EFFECT OF *LACTOBACILLUS SAKEI* AND *LACTOBACILLUS CURVATUS* ON CHANGES OF THE MICROBIAL COMMUNITY IN VACUUM-PACKAGED RAW BEEF DURING STORAGE

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Abstract –*Lactobacillus sakei* and *Lactobacillus curvatus* were separately incubated in vacuum-packaged raw beef as bio-protective cultures to inhibit the naturally contaminating microbial load. Changes of the microbial diversity on inoculated or non-inoculated (control) beef were monitored at days 0, 7, 13, 21, 28 and 38 at 4°C, using polymerase chain reaction-denaturing gradient gel electrophoresis. The initial contaminating bacteria on beef samples were various, predominated by indigenous *Lactobacillus sakei*. At longer storage times, the application of *L. sakei* and *L. curvatus* both inhibited the growth of contaminating bacteria, including *Enterbacteraceae*, *Pseudomonas fragi*, *Brochothrix thermosphacta*, and *Pseudomonas putida*. However, after day 13 of storage, the dominant position of the bio-protective culture in samples inoculated with *L. curvatus* was replaced by indigenous *L. sakei*. The inhibitory activity of bio-protective cultures was verified by plate count methods, and further demonstrated that the inhibition of spoilage-related bacteria obtained from *L. sakei* was greater than that from *L.curvatus*. Also, the inoculation of both cultures significantly reduced the level of total volatile basic nitrogen over time compared with controls. Thus, the application of both *L. sakei* and *L. curvatus* provided a potential hurdle to inhibit growth of spoilage-related bacteria and improve the shelf-life of vacuum-packaged raw beef.

Key Words – biopreservation, microbial diversity, raw beef, PCR-DGGE.

I. INTRODUCTION

Freshness, nutritional value, minimal processing, and low or no chemical additives prompts growing consumer interest towards chilled meat. However, such chilled product is also highly favorable for the growth of microorganisms, due to high water content and abundant nutrients in fresh meat [1]. In addition to traditional processes like salting and curing, mild preservative methods have been applied in chilled or fresh meat, including high hydrostatic pressure, pulsed electric field, irradiation, ozone, packaging and application of probiotics or targeted metabolites [2-4]. Addition of probiotics is termed bio-control or bio-preservation [5], using a natural microflora to increase safety and extend shelf life of food products without altering the sensory attributes [6]. Lactic acid bacteria (LAB) as a protective culture have been generally regarded as safe (GRAS) [7] and described as 'food grade' organisms by Bredholt et al. (1999) [8]. LAB often are naturally present in meat or meat products and act as powerful competitors to contaminating pathogenic or spoilage bacteria by producing a wide range of antimicrobial metabolites such as organic acids, bacteriocins and other metabolites [5-6, 9-11].

The inhibitory activity of the bio-protective cultures was evaluated by the reduced number of inoculated pathogenic bacteria or spoilage-related organisms in food [11-14]. However, little information is available about the dynamic changes of the naturally contaminating bacteria in meat during storage under the influence of such protective cultures at the species level, especially in vacuum-packed raw beef. It is essential to understand the overview of species changes in the microbial community during beef storage, to determine whether bio-cultures exert inhibitory activity at particular stages of storage, and to confirm how much suppression would be produced by the bio-cultures towards the main spoilage flora. Also, in spite of a large literature on the subject, there are few studies focusing on the comparable antibacterial properties of different LAB strains on raw beef, which is very important to select the most suitable strains for a specific food. Thus, the aim of this study was to determine the bacterial diversity and monitor the community dynamic changes during storage of vacuum-packaged sliced raw beef as affected by *L. sakei* or *L. curvatus*, using the polymerase chain reaction-denaturing gradient gel electrophoresis technique (PCR-DGGE), and to make a comparison between the two strains to select a better bio-culture for raw beef.

II. MATERIALS AND METHODS

A. Preparation of the bio-protective culture solutions and samples

The protective cultures used were commercial cultures Bactoferm™ B-2 (*Lactobacillus.sakei*) and SafePro® B-LC-48 (*Lactobacillus. curvatus*) supplied by Chr.

Hansen (Copenhagen, Denmark). Each culture was reconstituted according to the manufacturer's instructions. The final culture solution was set to deliver a concentration around $\log_{10}$7.5 CFU/g for the dipped beef samples.

Twelve conventionally segmented *longissimus lumborum* (LL, pH_{24h} 5.4-5.8) from six carcasses were selected from a cattle slaughter plant based in Shandong province, China. Muscles from the same animal were pair-packed in a plastic bag and placed in a foam box and transported to the laboratory within 2h. Every treatment was repeated three times on separate days; thus two carcasses were used for one trial.

Tendons were removed aseptically, and the LL was cut perpendicularly to the muscle fibers into 25 cm^2 steak samples approximately 2 cm thick. Fifty-four slices in total were collected from both sides of the LL from one carcass. Those slices were randomly assigned to dip into the. *L. sakei* solutions for 30s as Ls samples and another 18 slices into *L. curvatus* solutions as Lc samples, and then both treated samples were drained aseptically for 1min. The 18 cuts left without treatment were regarded as control samples. All the slices were separately packed in polyamide bags (PA)/ polyethylene (PE) membrane oxygen permeability (<15 $cm^3m^{-2}24h^{-1}$ at 25°C, 1 atm, 80μm thickness), and collected immediately after inoculation (day 0) and at 7, 13, 21, 28, 38 days storage at 4°C. Three packages of Ls, Lc or control cuts were used for the following analysis at each time interval.

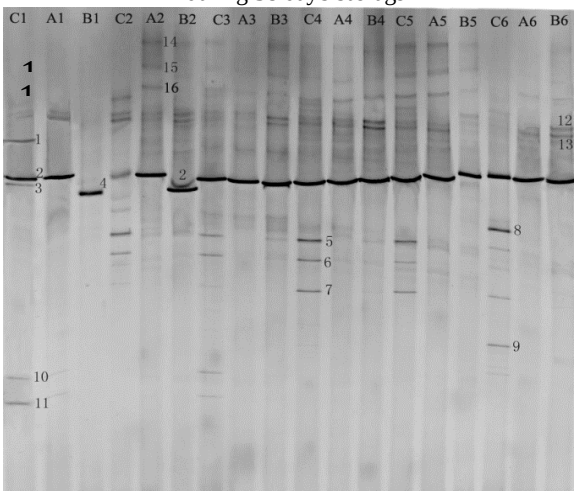
B. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) procedures

Total bacterial DNA was directly extracted from duplicate Ls, Lc or control samples at each designated time. Nested PCR and touchdown PCR reactions were followed as described by Rochelle (1995) and Hu et al (2008) [15-16]. PCR products were analyzed by DGGE by using a Bio-Rad D-code apparatus. Samples were applied to 8% (wt/vol) polyacrylamide gels in 0.5×TAE buffer. Parallel electrophoresis experiments were performed at 60°C by using gels containing a 30-60% urea-formamide denaturing gradient (100% corresponded to 7M urea and 40% (wt/vol) formamide). The gels ran for 16h at 75V, then were stained with ethidium bromide for 5min, rinsed for 15min in distilled water, observed and photographed by the Bio-Rad Gel Doc system (BioRad, Milano, Italy).Final samples were sent to Sangon Biotech Company (Shanghai, China) for sequencing. GenBank DNA data base was used for the identification of those sequences [17]. Sequences with more than 96% identity were considered to represent the same species.

III. RESULTS AND DISCUSSION

Dynamic changes of the microbial community profiles during 38 days storage were obtained from the raw beef inoculated with *L. sakei* or *L. curvatus* (Ls and Lc, respectively) by using the nested PCR-DGGE (Fig. 1). More than 20 different bands were found, of which 16 bands were identified by 16S rDNA sequencing. The GenBank accession numbers for the nucleotide sequences obtained from the DGGE bands are shown in Table 1.These sequences displayed a greater than 98% identity with sequences in the GenBank databases except for band 3, which showed 95% similarity.

Fig.1 Denaturing gradient gel electrophoresis (DGGE) profile of 16S rDNA PCR products recovered from vacuum-packaged raw beef with inoculation of protective cultures during 38 days storage



Lanes C0-C38 represent control samples; Lanes Ls0- Ls38 represent treatment with protective culture *Ls*-B-2 and Lanes Ls0- Ls38 represent treatment with protective culture *Ls*-B-LC-48 stored for time. Bands indicated by numbers 1-16 were excised, and after re-amplification, subjected to sequencing. Ls = samples incubated with *Lactobacillus sakei*; Lc = samples incubated with *Lactobacillus curvatus*. Control = beef samples without any treatment.

A. Microbial diversity of vacuum-packaged raw beef during the 38 days storage at 4°C (control samples)

Seven bands were detected at day 0 in the control samples, indicating a high initial bacterial diversity in the vacuum-packaged raw beef. *L.sakei* (band 2) was the main bacterial contaminant from the slaughter plant. Bands 12 and 13 were very common bacteria found in vacuum-packaged meat, belonging to LAB. Band 10 was identified as *Listeria sp*, which reflected that the hygiene conditions of the beef processing plant need to improve.

The bacterial diversity changed as the storage time increased. The inherent LAB (band 2) was not only the main components in the initial composition of contaminating bacteria, but also dominated throughout

the entire storage time in the vacuum-packaged raw beef, which is consistent with Metaxopoulos (2002) [18]. The other main microflora appeared in a relatively regular order during storage. Both *Enterbacteriaceae* (band 5) and *P. fragi* (band 6) bacteria emerged in the early storage days. *B. thermosphacta* (band 7) shared the dominant position during the mid-storage times, and at the end, *P. putida* (band 8) appeared and was the predominant bacteria. Previous studies have found that these bacteria all contribute to beef deterioration. *Enterobacteriaceae* and *B. thermosphacta* were the prevailing spoilage organisms in vacuum packaging [1,19] and *Pseudomonas spp.* were acknowledged as the most dominant genus owing to their capability for glucose and amino acid degradation even in the vacuum-packaged conditions, of which *P. fragi* was the species most frequently isolated from the early stage while *P. putida* showed up in the latter stage during the meat storage [20-21]. In these studies and the present study, one of the great advantages of using the DGGE molecular method is to determine the exact order of appearance of the dominant bacteria at the species level during storage, which is not possible by the traditional culture techniques.

Table 1
Strains identified by means of 16SrDNA sequencing fragments from DGGE bands of total bacterial community DNA directly extracted from beef

Band No.	Closest relatives	Identity %	Accession No.
1	Uncultured bacterium	99%	JF719396.1
2	*Lactobacillus sakei*	100%	AB671579.1
3	*Serratia sp.*	95%	HQ690889.1
4	*Lactobacillus curvatus*	100%	JF756310.1
5	*Enterbacteriaceae* bacterium	99%	JN571324.1
6	*Pseudomonas fragi*	100%	HQ824990.1
7	*Brochothrix thermosphacta*	100%	JF756334.1
8	*Pseudomonas putida*	100%	JF745568.1
9	Uncultured bacteria	99%	JN378768.1
10	*Listeria sp.*	100%	JF967622.1
11	Uncultured *Pseudomonas sp.*	99%	FN554308.1
12	*Lactobacillus graminis*	99%	GU470987.1
13	*Leuconostoc mesenteroides*	100%	JF756260.1
14	Uncultured *Lactobacillus*	99%	GU363936.1
15	*Lactobacillus fuchuensis*	100%	JF756333.1
16	*Leuconostoc camosum*	100%	JF756140.1

B. Effects of *L.sakei* and *L. curvatus* on the microbial community of vacuum-packaged raw beef during 38 days storage at 4°C

With the inoculation of *L.sakei* or *L. curvatus*, the initial bacterial composition in Ls and Lc samples were dominated by those two bio-protective cultures, respectively (Lanes Ls0 and Lc0). Obviously, *Enterbacteriaceae* bacterium, *P. fragi* and *B. thermosphacta* (bands 5, 6 and 7) were completely inhibited in Ls and Lc samples during the prolonged storage and *P.putida* was suppressed until it appeared late in the storage period. These results were supported by a number of other studies, of which Katikou et al., found two strains of LAB was beneficial in reducing *Enterbacteriaceae* bacterium and *Pseudomonas* spp. in vacuum-packaged beef [6]. Castellano et al. (2006) and Metaxopoulos et al. (2002) demonstrated that *L. curvatus* CRL705 and *L. curvatus* L442 reduced the *B. thermosphacta* population in meat discs and cured products at chill temperatures, respectively [12, 18].

Additionally, as shown in Fig. 1, for Ls samples, *L. sakei* was always the prevailing bacteria in the microbial community. However, in the Lc samples, unexpectedly, *L. sakei* emerged at day 7 and occupied *L. curvatus*`s predominant position in the following days, so that band 4 was hardly detected at day 13 and disappeared from day 21. In the study of Castellano et al. (2004), they found the bio-protective culture *L. casei* (*curvatus*) could not completely inhibit the growth of *L. sakei* CRL 1424 that was inoculated in the meat slurry at an initial concentration of 3.6 log CUF/ml [22]. In view of this, in the present study, band 2 shown in Ls samples may be the combination of the inoculated and the indigenous *L. sakei* strains. The reason why the inoculated bio-protective cultures were failing to compete with the indigenous *L. sakei* is uncertain. But it should note that the inoculated *L. sakei* and *L. curvatus* must play a major role in suppressing the spoilage-related bacteria that was observed in this study during the early storage time, as the indigenous *L. sakei* alone could not inhibit the growth of those bacteria shown in control samples.

The plate counts results (data not shown) obtained in the current study were consistent with the DGGE results, both showing an effectiveness of the inhibition towards *Enterbacteriaceae*, *Pseudomonas* spp. and *B. thermosphacta* by applying *L.sakei* and *L.curvatus* in raw beef, which was also in agreement with previous studies mentioned above [6, 12, 18].

No negative beef quality traits (data not shown) were found in the samples inoculated with *L. sakei* or *L. curvatus*. Both cultures improved the lightness at the latter stages of storage and decreased the content of TVBN throughout the entire store period.

IV. CONCLUSION

The application of *L. sakei* or *L. curvatus* effectively inhibited the growth of *Enterbacteraceae*,

P. fragi, *B. thermosphacta*, and *P. putida*, which successively peaked in the microflora of vacuum-packaged raw beef during 38 days storage at 4°C. The inoculation with *L.curvatus* was less efficient than that with *L.sakei* based on the culture-dependent methods. The inhibitory activity of those two bio-protective strains maybe associated with the indigenous LAB, as it was not possible to determine whether the inhibitory actions were derived from the inoculated culture alone or from both the inoculated and indigenous LAB, considering that the DGGE profiles showed growth suppression of inoculated *L. curvtaus* during storage. Future studies should take into account the interaction between inoculated bio-protective and the indigenous LAB in raw beef. Additionally, the inoculation of both cultures has no negative effects on the beef quality traits. In conclusion, the use of both *L. sakei* and *L. curvatus* provided a potential hurdle to improve the shelf life of the vacuum-packaged raw beef.

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REFERENCES

1. Ercolini, D., Russo, F., Torrieri, E., Masi, P., Villani, F., (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Applied and Environmental Microbiology 72: 4663-4671.
2. Behsnilian, D., Butz, P., Greiner, R., Lautenschlaeger, R., (2014). Process-induced undesirable compounds: Chances of non-thermal approaches. Meat Science 98: 392-403.
3.Zhou, G.H., Xu, X.L., Liu, Y., (2010). Preservation technologies for fresh meat-A review. Meat Science 86: 119-128.
4. Lücke, F.K., (2000). Utilization of microbes to process and preserve meat. Meat Science 56: 105-115.
5. 6. Katikou, P., Ambrosiadis, I., Georgantelis, D., Koidis, P., Georgakis, S.A., (2005). Effect of Lactobacillus-protective cultures with bacteriocin-like inhibitory substances' producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. Journal of Applied Microbiology 99: 1303-1313.
7. Adams, M.R., Marteau, P., (1995). On the Safety of Lactic-Acid Bacteria from Food. International Journal of Food Microbiology 27: 263-264.
8. Bredholt, S., Nesbakken, T., Holck, A., (1999). Protective cultures inhibit growth of *Listeria monocytogenes* and *Escherichia coli* O157: H7 in cooked, sliced, vacuum- and gas-packaged meat. International Journal of Food Microbiology 53: 43-52.
9. Castellano, P., Raya, R., Vignolo, G., (2003). Mode of action of lactocin 705, a two-component bacteriocin from *Lactobacillus casei* CRL705. International Journal of Food Microbiology 85: 35-43.
10. Champomier-Verges, M.C., Chaillou, S., Cornet, M., Zagorec, M., (2002). Erratum to "*Lactobacillus sakei*: recent developments and future prospects" [Research in Microbiology 152 (2001) 839]. Research In Microbiology 153: 115-123.
11. Jones, R.J., Zagorec, M., Brightwell, G., Tagg, J.R., (2009). Inhibition by *Lactobacillus sakei* of other species in the flora of vacuum packaged raw meats during prolonged storage. Food Microbiology 26: 876-881.
12. Castellano, P., Vignolo, G., (2006). Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins. Letters in Applied Microbiology 43: 194-199.
13. Castellano, P., González, C., Carduza, F., Vignolo, G., (2010). Protective action of *Lactobacillus curvatus* CRL705 on vacuum-packaged raw beef. Effect on sensory and structural characteristics. Meat Science 85: 394-401.
14. Chaillou, S., Christieans, S., Rivollier, M., Lucquin, I., Champomier-Vergès, M.C., Zagorec, M., (2014). Quantification and efficiency of *Lactobacillus sakei* strain mixtures used as protective cultures in ground beef. Meat Science 97: 332-338.
15. Rochelle, P.A., Will, J.A.K., Fry, J.C., Jenkins, G.J.S., Parkers, R.J., Turley, C.M., Weightman, A.J., (1995). Extraction and amplification of 16S rRNA genes from deep marine sediments and seawater to assess bacterial community diversity. In: Trevors, J.T., Elsas,J.D. (Eds.), Nucleic Acids in the Environment. Springer-Verlag, Berlin, pp. 219-239
16. Hu, P., Xu, X.L., Zhou, G.H., Han, Y.Q., Xu, B.C., Liu, J.C., (2008). Study of the *Lactobacillus sakei* protective effect towards spoilage bacteria in vacuum packed cooked ham analyzed by PCR-DGGE. Meat Science 80: 462-469.
17. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.
18. Metaxopoulos, J., Mataragas, M., Drosinos, E.H., (2002). Microbial interaction in cooked cured meat products under vacuum or modified atmosphere at 4 degrees C. Journal of Applied Microbiology 93: 363-373.
19. Fontana, C., Cocconcelli, P.S., Vignolo, G., (2006). Direct molecular approach to monitoring bacterial colonization on vacuum-packaged beef. Applied and Environmental Microbiology 72: 5618-5622.
20. Molin, G., Ternstrom, A., (1982). Numerical taxonomy of psychrotrophic pseudomonads. Journal of General Microbiology 128: 1249-1264.
21. Sutherland, J.P., Patterson, J.T., Murray, J.G., (1975). Changes in the microbiology of vacuum-packaged beef. Journal of Applied Bacteriology 39: 227-237.
22. Castellano, P.H., Holzapfel, W.H., Vignolo, G.M., (2004). The control of *Listeria innocua* and *Lactobacillus sakei* in broth and meat slurry with the bacteriocinogenic strain *Lactobacillus casei* CRL705. Food Microbiology 21: 291-298.