Analysis of Dominant Spoilage Bacteria in Beijing Sausages

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Abstract: Low temperature-heated meat products, which are popular among consumers because of their unique flavor, texture, and nutrition, have a relatively short shelf life due to the low sterilization temperature and are therefore prone to spoilage. The profile of bacteria associated with spoilage of Beijing sausages is here summarized through isolation, identification and spoilage microflora analysis. The main spoilage bacteria isolated and identified from Beijing sausages were one genus of *Pseudomonas sp.*, one genus of *Staphylococcus sp.* and two genera of *Brochothrix sp.* By denaturing gradient gel electrophoresis (PCR-DGGE) analysis, we identified 13 spoilage bacteria of Beijing sausages, of which *Pseudomonas sp.* was the most abundant.

1 INTRODUCTION

Beijing sausage is a low-temperature meat product which has the advantages of simple production technology, low sterilization temperature, slight protein denaturation, compact meat quality, elasticity, and delicious taste. However, due to the characteristics of the processing technology, the heating temperature of Beijing sausages fails to effectively sterilize the product, and the nutrition profile provides good conditions for the growth of microorganisms. Thus, Beijing sausages are prone to spoilage during storage, transportation, and sale, so the shelf life of these products is often short, greatly limiting the development of such products.

The key to extending the shelf life of meat products is to study the species and characteristics of the dominant bacteria involved in spoilage, and then select effective preservation measures. In recent years, the species and characteristics of spoilage bacteria in some meat products have been studied in domestic and foreign literature (Adams et al., 2007; Danilo et al., 2009; Doulgeraki et al., 2012; Liu et al., 2010; Peirson et al., 2003), collectively showing that the types of dominant bacteria involved in spoilage vary between meat products. Here, the bacterial distribution after spoilage in Beijing sausages was analyzed. The dominant bacteria causing the spoilage were isolated, purified, and identified to provide the theoretical basis for taking effective anti-spoilage measures and thus extending the shelf life of Beijing sausages.

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2 MATERIALS AND METHODS

2.1 Media

Nutrient Broth (NB) was made by dissolving 10 g peptone (Beijing Oboxing Biotechnology Co., Ltd, China), 3 g beef extract (Beijing Oboxing Biotechnology Co., Ltd, China), and 5 g sodium chloride in 1 L distilled water. pH was adjusted to 7.4 with 1 mol/L sodium hydroxide solution, then sterilized at 121 ºC for 15 min. Nutrient Agar (NA) consisted of NB with the addition of 1.5%-1.7% agar.

2.2 Samples and Sensory Observation

Beijing sausages produced on the same day were obtained from the Pengcheng food branch of BeijingShunxin Agricultural Limited Company, with the specification of weighing 350-400 g/piece. Ten Beijing sausages were put into an open homogeneous sack and stored at 4 ºC. The sensory indexes of the samples were observed and recorded in time during storage. According to the national standard SB/T 10481-2008 (Ministry of Commerce of the People's Republic of China, 2009), a sensory record table was set up. The results are shown in Table 1. After sensory evaluation, they were taken out for microbiological analysis.

Table 1: Sensory changes of Beijing sausages in storage.

	Storage days/d												
	1-3	4	5	6	7	8	9	10	11	12	13	14	15
Color	++++	++++	+++	+++	++	++	++	++	+	+	+	—	—
Texture	++++	+++	+++	++	++	++	++	+	+	+	+	—	—
Smell	++++	++++	+++	+++	+++	++	++	++	+	+	+	+	—

(Notes: ++++: very bright color, tight and resilient tissue, no mucus and mildew, no odor and rancidity, with meat flavor; +++: bright color, tight tissue, no mucus, and mildew, with meat flavor, no odor and rancidity; ++: dark color, whitened epidermis, soft tissue, no meat flavor; +: dark color, whitened epidermis, soft and inelastic tissue, mild rancidity odor; —: with white odor, juicy phenomenon, and rancid flavor.)

2.3 Analysis of the Dominant Spoilage Bacteria in Beijing Sausages

2.3.1 Sample Pretreatment

As shown in Table 1, when stored at 4 ºC, the samples had reached complete spoilage after 15 days. The spoiled sausages were evenly divided into two groups. Under sterile conditions, each sausage's epidermis and central part were evenly sampled with sterile scissors. The samples from each group were cut and mixed thoroughly.

2.3.2 Microbiological Culture

From each of the two mixed samples, 25 g was moved into the aseptic homogenization bag with filter membrane, 225 mL 0.85% sterile physiological saline solution added, and samples homogenized for 150 s by the beat nomogenizer. Next, 1 mL of filtrate was extracted with a pipettor, slowly injected into a test tube containing 9 mL sterile physiological saline solution along the tube wall, and shaken evenly on the oscillator to make 10^{-2} samples of homogenization solution. Finally, the required concentration was diluted 10 times in a turn, according to the estimation of sample contamination, 2-3 samples with the appropriate dilution were selected, and 100 μL of sample homogenate was injected into the surface of the medium by pipettor. Samples were allowed to set for 10 min then incubated at 36±1 ºC for 48 h.

2.3.3 Isolation, Purification, and Morphological Analysis of Dominant Spoilage Bacteria

Typical single colonies cultured on NA medium were selected and repeatedly streaked on the medium for separation and purification to obtain pure colonies. For the four isolated and purified individual colonies, colony morphology was observed and recorded, including shape, size, color, luster, transparency, edge shape, surface smoothness, wettability, and uplift degree. Single colonies were stained with safranin, and the morphology of the cells was observed by ZEISS Axioplan 2 optical microscope (10 times eyepiece × 100 times objective lens).

2.3.4 Identification of Dominant Spoilage Bacteria by 16S rDNA Sequence Analysis

The four strains purified as described above (numbered R1, R2, R3, R4) were inoculated in 5 mL corresponding medium and cultured at the appropriate temperature.

(1) Genomic DNA extraction: Briefly, bacterial solution (1.0 mL) was centrifuged at 13000 rpm for 2 min in a 1.5 mL centrifuge tube. The supernatant was discarded, the pellet transferred to a fresh tube, and 1.0 mL 0.85% NaCl was added. The tubes were then centrifuged at 13000 rpm for 2 min. The precipitate was suspended in 550 μL 1×TE. Then, 17 μL lysozyme (35 mg/mL), 3 μL proteinase K (20 mg/mL), and 30 μL 10% SDS were added, with 30 min incubation at 37 °C after each. 100 μL NaCl (5 mol/L) and 80 μL CTAB/NaCl solution were added and mixed well, and samples incubated at 65 °C in a water bath for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and samples mixed. After mixing, the tubes were centrifuged at 13000 rpm for 10 min at room temperature. The aqueous layer was transferred to a fresh tube, and 2 volumes of cold ethanol were added. The tubes were centrifuged at 13000 rpm for 10 min. The supernatant was discarded and placed at room temperature for 30 min to remove residual ethanol. DNA was dissolved in sterile water or TE.

(2) PCR amplification: Bacterial DNA extracted as described above was used as the template. PCR was performed with 16S rDNA universal primers (forward primer 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3', reverse primer 1541R: 5'-AAG GAG GTG ATC CAG CC-3').

Reaction conditions for PCR were as follows: 95 oC for 5 min; 30 cycles of 1 min at 95 °C, 1 min at 57 °C, and 1 min 20 s at 72 °C; 72 °C for 5 min.

The reaction mixture was 100 μL and consisted of 0.8 μL Taq (5 U/μL), 10 μL 10× PCR Buffer (Mg^{2+} Plus), 8 μL dNTP Mixture (2.5 mM/each), 2.5 ng template DNA, 2 μL each forward and reverse primers (10 μmol/L), and ddH_2O to 100 μL (ARa et al., 2006; Cheng et al., 2006).

(3) Sequence alignment: The PCR amplification products were purified and sequenced by Beijing Haocheng Mingtai Technology Co., Ltd. The 16S rDNA sequence of the strains with high homology were downloaded from NCBI and analyzed by MEGA 7 software.

(4) Phylogenetic analysis: Phylogenetic analysis was performed by MEGA 7.0.26 software. The phylogenetic tree was built using the neighbor-joining method and with 1000 bootstrapping replicates.

2.4 PCR-DGGE Analysis of Spoilage Bacteria in Beijing Sausages

Bacteria were identified using PCR denaturing gradient gel electrophoresis. Twenty-five grams of the pretreated sample described in Section 2.3.1 was packed in a sterile homogenization sack, added 225 mL 0.85% sterile physiological saline solution, and the sample homogenized for 150 s. Approximately 4 mL of sample was transferred to a sterile 5 mL centrifuge tube. After storage at -80 oC for 24 h, samples were sent to the Beijing Haocheng Mingtai Technology Co., Ltd for PCR-DGGE identification. The DGGE recovered bands were PCR amplified.

3 RESULTS

3.1 Identification Results and Analysis of 16S rDNA

Morphological characteristics of strains R1-R4 are shown in Table 2. The microscopic images of the safranin single dye are shown in Figure 1.

Table 2: Morphology characteristics of different strains.

Colonial morphology	R1	R2	R3	R4
Shape	circle	circle	circle	circle
Size	big	small	small	small
Color	light milky white	white	white	white
Luster	glossy	glossy	glossy	dim
Transparency	translucent	opaque	opaque	opaque
Edge shape	regular	regular	regular	regular
Sshirunshiruurface	smooth	smooth	smooth	smooth
Wettability	moist	dry	moist	moist
Uplift	raised	flat	raised	raised

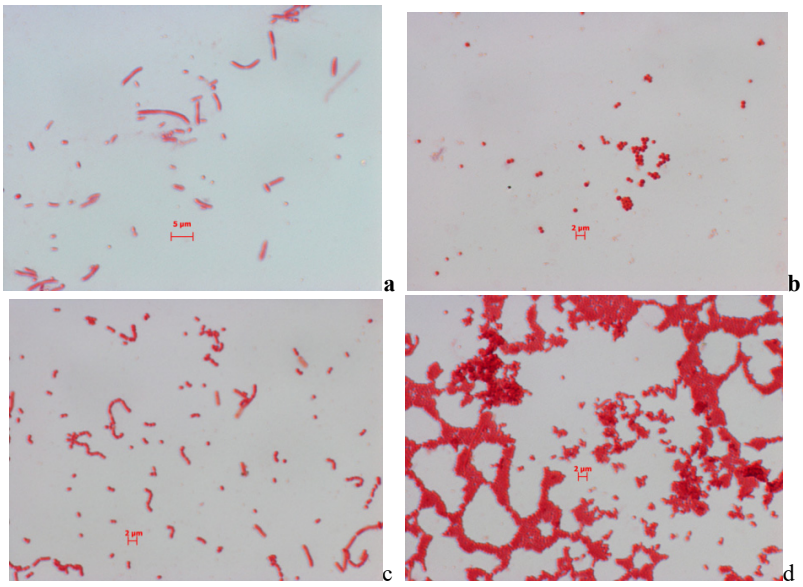

Figure 1: Microscope images of strains R1 (a), R2 (b), R3 (c) and R4 (d).

Four strains were isolated and purified for 16S rDNA sequence analysis. NCBI was used to identify the bacteria based on the sequences; analysis results are shown in Table 3.

Table 3: Result of NCBI sequence comparison analysis of four strains.

.	Strain label	Similarity	Population and login number of the strain with the highest similarity in GenBank	English name
1	R1	99.78%	*Pseudomonas lundensis* ATCC 49968(T)	*Pseudomonas sp.*
2	R2	100%	*Staphylococcus vitulinus ATCC51145(T)*	*Staphylococcus sp.*
3	R3	100%	*Brochothrix thermosphacta* ATCC 11509*(T)*	*Brochothrix sp.*
4	R4	100%	*Brochothrix thermosphacta* ATCC 11509*(T)*	*Brochothrix sp.*

3.2 Identification Results and Analysis of PCR-DGGE

The result of DNA extraction is shown in Figure 3. The result of PCR amplification is shown in Figure 4. DGGE patterns of bacterial DNA are shown in Figure 5. The primer was 16S rDNA corresponding to the universal primer 338F-534R, and the PCR product in the map was 250bp, which was judged as the target fragment. The PCR amplification results are shown in Figure 4.

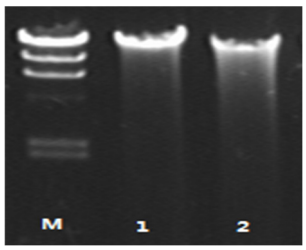

Figure. 3: Result of DNA extraction.

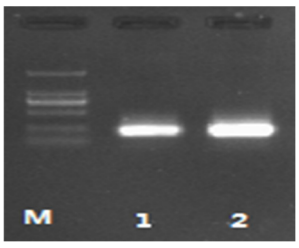

Figure. 4: Result of PCR amplification.

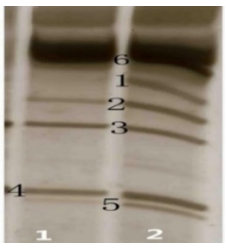

Figure. 5: DGGE patterns of bacterial DNA.

(Notes: The Arabic numerals in the figure indicate the number of the rubber recovery, with only one band cut at the same level. Due to the low brightness of band 6 in DGGE, the dominant bacteria were not considered, and the gray value analysis was not carried out.)

4 CONCLUSIONS

Two methods, microbial cultivation and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), were used to analyze the dominant spoilage bacteria in Beijing sausages. Using 16S rDNA sequence analysis, the main spoilage bacteria isolated and identified were one genus of Pseudomonas sp., one genus of Staphylococcus sp., and two genera of Brochothrix sp. PCR-DGGE analysis identified 13 species of bacteria, including Pseudomonas sp., Brochothrix sp., and Staphylococcus sp., with Pseudomonas sp. being the most dominant spoilage bacteria, followed by Brochothrix sp. and Staphylococcus. The results of the two methods are consistent with one another and with the results of other studies in the past two years identifying spoilage bacteria in low-temperature meat products (Samelis et al., 2000; Jenni et al., 2015).

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