Meat Science 88 (2011) 682–688

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Effect of high pressure treatment on microbial populations of sliced vacuum-packed cooked ham

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ARTICLE INFO

Article history:
Received 31 May 2010
Received in revised form 24 February 2011
Accepted 28 February 2011

Keywords:
High hydrostatic pressure
DGGE
Weissella viridescens
Cooked ham

ABSTRACT

In this study, culture-dependent and culture-independent approaches were used to reveal the microbial diversity and dynamic changes occurring in sliced vacuum-packed cooked ham after high pressure processing (HPP, 400 MPa or 600 MPa for 10 min at 22 °C) during refrigerated storage over 90 days. Direct extraction of genome DNA and total RNA from meat samples, followed by PCR-denaturing gradient gel electrophoresis (DGGE) and RT-PCR-DGGE on 16S rDNA V3 region, was performed to define the structure of the bacterial populations and active species in pressurized cooked ham. Results showed that HPP affected differently the various species detected. The predominant spoilage organisms 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 at any time during refrigerated storage. *Weissella viridescens* and *Leuconostoc mesenteroides* survived HPP at 600 MPa for 10 min at 22 °C and were responsible for the final spoilage. 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 600 MPa for 10 min at room temperature (22 °C) has a powerful inhibitory effect on the major spoilage bacteria of sliced vacuum-packed cooked ham. High pressure treatment may lead to reduced microbial diversity and improve the products' safety.

1. Introduction

High pressure processing (HPP) offers an alternative preservation method for processed meat and meat products. Unlike other forms of treatment relying on convection or conduction, a major benefit of HPP is its immediate and uniform effect throughout the entire product (Norton & Sun 2008; Raso, Góngora-Nieto, Barbosa-Cánovas, & Swanson 1998). When compared to thermal treatments, HPP causes little or no effects on nutritional or quality parameters, but micro-organisms can be inactivated at higher pressures (Cheftel, 1995).

Recent studies have clearly indicated that most vegetative micro-organisms in meat samples are largely inactivated at pressures of 400–600 MPa for several minutes at room temperature, especially when the initial microbial load of the products is very low (Garriga, Grèbol, Aymerich, Monfort, & Hugas 2004; Jofré, Aymerich, Grèbol, & Garriga 2009; Patterson, McKay, Connolly, & Linton 2010; Smith, Mendonca, & Jung 2009). However, it is also well documented for example, that when HPP is used in improving safety and prolonging the shelf life of meat and meat products, a large number of sub-lethally injured micro-organisms can revive and become fully functional in a favorable environment during subsequent storage (Bozoglu, Alpas, & Kaletunç 2004; Koseki, Mizuno, & Yamamoto 2008; Wu 2008; Yuste et al. 2003). This may cause problems to the safety and shorten the shelf-life of meat. Injured micro-organisms are not recovered, or could not be detected, in selective media, and cells present in low numbers are quite often inhibited by microbial population numerically more abundant (Hugenholtz, Goebel, & Pace 1998; Koseki et al. 2008; Wu & Fung 2001; Yuste et al. 2003). For these reasons, it is crucial to have tools that allow characterization of the predominant bacteria, and monitoring of the microbial populations without cultivation. This goal can be achieved by culture-independent methods such as PCR-denaturing gradient gel electrophoresis (DGGE).

Recently, several studies focusing on the soil environment suggested that direct DNA extraction did not accurately reflect environmental changes. Instead analyses targeting rRNA, in addition to, or instead of rDNA, were believed to be more suitable since metabolically active cells generally contain higher levels of intracellular 16S rRNA (Hoshino & Matsumoto, 2007). Other studies on bacteria from environmentally-contaminated soils also found that whilst some differences in physiological characteristics were evident, DNA-based bacterial community fingerprints failed to show any clear differences, and culturable bacterial numbers, and/or microbial biomass did not change (Duineveld, Kowalchuk, Keijzer, van Elsas, & van Veen 2001; Ellis, Morgan, Weightman, & Fry 2003; Engelen et al. 1998). By using

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doi:10.1016/j.meatsci.2011.02.029

DNA as a molecular marker, it is possible to determine the presence or absence of a particular bacterial species in the sample, but it is impossible to assure that whether these bacteria are viable (Diez et al., 2008). Where culture-independent methods have been used to study microbial ecology, RNA-based methods yielded more useful information on viable and metabolically active microbial populations *in situ* than did DNA-based methods, due to the fact that rRNA synthesis and bacterial cell growth are closely related (Wagner, 1994).

In this work, we investigated the use of denaturing gradient gel electrophoresis (DGGE) analysis of total RNA and genome DNA of bacteria in control and pressure-treated cooked ham. In addition, we used a culture-dependent method to isolate and identify the representative spoilage bacteria present following treatment.

2. Materials and methods

2.1. Preparation of cooked-smoked pork shoulder (ham)

Sliced cooked ham meats were prepared in a local meat factory using pork shoulder meat (commonly known as picnic or shoulder hams), according to conventional techniques without addition of any preservatives except for nitrite. Pork shoulder muscles were stripped of fat and mixed with the following additives (in g/Kg): salt, 23; pentasodium tripolyphosphate, 5; sodium ascorbate, 0.6; sodium glutamate, 2.5; sucrose, 12; flavoring additives, 2.25; soya isolate protein, 5; potato starch, 40; nitrite, 0.15 and water, 200. The raw materials were cured for 16 h at 4 °C under vacuum, and packed into an artificial casing and cooked until the core temperature reached 72 °C and then smoked for 2 h. After cooling by immersion in cold water, the products were maintained at 4 °C overnight, the hams were then aseptically sliced into 0.5 cm slices (Hu, Zhou, Xu, Li, & Han, 2009). 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.

2.2. High pressure treatment

Before high pressure processing, samples were vacuum packed within another polyethylene membrane layer (Beijing Huadun Xuehua Plastic Group Co., Ltd, China) to prevent contamination from the high-pressure transmission fluid (bis (2-ethylhexyl) sebacate, Li-Dong Precision Machinery Company, Shenzhen, China). Sliced cooked hams were subjected to high pressure in a 2 L vessel (52 Institute, Baotou, Neimeng, China) at 400 MPa or 600 MPa for 10 min at room temperature (22 °C). The pressure level and time of pressurization were controlled by a computer program (BTNMC for HPP Control 1.0). The temperature of high pressure transmission fluid inside the pressure chamber during pressurization was monitored through a K-type thermocouple. Pressure holding time reported in present study does not include pressure come-up or release times. The pressure come-up rate was 350 MPa/min and pressures were released instantaneously. The temperature increase in the compression fluid due to adiabatic heating in the HPP chamber was less than 2 °C/200 MPa.

2.3. 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 controls (non-treated samples, NT). Duplicate samples of hams collected at time 0 (before HPP), 1 (after HPP), 30 and 90 days were used for microbiological and molecular biology analysis.

2.4. pH measurements

Ten gram portions of each ham sample (two replicates) was homogenized in 10 mL distilled water for 2 min, and used for pH determination (Microprocessor pH meter, Hanna HI9025c, Portugal).

2.5. Microbiological analysis and pure cultures isolation

Twenty five gram of each sample (two replicates) was aseptically taken and homogenized in 225 mL of sterile peptone saline (1 g of peptone and 8.5 g of NaCl per liter). After shaking at 200 oscillations/m for 10 min in a stomacher, the suspension was serially diluted in triplicate (1:10) in peptone saline. Serial dilutions were plated onto appropriate culture media and the following microbiological analyses were carried out on HPP and NT samples (Table 1).

For the samples treated with HPP of 600 MPa for 10 min at 22 °C, immediately after counting, colonies developed on MRS medium were isolated and streaked three or more times to obtain the pure cultures. Pure cultures were initially selected and regrouped by their macroscopic, microscopic morphotype, Gram staining as well as using a catalase test. Representative isolates were then grown in MRS broth at 30 °C. Cells at the stationary phase (cultured for 12–16 h) were collected by centrifugation at $10000\times g$ for 2 min at 4 °C for further extraction of DNA, followed by DGGE analyses (Hu et al., 2009).

2.6. DNA extraction from pure culture isolates

For DNA extraction from cultures and isolates, 1 mL cultivated cells in duplicate were centrifuged at $10000\times g$ for 10 min (4 °C). The pellet was resuspended in 180 μL of lysis buffer (20 mM Tris, pH 8.0; 2 mM Na_2-EDTA; 1.2% Triton and 20 mg/mL lysozyme). Bacterial DNA was then extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd, China) according to the protocol, with purification of genomic DNA provided in the manufacturer's instructions. Finally, DNA was eluted with TE buffer (Tiangen) and stored at −20 °C.

2.7. Nucleic acids extraction directly from meat samples

Direct extraction of total bacterial nucleic acids from samples at each sampling point was undertaken as follows: 20 g each sample, in duplicate, was homogenized in a stomacher tube with 80 mL of saline peptone water and shaken for 30 min at 4 °C. The sediment was allowed to settle for 5 min, and then two 35 mL aliquots were transferred into two 50 mL sterile tubes, one for DNA and another for RNA extraction.

Table 1
Media and incubation conditions used in present study.

Microbe Species	Plate count media	Purchase co.	Incubation
Aerobic plate count	PCA[a]	Land Bridge(Beijing)	37 °C/48 h
Psychrotrophs count	PCA	Land Bridge(Beijing)	7 °C/10 d
Lactic acid bacteria	MRS[b]	Oxoid(England)	Double layer, 30 °C/48 h
Enterobacteriaceae	VRBDA[c]	Land Bridge(Beijing)	Double layer, 37 °C/24 h
Staphylococci	BP[d]	Land Bridge(Beijing)	37 °C/48 h
Brochothrix thermosphacta	STAA[e]	Oxoid(England)	25 °C/48 h
Pseudomonads	CFC[f]	Oxoid(England)	25 °C/48 h
Yeasts and Moulds	PDA[g]	Land Bridge(Beijing)	25 °C/5 d

[a] Plate Count Agar.
[b] de Man, Rogosa, Sharpe agar.
[c] Violet Red Bile Dextrose Agar.
[d] Baird-Parker Agar.
[e] Streptomycin Thallus Acetate Agar, with STA selective supplement without actidione.
[f] Cetrimide–Fucidin–Cephaloridine Agar.
[g] Potato Dextrose Agar.

Each of the two tubes was centrifuged (Avanti J-E, Beckman Coulter, American) for 10 min at 4000×*g* (4 °C). The supernatant (20 mL) was aseptically transferred into a 50 mL sterile centrifuge tube, and re-centrifugation was performed at 10,000×*g* for 20 min (4 °C). The pellet was transferred to a sterile 2 mL tube and then stored at −80 °C for nucleic acids extraction.

Bacterial DNA was extracted according to the protocol described above. Finally, DNA was eluted with TE buffer (Tiangen) and stored at −20 °C.

For RNA extraction, 1 mL RNAiso™ Plus reagent (TaKaRa Biotechnology Dalian Co., Ltd. China) was added into the tubes containing the bacterial cells and mixed immediately by vortexing for 2 min, and then incubated for 5 min at room temperature (15–25 °C). Then 200 μL of chloroform was added and mixed well. After extraction for 5 min, tubes were centrifuged at 12,000×*g* for 15 min at 4 °C. The supernatant was transferred into a 1.5 mL RNase-free tube and 400 μL isopropanol was added and then incubated at room temperature for 10 min prior to, re-centrifugation 12,000×*g* for 10 min (4 °C). The supernatant was removed and 1 mL 75% ethanol was added and the tube inverted several times and centrifuged at 12,000×*g* for 5 min (4 °C). The supernatant was decanted and residual supernatant was removed by gently dabbing the inverted tube once onto a paper towel. RNAs were suspended with 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. Purified RNAs were suspended with RNase Free dH_2O and stored at −80 °C.

2.8. 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. Firstly, 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), using 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. Following that, 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 (Ampe, ben Omar, Moizan, Wacher, & 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.

2.9. PCR

PCR was used for 16S rDNA-V3 amplification. Reactions were carried out in a final volume of 25 μL containing 20 pM primer pairs gc338f and 518r, each 0.25 μL, GoTaq Green Master Mix (Promega, USA) 12.5 μL, DNA template 1 μL (100–200 ng) and Nuclease-Free Water 11 μL. The template DNA was denatured for 5 min at 94 °C. To increase the specificity of the amplification and reduce the formation of spurious byproducts, a "touchdown" PCR was carried out. The initial annealing temperature of 65 °C was 10 °C above the expected annealing temperature and decreased 0.5 °C every second cycle until the touchdown temperature of 55 °C was reached whereupon, 15 additional cycles were carried out at 55 °C. A denaturation step of 94 °C for 1 min was used and extension was carried out at 72 °C for 3 min with a final extension of 10 min at 72 °C which completed the amplification cycle, and was then cooled to 4 °C. The PCR product (5 μL) was analyzed by electrophoresis in 1.2% agarose gel.

2.10. DGGE analysis

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, & Uitterlinden, 1993), with the following modifications according to the work of Hu et al. (2009). 7–8 μL PCR samples were loaded onto 8% (wt./vol.) polyacrylamide gels (acrylamide/bisacrylamide = 37.5:1) in 0.5× TAE with a 35–55% gradient of urea and formamide increasing in the direction of parallel electrophoresis. A 100% denaturant acrylamide was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 10 min at 200 V and then for 16 h at 85 V. Gels were stained with 0.5× TAE buffer containing ethidium bromide (0.5 mg/L) for 20 min, then rinsed twice for 10 min in milli-Q water and photographed under UV transillumination with the GelDoc 2000 system (BioRad).

The related strains in the marker used in present study were selected from our laboratory which were isolated from pork meat, and identified by Gram staining and catalase test belonging to lactic acid bacteria (LAB) group. Strains were identified by amplification using primers P1 and P4 as described by Klijn, Weerkamp, and de Voss (1991), targeting 700 bp of the V1–V3 region of the 16S rDNA. After purification, products were sent to a commercial facility for sequencing (Invitrogen Sequencing Department, Shanghai, China). Sequences were aligned in GenBank using the Blast program to determine the closest known relatives of the partial 16S rDNA sequence obtained. After identification, strains were amplified using PCR protocol with primers gc-338f and 518r as described above. Strain amplicons were mixed well and used as markers for DGGE analysis (Table 2).

2.11. Sequencing of DGGE bands

DNA fragments for nucleotide sequencing were excised with a sterile scalpel and eluted in 20 μL sterile water. DNA was allowed to diffuse into the water at 4 °C overnight. From the eluted DNA 2–4 μL was used as a template and re-amplified with the primers without the GC clamp. The PCR protocol used was described above. Sequencing was performed at Invitrogen Sequencing Department (Invitrogen Company, Shanghai, China). The partial sequencing was performed using the forward primer 338f without the GC clamp and 518r. The sequences recovered were aligned to 16S rDNA gene fragments available from the National Center for Biotechnology Information databases (NCBI) using searches in BLAST from GenBank 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.

2.12. Statistical analyses

Data were statistically analysed using one-way analysis of variance (ANOVA), and means were separated by Duncan's multiple-range test at 5% level. Data analyses were conducted using SAS 8.12 (SAS Inst. Inc., Cary, NC, 2001). The fingerprints of the DGGE profile were analyzed using Quantity one 1D Analysis software version 4.5 (Bio-Rad, USA).

Table 2
DGGE marker used in present study.

Bands	Closest relatives	Accession no.
a	*Weissella viridescens*	EU621989
b	*Weissella cibaria*	EU621988
c	*Leuconostoc mesenteroides*	EU621987
d	*Lactobacillus sakei*	EU621985
e	*Lactobacillus curvatus*	EU621984
f	*Carnobacterium divergens*	EU621983
g	*Lactococcus lactis* subsp. *lactis*	EU621982

3. Results

3.1. Meat pH and bacterial enumeration

The results of pH measurements are shown in Fig. 1. Initial pH values (before HPP) were above 6.38 for all samples (data not shown). After HPP, at day 1 of refrigerated storage, the mean pH value of samples decreased to 5.92, 6.28 and 6.37 for NT, high pressure treated at 400 MPa and 600 MPa samples, respectively. NT samples were characterized by a drop in pH values from above 6.38 (day 0) to just above 5 (day 90) (Fig. 1). In the case of samples treated at 600 MPa, no significant differences were observed during the first 30 days. Higher pH was observed in samples treated at 600 MPa, being significantly different from those at 400 MPa at the same sampling day during the whole storage period (Fig. 1).

Table 3 shows the microbial evolution of sliced cooked ham during refrigerated storage. During the entire storage period, LAB constituted the main flora in both treated and untreated samples. After HPP, a significant decrease was observed in LAB counts, both in samples treated at 400 MPa and 600 MPa. *Staphylococci* showed some resistance to pressure. The mean number of *Staphylococci* decreased from an initial value of 3.04 $\log_{10}$CFU/g to 1.90 $\log_{10}$CFU/g and then below the detection level (<1) immediately after high pressure treatment of 400 MPa and 600 MPa, respectively. Other microorganisms, such as *Enterobacteriaceae*, *Brochothrix thermosphacta*, Pseudomonads, and yeasts and moulds were only detected in NT samples.

3.2. Identification of the isolates by DGGE analysis and sequencing

A total of 84 colonies isolated from MRS media of HPP samples treated at 600 MPa at each sampling point were analyzed by conventional microbiology analysis. Finally, 18 isolates were selected by culture dependent analysis of bacterial populations (macroscopic, microscopic morphotypes, as well as catalase test). Each isolate exhibited on unique band in DGGE profile. However, only 2 different co-migrations exhibited, accounted for 18 unique bands. When all these 18 unique bands were excised, re-amplified and sequenced, they represented 2 different species. Sequences of 14 pure cultures were most similar to *Weissella viridescens*, whereas the other 4 were similar to *Leuconostoc mesenteroides*.

3.3. Direct analysis of sliced cooked ham by RNA-combined with DNA-DGGE

RNA- and DNA-DGGE profiles of direct analysis of sliced cooked ham are shown in Figs. 2 and 3. A high microbial diversity at the commencement of the storage was observed, as indicated by the presence of multiple bands in NT samples (Fig. 2). However, the microbial diversity of HPP samples was extremely simple, with only 1

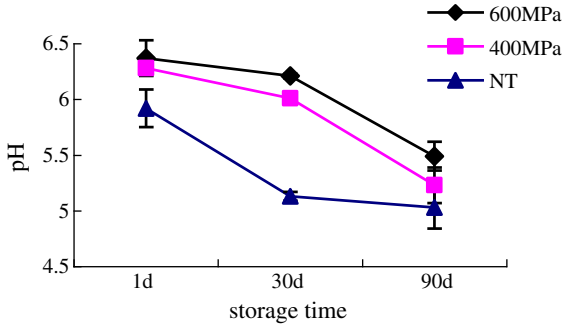

Fig. 1. Changes of in pH values ofin sliced vacuum-packaged cooked ham during storage at 4 °C. NT: not treated; and 400 MPa and 600 MPa: high pressure treated at 400 MPa and 600 MPa for 10 min at 22 °C, respectively.

Table 3
Microbial evolution in sliced vacuum-packed cooked ham during storage at 4 °C.

Time (days)	NT	400 MPa	600 MPa	NT	400 MPa	600 MPa	NT	400 MPa	600 MPa	NT	400 MPa	600 MPa
	Aerobic plate count			Psychrotrophs count			Lactic acid bacteria			*Enterobacteriaceae*		
0	4.70 ± 0.23^{b}	4.70 ± 0.23^{c}	4.70 ± 0.23^{b}	4.90 ± 0.04^{c}	4.90 ± 0.04^{c}	4.90 ± 0.04^{b}	4.65 ± 0.24^{c}	4.65 ± 0.24^{c}	4.65 ± 0.24^{b}	1.60 ± 0.36	1.60 ± 0.36	1.60 ± 0.36
After HPP	NA	$_{A}3.58 \pm 0.16^{d}$	$_{B}2.48 \pm 0.09^{c}$	NA	$_{A}3.64 \pm 0.09^{d}$	$_{B}2.17 \pm 0.41^{c}$	NA	$_{A}3.66 \pm 0.08^{d}$	$_{B}2.81 \pm 0.04^{c}$	NA	<1	<1
30	$_{A}7.66 \pm 0.31^{a}$	$_{B}6.37 \pm 0.33^{b}$	$_{C}4.92 \pm 0.31^{b}$	$_{A}8.53 \pm 0.22^{a}$	$_{B}6.93 \pm 0.16^{b}$	$_{C}5.01 \pm 0.33^{b}$	$_{A}8.72 \pm 0.08^{a}$	$_{B}6.06 \pm 0.13^{b}$	$_{C}5.53 \pm 0.26^{b}$	<1	<1	<1
90	$_{B}7.28 \pm 0.05^{a}$	$_{A}8.33 \pm 0.41^{a}$	$_{A}8.76 \pm 0.11^{a}$	$_{A}8.30 \pm 0.08^{a}$	$_{A}8.06 \pm 0.21^{a}$	$_{A}8.59 \pm 0.11^{a}$	$_{B}7.27 \pm 0.05^{b}$	$_{A}8.16 \pm 0.09^{a}$	$_{A}8.71 \pm 0.52^{a}$	<1	<1	<1
	Staphylococci			*Brochothrix thermosphacta*			Pseudomonads			Yeasts and Moulds		
0	3.04 ± 0.47	3.04 ± 0.47^{a}	3.04 ± 0.47	4.31 ± 0.17	4.31 ± 0.17	4.31 ± 0.17	2.59 ± 0.41	2.59 ± 0.41	2.59 ± 0.41	1.58 ± 0.51	1.58 ± 0.51	1.58 ± 0.51
After HPP	NA	1.90 ± 0.31^{b}	<1	NA	<1	<1	NA	<1	<1	NA	<1	<1
30	<2	<2	<1	<2	<2	<1	<2	<2	<1	<2	<2	<1
90	<2	<2	<1	<2	<2	<1	<2	<2	<1	<2	<2	<1

NT: nontreated; 400 MPa and 600 MPa: high pressure treated at 400 MPa and 600 MPa for 10 min at 22 °C, respectively; NA: nonapplicable.
Values are mean of triplicate ± standard deviation.
Data are expressed in $\log_{10}$ CFU/g where "<1" and "<2" refer to there are no clone at 10^{1} and 10^{2} dilution, respectively.
a–d: averages with different letters in the same column are different ($p<0.05$).
A–B: averages with different letters in the same row for each microorganism group are different ($p<0.05$).

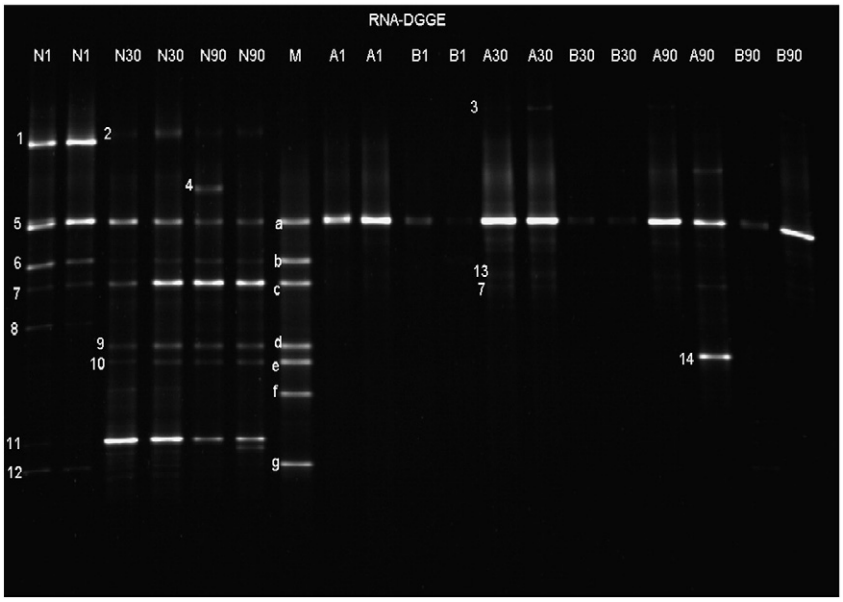

Fig. 2. RNA-DGGE profile of bacterial community populations in pressure treated and non-treated samples at storage time 1, 30 and 90 days. M: marker; A: 400 MPa/10 min/22 °C; B: 600 MPa/10 min/22 °C; and N: not treated.

or 3 bands present during the various storage periods (Fig. 2). Table 4 shows the identification results of the DGGE bands by 16S rRNA gene sequencing. It is obvious that HPP significantly inactivated spoilage species including *Lactococcus garvieae* (Fig. 2. band 1), *Weissella cibaria* (Fig. 2. band 6), *Lactobacillus sakei* (Fig. 2. band 9), *Lactobacillus curvatus* (Fig. 2. band 10) and *Leuconostoc carnosum* (Fig. 2. band 11). In the samples treated at 400 MPa, *Weissella minor* (Fig. 2. band 3), *Leuconostoc mesenteroides* (Fig. 2. band 7) and uncultured *Weissella sp.* (Fig. 2. band 13) appeared from day 30 and remained until the end of storage, whereas *Lactobacillus coryniformis* (Fig. 2. band 14) only appeared at the end of the storage (90 day). During the entire period of storage, *Weissella viridescens* (Fig. 2. band 5) was present even in the samples treated at 600 MPa.

The major species of bacteria present were detected both by RNA-based and DNA-based DGGE analysis (Figs. 2 and 3). A higher microbial diversity was observed in RNA-DGGE profile than in DNA-DGGE profile whether at the beginning or during the entire refrigerated storage period in NT samples. *W. cibaria* (Fig. 2. band 6) only appeared in RNA-DGGE profile during storage at day 30 and day 90. It is interesting that *L. curvatus* (Fig. 3. band H) was only detected by DNA-based DGGE method in both treated and non-treated samples, whereas *Streptococcus* sp. (Fig. 3. band I) only appeared in pressurized samples in DNA-DGGE profile during the whole refrigerated storage.

4. Discussion

Cooked ham is a highly nutritious food, which spoils quickly with cross contamination after heat processing. The higher the initial microbial load on the meat the shorter the shelf-life (Holley, 1997).

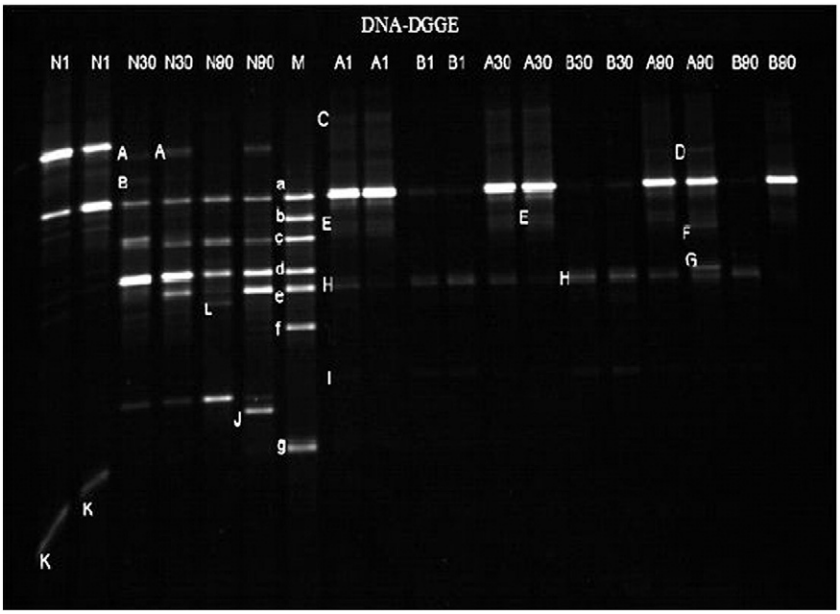

Fig. 3. DNA-DGGE profile of bacterial community populations in pressure treated and non-treated samples at storage time 1, 30 and 90 days. M: marker; A: 400 MPa/10 min/22 °C; B: 600 MPa/10 min/22 °C; and N: not treated.

Table 4
Identification of the bands excised from RNA- and DNA-DGGE gels.

Isolate bands	Closest relatives	ID (%)	Accession no.
From RNA-DGGE gel			
1	*Lactococcus garvieae*	100	GU363927
2	Uncultured *Lactobacillus* sp.	99	GU363928
3	*Weissella minor*	100	GU363926
4	*Lactobacillus plantarum*	100	GU363929
5	*Weissella viridescens*	100	GU363930
6	*Weissella cibaria*	100	GU363931
7	*Leuconostoc mesenteroides*	99	GU363932
8	*Weissella paramesenteroides*	99	GU363934
9	*Lactobacillus sakei*	100	GU363935
10	*Lactobacillus curvatus*	100	GU363936
11	*Leuconostoc carnosum*	99	GU363937
12	*Lactococcus lactis* subsp. *lactis*	100	GU363938
13	Uncultured *Weissella sp.*	100	GU458347
14	*Lactobacillus coryniformis*	100	GU458346
From DNA-DGGE gel			
A	Uncultured *Lactobacillus* sp.	100	HM359074
B	*Lactobacillus plantarum*	100	HM359075
C	*Weissella minor*	100	HM359076
D	*Weissella sp.*	100	HM359077
E	Uncultured *Weissella* sp.	100	HM359078
F	*Leuconostoc mesenteroides*	99	HM359079
G	*Lactobacillus coryniformis*	100	HM359080
H	*Lactobacillus curvatus*	100	HM359081
I	*Streptococcus* sp.	99	HM359082
J	Uncultured bacterium	99	HM359083
K	*Lactococcus lactis* subsp. *lactis*	100	HM359084

Several studies (Cheftel 1995; Garriga et al. 2004; Patterson et al. 2010; Yuste, Pla, Capellas, Ponce, & Mor-Mur 2000) clearly indicated that HPP applied in the range of 400–600 MPa for several minutes would effectively inactivate spoilage bacteria in meat to below the detection limit, especially when the initial microbial load of the products is very low. In present study, LAB constituted the main flora in high pressure treated sliced vacuum-packed cooked ham. During the storage period, all other micro-organisms were inactivated, either by HPP or by pH decrease, and with increasing of LAB counts, the pH values decreased simultaneously. These results suggested that LAB was the predominant biota associated with spoilage of pressurized sliced vacuum-packed cooked ham.

Concerning the bacterial ecology as determined by DGGE profiles, species of *W. viridescens* (band 5), *W. cibaria* (band 6), *L. mesenteroides* (band 7), *L. sakei* (band 9), *L. curvatus* (band 10) and *L. carnosum* (band 11) made up the major group of spoilage bacteria developing on NT samples. However, after HPP at 600 MPa, only *W. viridescens* (band 5) was still viable, and *L. mesenteroides* (band 7) appeared as weakly evident at the end of storage time (90 days). Results by DGGE analysis of the strains isolated from 600 MPa samples also showed that *W. viridescens* and *L. mesenteroides* are the two main species in pressurized samples. The results presented here indicated that HPP affected differently the various species detected, and the microbial diversity in HPP samples was reduced during the storage period, as already suggested by other authors (Diez, Santos, Jaime, & Rovira 2009; Diez et al. 2008; Patterson et al. 2010; Tahiri, Makhlouf, Paquin, & Fliss 2006).

The use of HPP of 600 MPa for 10 min at room temperature (22 °C) displayed an effective inactivation on LAB, the major group of spoilage bacteria on various types of vacuum-packed meat and meat products (Borch, Kant-Muermans, & Blixt 1996; Hu et al. 2009; Samelis, Kakouri, & Rementzis 2000). Compared the DGGE profile of samples treated at 400 MPa and 600 MPa we found that, the inactivation of high pressure towards spoilage bacteria was enhanced with the increasing of pressure level. It is obvious that *W. minor* (band 3), uncultured *Weissella* sp. (band 13) and *L. coryniformis* (band 14) survived high pressure of 400 MPa, whereas *W. viridescens* (band 5) and *L. mesenteroides* (band 7) survived high pressure of 600 MPa. These results are consistent with the findings reported by others (Diez, Jaime, & Rovira 2009; Patterson et al. 2010), who observed that *W. viridescens* was the dominant micro-organism in pressure-treated meat samples.

In the present study, we used a combination of RNA- and DNA-based DGGE analysis to detect and identify those micro-organisms that were responsible for the final spoilage of HPP meat samples. Results revealed that RNA-based DGGE methods, which were developed in the present study, are useful and effective in detecting active species and monitoring the actual microbial dynamics. Obviously, *W. viridescens* (band 5) and *L. mesenteroides* (band 7) were the major spoilage species in pressurized cooked ham. Comparing DNA-DGGE and RNA-DGGE profiles we found that in pressurized samples, *L. curvatus* (band H), was weakly evident during the whole refrigerated storage period, only appearing in the DNA-DGGE profile. These results suggest that this specie may be killed during HPP but the genome DNA fractions of the bacterium were not degraded at that time. In addition, *Streptococcus* sp. (band I) was only detected by DNA-based DGGE methods, indicating that total RNA extraction may lose some template from the bacterial populations compared to that extracted from the genome DNA. *L. coryniformis* (band 14), one of the less studied *Lactobacillus* species, is usually associated with fermented products. In our work, this bacterium was found to be a pressure-resistant species. *W. minor* (band 3) and uncultured *Weissella* sp. (band 13) were detected in samples pressurized at 400 MPa show that, *Weissella* may be the most pressure-resistant genus in cooked ham.

Acknowledgements

The authors would like to thank Yulong Gao in Nanjing University of Finance and Economics for providing high pressure instrument. This work was financially supported by the College Postgraduate Research and Innovation Project in Jiangsu province (CX10B_310Z).

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