The spoilage of air-packaged broiler meat during storage at normal and fluctuating storage temperatures

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ABSTRACT Bacterial diversity and the major flora present on air-packaged broiler meat during storage at normal (4°C) and fluctuating storage temperatures (0–4°C and 4–10°C) were investigated using culture-dependent and culture-independent approaches. Culture-dependent analysis revealed that the growth of microflora was retarded when broiler meat was stored at lower temperatures (0–4°C). Denaturing gradient gel electrophoresis profiles showed that *Staphylococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Carnobacterium* spp., *Aeromonas* spp., and *Weissella* spp. were the dominant bacteria throughout all storage conditions. *Enterobacteriaceae* only appeared in samples subjected to storage with high temperature abuse, whereas *Shewanella* spp. and *Psychrobacter* spp. were only detected in samples stored below 4°C. Our results provide evidence that, compared with storage at a standard fixed temperature (4°C), fluctuations in temperatures induce a more complex bacterial diversity in the air-packaged broiler.

Key words: temperature abuse, temperature fluctuation, broiler, PCR-denaturing gradient gel electrophoresis, spoilage

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INTRODUCTION

Poultry meat is consumed all over the world and is a highly perishable food. Microbial spoilage is of great concern for fresh poultry meat as it only has a storage life under refrigerated conditions (0–10°C; Chouliara et al., 2008). To delay the growth of spoilage microorganisms and extend shelf life, the cold chain is widely used in the fresh poultry trade (James, 1996; Likar and Jevsnik, 2006). However, temperature during storage, transport, distribution, and retail is out of the manufacture's direct control and often deviates from specification (James, 1996; Nychas et al., 2008). Moreover, the temperature of domestic and retail store refrigerators, which are considered to be critical points of the cold chain, often range from −1 to 15°C (Bovill et al., 2001; James et al., 2008). Temperature is one of the most crucial parameters that affects microbial growth (Nedwell, 1999; Smolander et al., 2004; Karadag and Puhakka, 2010). As a result, the composition of the microbial community changes according to varying temperatures (Karadag and Puhakka, 2010; Franciosi et al., 2011).

Numerous objective tests for meat spoilage have been suggested. Total volatile basic nitrogen (**TVB-N**) is one of the main chemical parameters related to the microbial growth of microorganisms, such as *Pseudomonas* spp. (Fraqueza et al., 2008; Boziaris et al., 2011). The TVB-N has been shown to correlate well with the microbial growth of spoilage microorganisms in dark turkey meat under aerobic packaging (Fraqueza et al., 2008). In addition, Hernández-Herrero et al. (1999) suggested that the determination of pH was a valuable indicator related to the growth of lactic acid bacteria (**LAB**). Therefore, TVB-N and pH are 2 indicators of microbial food spoilage (Hernández-Herrero et al., 1999; Fraqueza et al., 2008).

Culture-independent methods have become a valid support to culture-dependent techniques because they are believed to overcome problems associated with selective cultivation and isolation of bacteria from natural samples (Ercolini et al., 2010). Bacterial identification based on molecular methods, especially those including the sequencing of genes coding for ribosomal 16S rDNA, has become a very important tool in studying bacterial communities in meat (Li et al., 2006; Ercolini et al., 2010; Lu et al., 2010). In this study, the exploration of the bacterial community in broiler carcasses

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subjected to different storage temperatures was performed using the PCR-denaturing gradient gel electrophoresis (**DGGE**) technique.

To our knowledge, most research on poultry spoilage has been carried out under ideal temperature storage conditions rather than conditions of temperature abuse (Smolander et al., 2004; Chouliara et al., 2008). Hence, there was a need to perform a simulated experiment on the basis of temperatures found in some domestic and retail store refrigerators. The purpose of the present work was to investigate the effect of temperature fluctuation on the spoilage of air-packaged broilers under chiller conditions.

MATERIALS AND METHODS

Sample Preparation and Storage Conditions

Seventy-eight broiler chicken carcasses (733–855 g) were obtained from a local processing plant immediately after evisceration and were placed into sterile containers and transported on ice to the laboratory within 45 min. Seventy-two carcasses were then individually packaged on polypropylene trays and wrapped with polyethylene film. The oxygen- and moisture vapor-impermeable trays had been sterilized in an autoclave at 121°C for 15 min. The oxygen, carbon dioxide, and water vapor transmission rates of the film were 14,483 $cm^3/(m^2 \times 24\ h \times atm)$; 63,683 $cm^3/(m^2 \times 24\ h \times atm)$; and 54 $g/(m^2 \times 24\ h)$, respectively. After packaging, the carcasses were randomly divided into 3 groups and stored at different temperatures: (a) normal temperature of 4°C; (b) fluctuating temperature between 0 and 4°C; and (c) fluctuating temperature between 4 and 10°C. Each fluctuation was achieved by transferring the product among constant-temperature culture chests, each with precise thermostatic control of temperature, at 4, 0, or 10°C every 24 h. The temperature of the center of the breast meat was monitored using a temperature-logging device (Testo 735-2, Testo, Germany) and recorded at one-minute intervals throughout the whole storage period (Figure 1). Three replicate samples were collected at time 0 (before packaging) and then after 1, 2, 3, and 4 d of storage for physicochemical and microbiological analyses.

pH Measurement

Five grams of breast muscle, free of fat and skin, was thoroughly homogenized with 45 mL of distilled water, and the homogenate was used for pH determination. The pH was measured using a digital pH meter (PHS-4A, Jingke, China) at room temperature. Each value is the mean of 3 measurements.

TVB-N

Ten grams of breast muscle, free of fat and skin, was minced and extracted with 90 mL of distilled water for 30 min. The mixture was then filtered, and 50 mL of filtrate [containing 5 mL of 1% MgO (wt/vol)] was used to determine the TVB-N value using a Foss 2300 Kjeltec Analyzer Unit (Foss, Denmark). The results are expressed as milligrams per 100 g.

Microbiological Analyses

Bacteria were obtained from carcasses using the whole carcass rinse procedure. Broilers were placed in sterile stomacher bags containing 300 mL of sterile saline-peptone dilution water (**SPW**; containing 0.85% NaCl and 0.1% peptone). After shaking at 250 rpm for 20 min on an orbital shaker (WSZ-160A, YiHeng Technical Co., China) at 4°C, the homogenate was collected and used for bacterial determination and PCR-DGGE analyses. Three replicates were performed on each sample.

For the determination of bacterial counts, the homogenate was serially diluted in triplicate (1:10) in SPW. Serial dilutions were plated onto appropriate culture media, and the following microbial analyses were carried out on samples stored under the different conditions: (a) for total viable counts (**TVC**), plate count agar (Lu Qiao Co., Beijing, China) was used and incubated at 37°C for 48 h; (b) for *Pseudomonas*, cetrimide-fucidin-cephaloridine agar (Oxoid Company, Cambridgeshire, UK) was used and incubated at 25°C for 48 h; (c) for LAB, de Man, Rogosa, Sharpe agar (Lu Qiao Co.) was used and incubated at 30°C for 48 h; and (d) for *Enterobacteriaceae*, violet red bile glucose agar (Lu Qiao Co.) was used and incubated at 37°C for 48 h.

All plates were examined visually for typical colony types. The surface areas of samples were determined according to Gill and Badoni (2005). Microbiological data were transformed into logarithms of the number of cfu/cm^2.

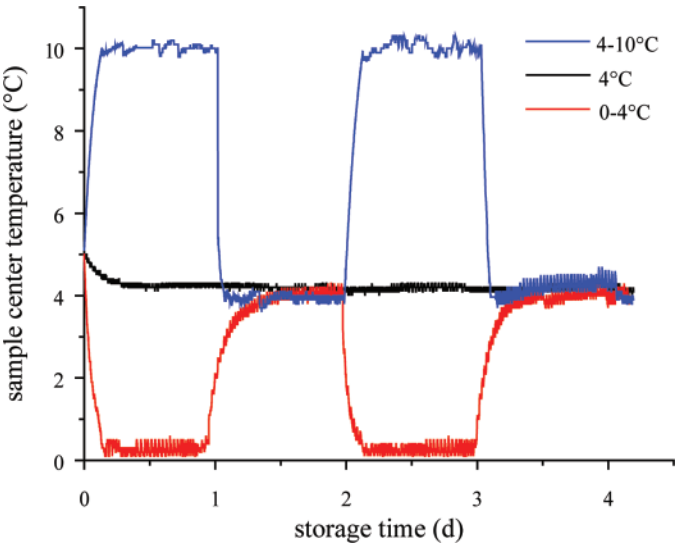

Figure 1. Changes of temperatures in broiler meat monitored by using a temperature-logging system. Color figure available in online PDF.

PCR-DGGE Analysis

Direct extraction of genome DNA from samples at each sampling point was undertaken as follows: 70 mL of homogenate (see Microbiological Analyses section) was aseptically collected and centrifuged at 2,000 × *g* at 4°C for 10 min (Avanti J-E, Beckman Coulter, Brea, CA). The supernatant (about 50 mL) was aseptically transferred into the sterile centrifuge tube and recentrifuged at 12,000 × *g* at 4°C for 10 min. The upper layer was discarded and the pellet was resuspended in 1 mL of sterile distilled water for DNA extraction. Total bacterial DNA was extracted using GenEluteTM Kit (Tiangen Biotech, Beijing, China) following the manufacturers' instructions. Finally, the DNA was suspended in 80 μL of TE buffer (10 m*M* Tris-HC1, 1 m*M* EDTA, pH 8.0) and stored at −20°C.

Primers U968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401 (5′-GCG TGT GTA CAA GAC CC-3′) were used to amplify the V6 to V8 regions of the bacterial 16S rDNA. The PCR reactions were performed in a total volume of 25 μL and included GoTaq Green Master Mix 12.5 μL (Promega, Fitchburg, WI), 0.5 μL (10 pmol/mL) of each primer, 1 μL of DNA template, and 10.5 μL of dH_2O. The following PCR program was used: 94°C for 5 min, 35 cycles of 94°C for 1 min, 56°C for 30 s, and 72°C for 1 min. Finally, the reaction was stopped with an extension step at 72°C for 7 min. The PCR products (5 μL) were analyzed in 1.2% agarose gel electrophoresis in 0.5 × TAE buffer (20 m*M* Tris, 10 m*M* acetic acid, and 0.5 m*M* EDTA; Lu et al., 2010).

The PCR products were analyzed by DGGE using a Bio-Rad DCode apparatus (Bio-Rad, Richmond, CA). Electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37.5:1)] containing a 35 to 55% urea-formamide denaturing gradient [100% corresponds to 7 *M* urea and 40% (wt/vol) formamide]. The gel was subjected to a voltage of 200 for 10 min and then 85 V for 16 h in 0.5× TAE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/L) for 20 min and analyzed under UV illumination using the GelDoc 2000 system (Bio-Rad).

Single bands from the DGGE gels were excised with a sterile scalpel and placed in 20 μL of sterile water. The PCR fragments were recovered in the solution by passive diffusion at 4°C for 16 h. Then 2 μL of the eluate was used as a template for re-amplification using PCR with the same primers and conditions as described (Lu et al., 2010). The PCR products were analyzed by DGGE to confirm that they migrated as a single band to the same position. Excised bands were then reamplified with the primer without the GC clamp and sent for sequencing to a commercial facility (Sangon Company, Shanghai, China). Searches in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the BLAST program were performed to determine the closest known relatives of the partial 16S rDNA sequences (400–600 bp) obtained (Altschul et al., 1997).

Statistical Analysis

Data were analyzed by ANOVA using SPSS 16.0 (SPSS Inc., Chicago, IL). Means and SD were calculated, and significant differences were determined at $P < 0.01$.The fingerprints of the DGGE profile were analyzed by Quantity one 1D Analysis software version 4.6.2 (Bio-Rad). Similarity indices were calculated for the DGGE profiles by the unweighted pair group method with arithmetic means.

RESULTS AND DISCUSSION

Table 1 shows changes in the TVB-N and pH values of broiler meat during storage. The TVB-N values increased significantly from the initial values of 7.69 mg/100 g to 12.88, 10.30, and 16.64 mg/100 g for groups 4, 0 to 4, and 4 to 10°C, respectively, but did not exceed 20 mg/100 g, which is the indicative limit for deterioration (Pearson, 1968). No significant differences ($P > 0.01$) among the 3 groups were found during the first 2 d. On d 3, the 4 to 10°C group showed significantly higher TVB-N than that of broiler meat stored at a lower temperature. By d 4, there were significant differences between the 3 groups. The data indicate that TVB-N increased significantly with the rise in storage temperature. The TVB-N is a chemical spoilage indicator of microbial growth (Boziaris et al., 2011). Fraqueza et al. (2008) suggested that numbers of microorganisms, especially *Pseudomonas* spp. and *Enterobacteriaceae*, could explain the variation observed in the TVB-N. Hence, in this study, it is likely that the formation of TVB-N was related to the growth of bacteria. Because low temperature delays the growth of bacteria, TVB-N only increased slowly when the temperature decreased. In addition, there was no significant difference between the pH in any of the 3 groups ($P > 0.01$). This may be due to the following reasons. First, as TVB-N compounds (such as NH_3) accumulate, the pH would increase (Fraqueza et al., 2008; Boziaris et al., 2011). Second, a negative correlation between pH value and the log of bacterial number was observed, as a result of metabolism by LAB, and the conversion of available glucose to organic acids (Hernández-Herrero et al., 1999; Fraqueza et al., 2008).

Pseudomonas, LAB, and *Enterobacteriaceae* have been considered as the dominate spoilage microorganisms in poultry meat (Chouliara et al., 2008; Patsias et al., 2008). The present study focuses on the monitoring of TVC, *Pseudomonas*, LAB, and *Enterobacteriaceae*.

The TVC for broilers stored at different temperatures are given in Figure 2a. The initial TVC was much lower than that reported by Göksoy et al. (2004). The TVC of samples stored at 0 to 4°C and at 4°C increased initially from 4.60 log cfu/cm^2 to 5.38 and 6.38 log cfu/cm^2, respectively. In contrast, samples stored at 4 to

Table 1. Changes in total volatile basic nitrogen (TVB-N) and pH values of broilers stored at normal temperature (4°C) and with temperature abuse (0–4°C and 4–10°C) for 0, 1, 2, 3, and 4 d[1]

Storage time (d)	TVB-N (mg/100 g)			pH		
	4°C	0–4°C	4–10°C	4°C	0–4°C	4–10°C
0	7.69 ± 0.16^{a}	7.69 ± 0.16^{a}	7.69 ± 0.16^{a}	6.16 ± 0.30^{a}	6.16 ± 0.30^{a}	6.16 ± 0.30^{a}
1	$9.04 \pm 0.42^{A,b}$	$8.80 \pm 0.35^{A,ab}$	$9.63 \pm 0.82^{A,b}$	$6.05 \pm 0.08^{A,a}$	$6.08 \pm 0.16^{A,a}$	$6.09 \pm 0.12^{A,a}$
2	$10.01 \pm 0.7^{A,b}$	$9.29 \pm 1.23^{A,ab}$	$10.54 \pm 0.48^{A,b}$	$5.98 \pm 0.04^{A,a}$	$6.15 \pm 0.22^{A,a}$	$6.05 \pm 0.14^{A,a}$
3	$11.29 \pm 0.36^{A,c}$	$9.99 \pm 1.09^{A,bc}$	$14.05 \pm 0.91^{B,c}$	$5.94 \pm 0.05^{A,a}$	$6.16 \pm 0.23^{A,a}$	$5.92 \pm 0.02^{A,a}$
4	$12.88 \pm 1.00^{B,d}$	$10.3 \pm 0.44^{A,c}$	$16.64 \pm 1.68^{C,d}$	$5.86 \pm 0.02^{A,a}$	$5.96 \pm 0.06^{A,a}$	$6.11 \pm 0.13^{A,a}$

[A–C]Different superscripts in the same row indicate values are significantly different ($P < 0.01$).

[a–d]Different superscripts in the same column indicate values are significantly different ($P < 0.01$).

[1]Values are the mean of triplicate ± SD (SD range: 0.04–1.68).

10°C reached 7.55 log cfu/cm^2; this exceeded the upper limit of microbiological acceptability (Mielnik et al., 1999; Tuncer and Sireli, 2008) after 2 d as a consequence of the temperature effect on bacterial growth rate. Bovill et al. (2001) also suggested that the rate of change of temperature might affect growth of bacteria.

Pseudomonas, the dominant microbial species in air-packaged poultry, are considered to be the prevalent cause of spoilage of poultry meat and meat products (Sundheim et al., 1998; Mielnik et al., 1999; Patsias et al., 2008). The behavior of the growth of *Pseudomonas* at different storage temperatures is shown in Figure 2b. Findings show that there was no significant difference between samples stored at 4°C and those fluctuating between 0 and 4°C, whereas significantly higher ($P < 0.05$) counts of *Pseudomonas* (approximately 0.94–1.78 cfu/cm^2) were recorded in samples stored at 4 to 10°C after 1 d. The results also show that temperature abuse

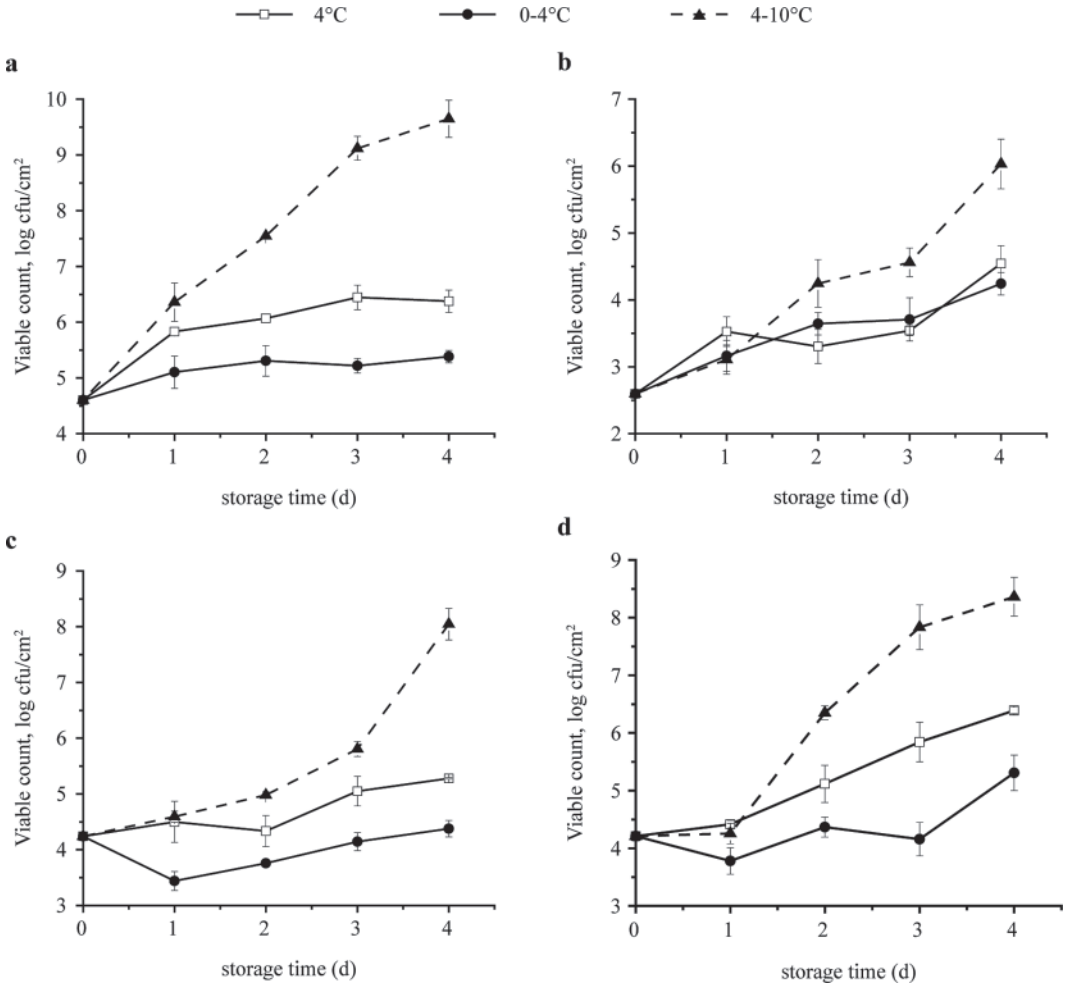

Figure 2. Microbial evolution in broilers during storage at normal temperature (4°C) and with fluctuating temperatures (0–4°C and 4–10°C) for 0, 1, 2, 3, and 4 d. (a) Total viable count; (b) *Pseudomonas* sp.; (c) lactic acid bacteria; and (d) *Enterobacteriaceae*. [a–c]Values on the same day of storage not sharing a common letter were significantly different ($P < 0.01$). Error bars represent mean values of triplicate ± SD.

Table 2. Identification of the bands excised from denaturing gradient gel electrophoresis (DGGE) gels

Band of DGGE	Sequence length (bp)	Closest relative	Similarity (%)	GenBank accession no.
1	439	*Staphylococcus* sp.	99	HQ174892
2	430	*Staphylococcus* sp.	99	HQ174893
3	430	*Enterobacteriaceae* sp.	99	HM234014
4	437	*Shewanella* sp.	99	HM234016
5	440	*Psychrobacter* sp.	99	HM234015
6	438	*Shewanella* sp.	99	HM234017
7	431	*Acinetobacter* sp.	99	HM234013
8	439	*Pseudomonas* sp.	99	HM234018
9	436	*Carnobacterium* sp.	99	HQ174895
10	437	*Aeromonas* sp.	99	HQ174894
11	438	*Weissella* sp.	100	HQ174896
12	586	*Weissella* sp.	99	HQ174897
13	586	*Weissella* sp.	99	HQ174898

had significant effects on surviving populations of *Pseudomonas* in poultry products when storage temperatures were above 4°C. These results are consistent with the findings reported by Davis and Conner (2007).

During storage, LAB increased from an initial value of 4.24 to 5.28, 4.38, and 8.04 log cfu/cm^2 for groups 4, 0 to 4, and 4 to 10°C, respectively (Figure 2c). Temperature abuse had significant effects on the counts of LAB. As expected, the growth rates of LAB for the 3 groups were different (4–10°C > 4°C > 0–4°C). From the above results it can be concluded that LAB was able to adapt to the environment when temperature decreased, and the growth was highly dependent on temperature. It was noteworthy that for some products, at the end of storage, temperature abuse did not increase spoilage (Cayré et al., 2003).

Enterobacteriaceae numbers are considered to be a hygiene indicator and have a high correlation with the sensory odor of the poultry (Smolander et al., 2004). During storage, the number of *Enterobacteriaceae* increased from 4.21 to 6.39., 5.31, and 8.36 log cfu/cm^2 for groups 4, 0 to 4, and 4 to 10°C, respectively (Figure 2d). *Enterobacteriaceae* numbers on d 1 were lower than on d 0, and on d 3 they were lower than on d 2. This suggested that *Enterobacteriaceae* actually decreased when stored at 0°C. These results indicate that *Enterobacteriaceae* were more dependent on storage temperature than were aerobic mesophilic and psychrotrophic bacteria (Smolander et al., 2004).

Denaturing gradient gel electrophoresis of V6 to V8 amplicons was used to investigate the microbial diversity of broilers during storage. It is based on the separation of PCR amplicons of the same size but of different sequences (Li et al., 2006; Lu et al., 2010). The PCR-DGGE fingerprints obtained from DNA directly extracted from broilers stored at different temperatures for 1, 2, 3, and 4 d are presented in Figure 3, whereas the results of the band sequencing are shown in Table 2. *Staphylococcus* sp., *Shewanella, Psychrobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Carnobacterium, Aeromonas* sp., and *Weissella* sp. were identified with lengths of 400 to 600 nucleotides, and the similarity to database sequences was more than 99%. The bacteria identified above are part of the microflora of raw meat and poultry (Li et al., 2006; Russell, 2008; Pennacchia et al., 2011) and these species are usually capable of growing at low temperatures (Ercolini et al., 2010). In particular, 2 bands (bands 1 and 2) for *Staphylococcus*, 2 bands (bands 4 and 6) for *Shewanella*, and 3 bands (bands 11–13) for *Weissella* were identified (Figure 3).

During the entire storage period, *Staphylococcus* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Carnobacterium* sp., *Aeromonas* sp., and *Weissella* sp. were present in all samples and were found to be the dominant bacteria at the end of the storage period. Figure 3 shows that temperature fluctuations did not affect the composition of these members of spoilage bacteria. In meat

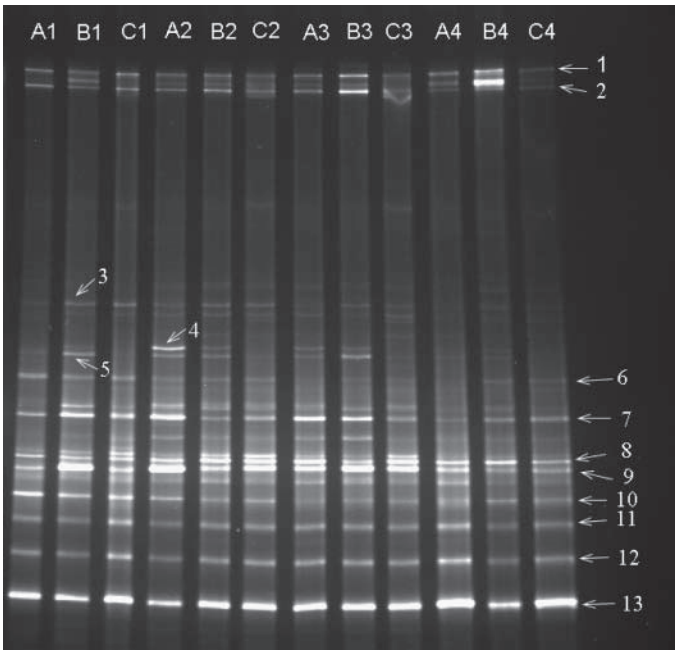

Figure 3. Denaturing gradient gel electrophoresis profiles of 16S rDNA amplicons obtained from broilers. Lanes A1 to A4: broiler meat stored at 4°C for 1, 2, 3, and 4 d; lanes B1 to B4: broiler meat stored at fluctuating temperature (0–4°C) for 1, 2, 3, and 4 d; lanes C1 to C4: broiler meat stored at fluctuating temperature (4–10°C) for 1, 2, 3, and 4 d. Numbers 1 through 13 are bands that were excised for sequence analysis.

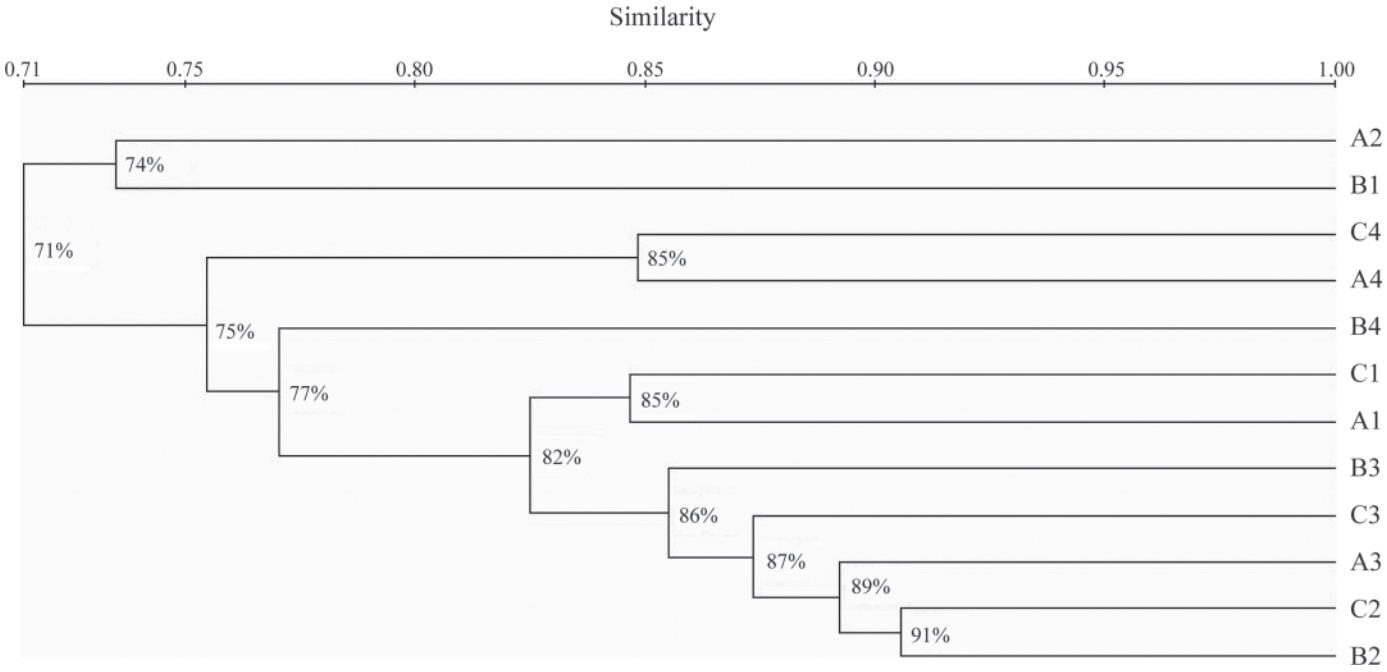

Figure 4. Cluster analysis of molecular banding patterns generated by PCR-denaturing gradient gel electrophoresis of Figure 3.

stored aerobically, spoilage organisms belong primarily to *Pseudomonas,* which attach more rapidly to meat surfaces than other spoilage bacteria (Li et al., 2006; Pennacchia et al., 2011). The other major members of spoilage bacteria, *Staphylococcus* sp., *Acinetobacter* sp., *Carnobacterium* sp., *Aeromonas* sp., and *Weissella* sp., usually play a minor role in the spoilage process (Li et al., 2006; Russell, 2008). The *Weissella* identified in this study were not able to be cultured (data not shown). Nieminen et al. (2011) reported that culture-independent methods were useful in identifying *Weissella* in raw meat.

Results from culture-dependent and -independent methods both provided evidence that decreasing temperature has a considerable effect on the growth of *Enterobacteriaceae* (band 3). In fact, compared with the constant temperature at 4°C, *Enterobacteriaceae* appeared more frequently in samples stored at fluctuating temperatures.

The intensity of bands 4 and 6 identified as *Shewanella* sp. decreased with storage duration. Moreover, *Shewanella* disappeared more quickly in samples stored at 4 to 10°C. Gram (1993) reported that the decrease of *Shewanella* at the end of storage was probably because of inhibition of *Pseudomonas*. The culture-dependent analysis has revealed that the growth of *Pseudomonas* was accelerated when broilers were stored at higher temperatures. Therefore, the increase of temperature may accelerate the disappearance of *Shewanella*. *Shewanella putrefacien* were considered as the principal cause of spoilage of poultry (Russell, 2008; Ólafsdóttir et al., 2006). The processing equipment may be an important source of *Shewanella putrefaciens* because of its ability to adhere to and form biofilms on food processing surfaces (Bagge et al., 2001).

Figure 3 also shows that *Psychrobacter* sp. (band 5) appeared only in samples stored at 4°C and between 0 to 4°C, and then they gradually disappeared over time. This result is consistent with the finding of Pennacchia et al. (2011) who reported that *Psychrobacter* sp. were the predominant microorganisms but were not found at longer storage times. In addition, it was found that the intensity of band 5 in samples stored at 4°C was weaker than that found at 0 to 4°C. It is well-established that *Psychrobacter* sp. appear more frequently at low temperatures (Gounot, 1991).

Figure 4 shows that the similarity indices of the DGGE profiles were above 70%. By comparing the microbial population of broilers stored at 4, 0 to 4, and 4 to 10°C, differences were highlighted. From Figure 4, the similarity of the bacterial community of samples stored at 4°C was 71% among the first 1 to 3 d (A1, A2, and A3) and 75% between the last 2 d (A3 and A4). The similarity indices increased with time of storage. This supports the conclusion that storage time has a strong affect on microbial communities and that microbiological diversity decreases with storage time (Li et al., 2006). However, for the samples stored under conditions of temperature fluctuation, the similarity increased during the first 3 d, and then it decreased on d 4. The similarity of samples stored at 0 to 4°C (B group) was 71% between d 1 (B1) and d 2 (B2), 86% between d 2 and d 3 (B3), and 77% between d 3and d 4 (B4). For the samples stored at 4 to 10°C (C group), the similarity increased from 82 to 87% during the first 1 to 3 d (C1, C2, and C3), but it decreased to 75% on d 4 (C4). It is obvious that temperature abuse affected the composition of the microflora and induced a more complex bacterial diversity, thus supporting the findings of Borch et al. (1996). The PCR-DGGE is a use-

ful tool to monitor and compare the effects of storage conditions and time on microflora.

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