APPLICATION OF HIGH PRESSURE TO INACTIVATE SPECIFIC SPOILAGE ORGANISMS OF COOKED HAM

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Abstract— **In this study, 16S rDNA DGGE fingerprinting was used to reveal the inhibitory effectiveness of high pressure processing (HPP, 400MPa or 600MPa for 10min at 22°C) targeted the specific spoilage organisms (SSOs) of sliced vacuum-packed cooked ham. Total bacterial RNA was directly extracted from cooked ham, followed by a two step RT-PCR protocol to amplify the V3 region of the 16S rDNA. Results by analysis of the population dynamic obtained from the DGGE profiles showed that, HPP affected differently the varoius species detected. SSOs of cooked ham such as *Lactobacillus sakei* and *Lactobacillus curvatus* were found to be very sensitive to pressure as they were unable to be detected in HPP samples during the entire period of refrigerated storage. *Leuconostoc mesenteroides* was completely inactivated by high pressure when treated at 600MPa. The most pressure-resistant bacteria was found to be *Weissella viridescens*, which induced the final spoilage of presurized cooked ham.**

Index Terms— **Cooked ham, High pressure processing, Specific spoilage organsims, RNA extraction**

I. INTRODUCTION

High pressure processing (HPP) is used in the microbiological stabilization of various food products. With HPP, the thermal damage induced by traditional methods of preservation can be avoid or limited (Carpi, Squarcina, Gola, Pedrielli, Bergamaschi and Rovere, 1999; Aymerich, Picouet and Monfort, 2008). Microorganisms are the major cause of spoilage of most cooked meat products. However, only a few species, the specific spoilage organisms (SSOs), give rise to the offensive off-flavours associated with meat spoilage (Gram and Dalgaard, 2002). Knowledge about the product's micro-flora and SSOs can ultimately be useful in microbiological inspections, shelf-life predictions and design of new preservation or production methods (Dalgaard, 1995). It is well documented that the most frequently isolated organisms from spoiled cooked meats are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Leuconostoc mesenteroides* (Dykes, Cloete and von Holy, 1995; Borch, Kant-Muermans and Blixt, 1996; Nychas, Skandamis, Tassou and Koutsoumanis, 2008; Hu, Zhou, Xu, Li and Han, 2009). A better understanding of the inactivation of SSOs by HPP may lead to improvements in application to meat preservation.

HPP may result in one population of microorganisms being killed, another population unaffected (non-injured) and a third population maybe sublethally injured (Wu, Fung, Kang and Thompson, 2001). Stressed or injured cells are not recovered in selective media and cannot be detected by culture-dependent methods (Wu, 2008). However, residual DNA templates from dead and injured microorganisms still exist in the samples, and therefore DNA-base culture-independent method could not distinguish active from dead organisms (Hoshino and Matsumoto, 2007). When culture-independent methods are used to study the microbial ecology, RNA-based methods are likely to yield more useful information on viable and metabolically active members of microbial communities *in situ* than DNA-based methods, due to the fact that rRNA synthesis and bacterial cell growth are closely related (Wagner, 1994). The aim of this study was to investigate the inhibitory effectiveness of HPP towards SSOs of sliced vacuum-packed cooked ham by using RNA-based DGGE methods.

II. MATERIALS AND METHODS

1. Preparation of cooked ham

Sliced cooked hams were prepared in a local meat factory according to the conventional techniques without addition of any preservative except for nitrite. The ham was made with pork meats, sodium chloride, pentasodium tripolyphosphate, sodium ascorbate, sodium glutamate, sucrose, flavoring additives, soya isolate protein, potato starch, nitrite and water. After vacuum packaging with polyamide (PA)/polyethylene (PE) membrane (oxygen permeability < 24 cm^3/m^2/day at 20°C, 120 μm thickness with PA/PE ratio 20/100), 300 packages (100 g sliced hams, per package) were stored at 4°C for sampling use.

2. High pressure treatment

Before high pressure processing, samples were vacuum packaged within another polyethylene membrane layer (Beijing Huadun Xuehua Plastic Group Co., Ltd, China) to prevent contamination from the high-pressure transmission fluid, an

oil, bis (2-ethylhexyl) sebacate (Li-Dong precision machinery company, Shenzhen, China). Sliced cooked hams were subjected to high pressure (52 Institute, Baotou, Neimeng, China) at 400MPa or 600MPa for 10min at room temperature (22°C). Pressure come-up times were approximately 1 min and 1.5 min for 400 and 600 MPa respectively. Pressures were released instantaneously.

3. Sample storage and examination

Immediately after high pressure processing, the outer package was removed, and then the HPP samples were stored at 4°C along with control (untreated samples, NT). Duplicate samples of hams were collected at time 0 (before HPP), 1, 15, 30, 60 and 90 days (after HPP) and were used for molecular analysis.

4. RNA extraction

Direct extraction of total bacterial RNA was made from samples at each sampling point. Ten g samples, in duplicate were homogenized in a stomacher tube with 50 mL of saline peptone water. The sediment was allowed to settle for 5 min and then 35 mL solution was transferred into another 50 mL sterile tube and centrifuged for 10 min at 4000 × g. Twenty 20 mL of the supernatant was aseptically transferred into a 50 mL sterile centrifuge tube and re-centrifugation was performed at 10000 × g for 20 min. The resulting pellet was transferred to a sterile 2 mL tube to which 1 mL RNAiso^{TM} Plus reagent (TaKaRa Biotechnology Dalian Co., Ltd. China) was added, followed by 200 µL of chloroform . Following incubation for 5 min, centrifugation was performed at 12000 × g for 15 min. The supernatant was transferred into a 1.5 mL RNase-free tube and 400 µl isopropanol was added, incubated at room temperature for 10 min and then re-centrifugated at 12000 × g for 10 min. The supernatant was decanted and 1 mL 75% ethanol was added to the pellet and then centrifuged at 12000 g for 5 min. The ethanol was removed and the RNAs were suspended in RNase Free dH_2O (TaKaRa) and 1 µl RNase Free DNase I (5 U/µl, TaKaRa) was added and incubated at 37°C for 20-30 min to digest DNA.

5. RT-PCR

RNA amplification was performed using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology Dalian Co., Ltd. China) according to the manufacturer's instruction with the following modifications. First strand cDNA was synthesized with 1 µl total RNA (100-300 ng). The primer used was Random 9 mers. Reactions were carried out in a Mastercycler ep cycler (Eppendorf, Germany), under the following conditions: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, 5°C for 5 min and cooling to 4°C. Then, cDNA was used for PCR using primers gc338f (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3'), spanning the V3 region of the 16S rDNA, as previously described by (Ampe, ben Omar, Moizan, Wacher and Guyot, 1999). The following PCR program was used: Initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 1 min, then cooling to 4°C. The PCR product (5 µl) was analyzed by electrophoresis in 1.2% agarose gel.

6. DGGE analysis and Sequencing

The Dcode™ Universal Mutation Detection System (BioRad, Richmond, CA) was used for DGGE analysis. Specific separations of PCR amplificons (approximately 230 bp) were performed according to the procedures described by (Muyzer, de Waal and Uitterlinden, 1993). The related strains in the marker used in present study were isolated from our samples, and identified by gram staining and atalase test belonging to lactic acid bacteria (LAB) group. Pure strains were also amplified using primers gc-338f and 518r as described above. Strain amplificons were mixed well and used for DGGE analysis.

DNA fragments to be nucleotide sequenced were excised with sterile scalpel and eluted in 20 µl sterile water. DNA was allowed to diffuse into the water at 4°C overnight. From the eluate 2-4 µl was used as a template and re-amplified with the primer without the GC clamp. Sequencing was performed at Invitrogen Sequencing Department (Invitrogen Company. Shanghai. China). The sequences recovered were aligned to 16S rDNA gene fragments available from the National Center for Biotechnology Information databases (NCBI), Searches in BLAST from GenBank were used to find the closest known relatives to the partial 16S rDNA sequences. Sequences with 99% or higher identity were considered to represent the same species. The fingerprints of the DGGE profile were analyzed using Quantity one 1D Analysis software version 4.6 (Bio-Rad, USA).

III. RESULTS AND DISCUSSION

Figure 1 shows the results of high pressure inactivation of SSOs in cooked ham. Table 1 shows the relative identification obtained by alignment in GenBank and accession numbers for the submitted sequences. At the beginning of the storage, a high microbial diversity in NT samples was observed, which is seen by the presence of multiple bands. In HPP treated samples, most bands disappeared. *Weissella viridescens* (band a) was detected, with intense bands, during the whole storage. *Leuconostoc mesenteroides* (band 4, 6) can be detected in the samples treated at 400MPa. When pressure was increased to 600MPa, it was completely inactivated. *Lactobacillus sakei* (band 7) and *Lactobacillus curvatus* (band 8) were not found in treated samples during the entire refrigerated storage. In our previous study (Hu et al., 2009), the SSOs of cooked ham were found to be *Lactobacillus sakei*, *Lactobacillus curvatus* and members of the genus *Leuconostoc* (*Leuconostoc mesenteroides* and uncultured *Leuconostoc*). The results presented here show that HPP is effective in inactivating the SSOs of sliced cooked ham, as already suggested by other authours (Diez, Urso, Rantsiou, Jaime, Rovira and Cocolin, 2008; Patterson, McKay, Connolly and Linton, 2009).

Concerning the bacterial ecology as determined by DGGE profiles, species of *Weissella viridescens* (band a),

Leuconostoc mesenteroides (band 4, 6), *Lactobacillus sakei* (band 7) and *Leuconostoc carnosum* (band 9, 10) made up the major group of spoilage bacteria developing on NT samples. However, after HPP at 600MPa, only *Weissella viridescens* (band a) still viable. The microbial diversity in HPP samples was reduced during the storage period. These results suggest that high pressure treatment is a suitable choice for minimising cross contamination problems in cooked meat products. We also conclude that high pressure treatment may lead to reduced microbial diversity and improve the products' safety.

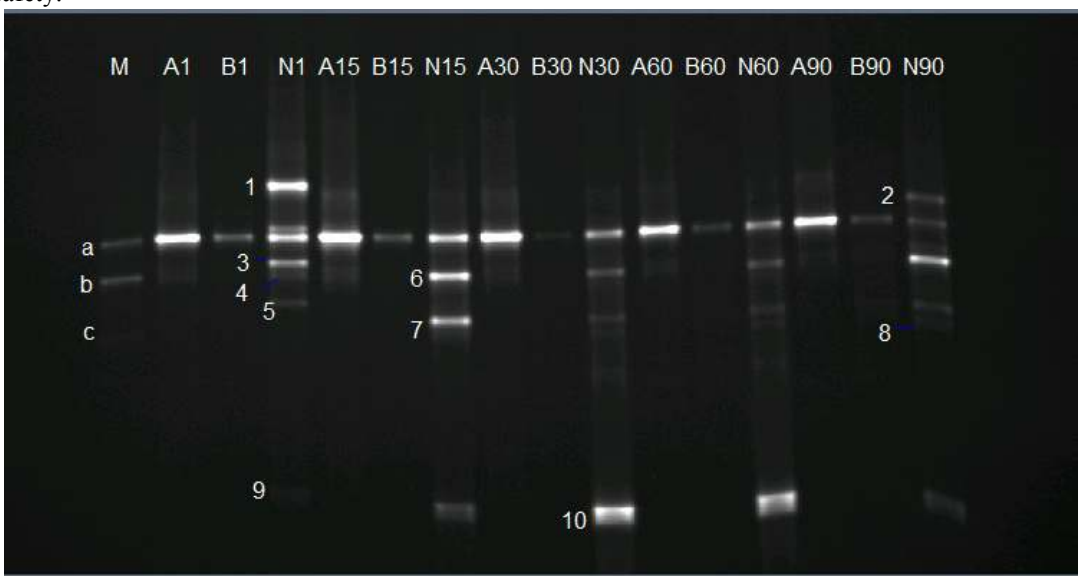

Fig. 1. RNA-DGGE profile of bacterial population in pressure treated and non-treated samples at storage time 1, 15, 30, 60 and 90 days.

M: marker; A: 400MPa/10min/22°C; B: 600MPa/10min/22°C; N: not treated

Table 1 Identification of the bands excised from DGGE gels

Isolate bands	Closest relatives	ID (%)	Accession No.
a	*Weissella viridescens*	100	GU363930
b	*Leuconostoc mesenteroides*	99	GU363932
c	*Lactobacillus sakei*	100	GU363935
1	*Lactococcus garvieae*	100	GU363927
2	*Lactobacillus plantarum*	100	GU363929
3	*Weissella cibaria*	100	GU363931
4	*Leuconostoc mesenteroides*	99	GU363932
5	*Weissella paramesenteroides*	99	GU363934
6	*Leuconostoc mesenteroides*	99	GU363932
7	*Lactobacillus sakei*	100	GU363935
8	*Lactobacillus curvatus*	100	GU363936
9	*Leuconostoc carnosum*	99	GU363937
10	*Leuconostoc carnosum*	99	GU363937

IV. CONCLUSION

An RNA-based DGGE approach clearly has potential for the analysis of active species that have survived in pressurized cooked ham. High pressure processing at 400 or 600MPa for 10min at room temperature (22°C) has a powerful inhibitory effect on specific spoilage organisms of sliced vacuum-packed cooked ham.

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