccoccus caseolyticus strain B03_06	KX681464	99%
22	Uncultured Macroccoccus sp. clone S5_E04	KP181835	85%
23	Streptococcus parauberis strain HFTC0135	MF574721	95%
24	Acinetobacter sp. strain MSRC26	MF924336	99%
25	Macroccoccus sp. IME1552 chromosome	CP017156	95%
26	Enterococcus ratti strain S3B	KX830982	95%
27	Aeromonas sp. NCCP-1748	LC270183	97%
28	Streptococcus dysgalactiae strain 14I	MF113285	92%
29	Staphylococcus epidermidis strain elva3	KU376392.1	96 %
30	Aeromonas sp. strain G3	MF101687	100%
31	Aeromonas sp. strain G3	MF101687	100%
32	Salmonella enterica subsp. arizonae	AB273736	99%
33	Enterobacter sp. strain GC4	MF928411	100%
34	Enterobacter sp. strain SA187	KX694407	99%
35	Aeromonas sp. strain G3	MF101687	100%
36	Enterobacter sp. strain MSRC72	MF924349	97%
37	Aeromonas veronii strain AVZ01	MF521598	100%

The dominant genus *Streptococcus* in raw goose meat was not detected after marinating and *Aeromonas* became the dominant bacteria in R0, 2h and 1d with the gradually declined content during storage process. *Weissella* started

to be detected from 2h and it was the dominant bacteria till the end of the storage. The result was consistent with that from the conventional microbial culture test. Through conventional microbial culture, *Lactobacillus* were

detected at 1d of storage, which were the dominant bacteria from 1d to 5d. *Weissella* is the dominant lactic acid bacteria in fermented foods such as white wine and soy sauce, which play a beneficial role in increasing the content of flavor substances and improving food safety during food fermentation[17-21]. *Weissella* can synthesize and release a class of polypeptides or precursor polypeptides with antibacterial activities, so as to effectively inhibit the growth of spoilage microorganisms and pathogenic bacteria [22]. *Weissella* in spiced goose can not only increase the flavor of goose meat, but also inhibit the growth of microorganisms. At the end of storage, the number of pathogenic bacteria such as *Enterococcus*, *Staphylococcus*, *Salmonella* and other pathogenic bacteria gradually increased and become dominant spoilage bacteria. Similar results are achieved by Keping Ye, which 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, PCR-DGGE analysis revealed that *Staphylococcus equorum*, *Weissella* sp., *Leuconostoc mesenteroides* became the dominating bacteria of PSDW at the end of storage[23].

3.2.3 Analysis on similarity and diversity of microbial flora structure in spiced goose during processing and storage

Based on the similarity of the PCR-DGGE profiles, a cluster analysis map was constructed to analyze the similarity of the microbial flora composition in spiced goose during processing and storage. The results are shown in Fig. 3. It can be seen that the similarity between two parallel samples were higher, while the lowest similarity was discovered in group 2h (83%) among three samples at the same sampling time point. The similarity in the other groups was greater than 90 %. The community structure of the R0 group presented significant differences with other groups, indicating that most of the microorganisms in the raw goose were killed during marinating. The microorganisms of spiced goose during the storage process mainly came from the environment in which the spiced goose was cooled and the microorganisms carried by the operators. The similarity of the colony structure between 2h and 1d was 76%, indicating that the microorganisms in the spiced goose had not multiplied. At 3d, the colony structure changed greatly compared with 2h and 1d, suggesting that the microorganisms began to multiply at this time. At 5d, the spiced goose was severely spoiled, and the similarity of colony structure between 3d and 5d was 69%.

Fig. 3. Colony DGGE cluster analysis of spiced goose during processing and storage

The Shannon index (H) was employed to evaluate the diversity of the microbial community. The larger the H, the more the spices and numbers of microorganisms. The Simpson's index (D) indicated the degree of uniformity of the microbial distribution. The richness index (S) equates to the number of microbial species. According to Table 3, during the processing and storage of spiced goose, the H and S of microorganisms increased during storage. The species and quantity of microorganisms in raw goose meat (R0) were the lowest, and the highest types and quantities of microorganisms were achieved at 5 d. The difference of H and S value was not significant between 2h and 1d. After high temperature cooking, the microorganisms carried in the raw goose were killed, and the microorganisms in the environment during the cooling did not start to multiply in large quantities. From 1d to 3d, the microorganisms began to multiply, and the number of previously dominant *Aeromonas* began to decrease remarkably due to inhibition by other dominant strains. At the same time, nutrients such as proteins in the goose were not degraded into available nutrients for microorganisms and the reproduction of other dominant bacteria had not reached the peak, and the H and S values were reduced at 3d. At 5d, the microorganisms multiplied and the number and types of microorganisms increased significantly, so the largest H and S values were achieved.

Table 3. Analysis on the microbial diversity of spiced goose during processing and storage

Grouping	Shannon index (H)	Simpson index (D)	Richness index (S)
R0	2.98±0.08 ^d	0.75±0.02 ^d	19.67±1.53 ^d
2h	3.28±0.10 ^{bc}	0.83±0.02 ^{bc}	26.67±2.52 ^{bc}
1d	3.37±0.03 ^{ab}	0.85±0.01 ^{ab}	29.00±1.00 ^{ab}
3d	3.22±0.04 ^c	0.81±0.01 ^c	25.00±1.00 ^c
5d	3.44±0.07 ^a	0.87±0.02 ^a	31.33±2.08 ^a

Note: The single factor sample analysis method was taken as the analysis method and the significant level was 0.05, n=3; the datas were expressed as mean±SD, and the different letters indicated the significant difference.

4 Conclusions

In this paper, the conventional microbial culture method and PCR-DGGE technology were combined to analyze

the microbial flora changes in spiced goose during processing and storage.

Through conventional microbial culture methods, the colonies in raw goose meat (R0) were 4.66 log CFU/g. The total number of colonies reached 9.79 log CFU/g at 5d. The dominant spoilage microorganisms in R0 were mainly Enterobacteriaceae, *Pseudomonas*, *Lactobacillus*, mold and yeast. The dominant spoilage organisms at 5d were Enterobacteriaceae, *Lactobacillus* and *Pseudomonas*.

Based on the results of PCR-DGGE detection, the number and types of microorganisms of spiced goose generally increased during processing and storage. The dominant bacteria of R0 and 2h were *Aeromonas* and *Acinetobacter*, while at 1d they were *Weissella*, *Acinetobacter*, *Aeromonas* and *Enterobacter*. *Weissella*, *Carnobacterium*, *Enterococcus* and *Enterobacter* became the dominant bacteria at 3d, and the dominant bacteria on 5d were *Weissella*, *Enterococcus* and *Staphylococcus*.

There was no microorganism was detected at 2 h using traditional microbial culture method, however *Aeromonas* and *Staphylococcus* were detected via PCR-DGGE technology. The reason is that PCR-DGGE technology can detect dead bacteria as well as live bacteria while traditional microbial culture method just detect live bacteria.

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