KKU Res. J. 2014; 19(Supplement Issue): 19-25
http : //resjournal.kku.ac.th

Diversity of lactic acid bacteria in Thai fermented pork (*nham*) during fermentation assessed by Denaturing Gradient Gel Electrophoresis

Umpika Burintramart, Kanittha Chantarasakha and Nipa Chokesajjawatee[*]

Food Biotechnology Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Pathum Thani 12120, Thailand

**Correspondent author: nipa.cho@biotec.or.th*

Abstract

This work presents the study of lactic acid bacteria (LAB) diversity in Thai fermented pork (*nham*) at different time points of the fermentation by denaturing gradient gel electrophoresis (DGGE) technique. Total DNA extracted from two *nham* samples, fermented with and without *Lactobacillus plantarum* starter culture, were amplified using primers targeted at V3 region of the 16S rRNA gene of bacteria. DNA bands appeared on DGGE gels were cloned and sequenced for species identification. The results showed that the LAB species in both *nham* samples at the beginning of fermentation was diverse and at least 15 species were detected. The number of species was then reduced dramatically during the first 2 days of fermentation. Two main species, i.e. *Lactococcus garvieae* and *Lactococcus lactis* were found from day 2 to day 15. In *nham* fermented with starter culture, the DGGE band corresponding to the *Lb. plantarum* was evident from the beginning to the end of the test period. However, in *nham* without starter culture, although no *Lb. plantarum* was added and no corresponding band appeared at the beginning of fermentation, the band gradually appeared as faint band on day 3 and increased in intensity as fermentation progressed. Similar pattern was also observed on the band corresponding to *Pediococcus pentosaceus.* This phenomenon suggested that *Lb. plantarum* and *P. pentosaceus* can be established successfully and may play an important role in *nham* fermentation system. This study also reported co-migration of different DNA species to the same DGGE-band position as well as fragments with the same DNA sequence may also migrated to different position on the DGGE gel. Therefore, the number of DGGE bands could not be used to identify the number of bacterial species in the sample accurately.

Keywords: *nham*, bacterial diversity, DGGE

1. Introduction

A Thai fermented pork product (*nham*) is a traditional food popularly consumed throughout Thailand and neighboring countries. Fermentation of *nham* often relies on naturally present microflora from raw materials. Several studies have shown that lactic acid bacteria (LAB) are the predominant microorganisms which play a crucial role during *nham* fermentation (1, 2). Traditionally, a culture-based method was used to identify microbial community in food. However, the culture-based method has some limitation, for example, stressed or weakend cells and non-culturable microbial population might not be able to grow under the selected laboratory conditions, and therefore can not be detected with the culture-based method. In this study we selected culture-independent method based on polymerase chain reaction coupled with denaturing gradient gel electrophoresis (DGGE) to study microbial population in *nham*. The DGGE technique is the widely used culture-independent method for investigating microbial diversity in many systems including food fermentation system (3). The DGGE technique is based on the separation of double-stranded DNA fragments that have the same size but different sequences. Separation of DNA fragment in denaturing gradient gel relies on the differential denaturation profile of each DNA fragment (4). In this work, we present the LAB diversity in *nham* fermented with and without starter culture at different time points of the fermentation using DGGE technique. The DGGE profile was obtained by amplifying the V3 region of the 16S rRNA gene of bacteria. Study of the LAB community during *nham* fermentation will give us understanding and insight in the role of each LAB at each stage of the fermentation. This knowledge can be used to understand *nham* fermentation system and design of new and effective starter cultures.

2. Materials and Methods

2.1 Starter culture

The *nham* starter culture, *Lactobacillus plantarum*, was obtained from BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

2.2 *Nham* preparation

Two batches of *nham* were prepared by mixing ground pork (52.1%), cooked pork rind (34.7%), garlic (4.3%), cooked rice (4.3%), whole bird chili (1.7%), curing salt (1.9%), sodium tripolyphosphate (0.2%), erythorbate (0.2%), sucrose (0.3%), and monosodium glutamate (0.2%). During mixing of the ingredients, the starter culture was added at the level of 10^4 CFU/g to one batch of the sample. The mixture was stuffed into a plastic casing of diameter 3.0 cm (approximately 200 g each). The *nham* samples were incubated at 30°C and were analyzed for LAB community after fermentation for 0, 2, 3, 5, 7, 10 and 15 days.

2.3 DNA extraction

Twenty-five grams of *nham* samples were mixed with 50 ml of saline peptone water (0.85% NaCl, 0.1% Peptone) in sterile filter bag and then homogenized for 1 min in a stomacher. The filtrate was transferred to a 50 ml centrifuge tube and centrifuged at 800×g for 1 min. And then 2 ml of the supernatant was transferred into a collection tube and centrifuged at 13000×g for 1 min to collect the cell pellet. Total DNA was extracted using Power Food Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's protocol.

2.4 PCR amplification

Primers Lac1 (5'-AGCAGTAGGGAATCTTCCA-3'), Lac2-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGGATTYCACCGCTACACATG-3'), and Lac3 (5'-AGCAGTAGGGAATCTTCGG-3') were used to amplified the V3 region of the 16S

rRNA gene (5, 6). PCR amplification was performed in a 50 μl reaction mixture containing 1× Ex Taq buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.5 μM of each primer, 1.25 U of *Taq* DNA polymerase (ExTaq, Takara Bio Inc., Japan), and 100 ng of DNA template. The amplification cycles consisted of 94°C for 2 min and 35 cycles of 94°C for 30 s, 61°C for 1 min, and 68°C for 1 min, followed by 68°C for 7 min (5).

2.5 DGGE analysis

DGGE analysis was performed on 8% polyacrylamide gels with a denaturing gradient of 35% to 55% (100% denaturants correspond to 7 M urea and 40% formamide) using DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., USA). The electrophoresis was carried out at a constant voltage of 80 V for 16 h at 60°C in 1× TAE running buffer. Gels were stained with 1× SYBR® Safe DNA gel stain (Invitrogen, USA) for 30 min and photographed on Ultra-slim LED machine (Gellex International Co. Ltd, Japan).

2.6 Identification of DGGE bands

The DGGE bands were excised from the gels and the DNA was eluted by submerging in 50 μl TE buffer overnight at 4°C. The eluted DNA was then re-amplified using the same primer set without GC clamp. The PCR products were cloned into a plasmid vector pDrive using QIAGEN PCR Cloning kit (QIAGEN, Germany) and transformed into *Escherichia coli* DH5α. Two to six clones from each DGGE band were subjected for sequencing analysis. Analysis of sequence data was performed using the SEQMATCH function supplied by the ribosomal data-base project (http://rdp.cme.msu.edu/).

3. Results and Discussion

The DGGE profile of LAB community in *nham* fermented with and without starter culture during fermentation was shown in Fig. 1. The bands A – U were cloned and sequenced for species identification. The result of the identification was shown in Table 1.

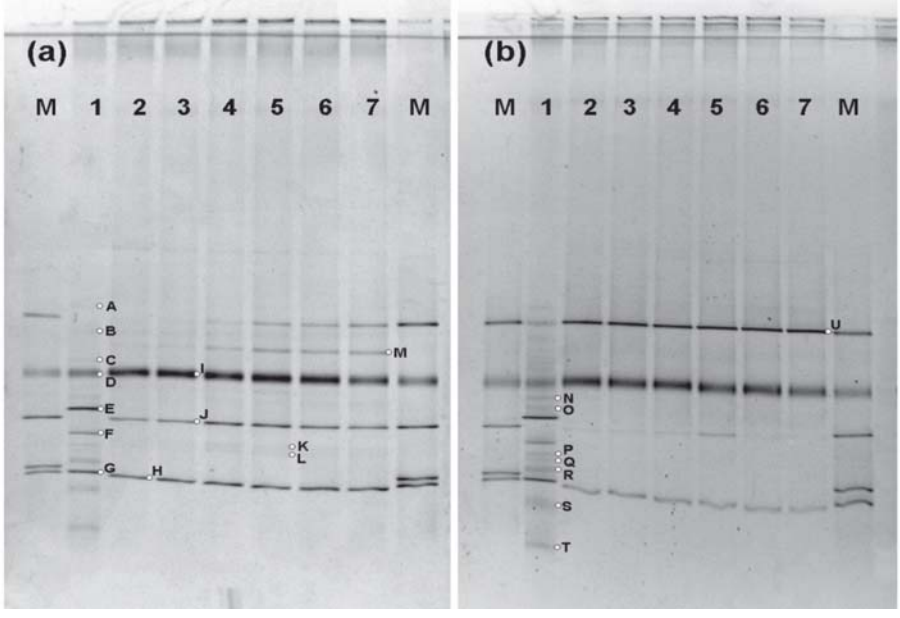

Figure 1. DGGE profiles of the 16S rRNA gene amplified from *nham* samples. (a) *nham* without starter culture, (b) *nham* with starter culture. Lane M: Marker, Lane 1-7: 0, 2, 3, 5, 7, 10 and 15 days of fermentation, respectively. The bands labeled in this figure (A-U) were subjected to sequencing for species identification.

Table 1. Species identification of DGGE band

Band ID		Band description
A	Mix 3 species:	- *Macrococcus brunensis* - *Macrococcus equipercicus* - *Lactococcus garvieae*
B	Mix 2 species:	- *Lactococcus garvieae* - *Streptococcus dysgalactiae*
C	Mix 3 species:	- *Streptococcus dysgalactiae* - *Vagococcus teuberi* - *Vagococcus carniphilus*
D	1 species:	- *Lactococcus garvieae*
E	Mix 4 species:	- *Macrococcus equipercicus* - *Streptococcus dysgalactiae* - *Streptococcus porcinus* - *Vagococcus fessus*
F	Mix 4 species:	- *Lactococcus garvieae* - *Macrococcus equipercicus* - *Vagococcus fluvialis* - *Streptococcus dysgalactiae*
G	Mix 2 species:	- *Streptococcus minor* - *Streptococcus dysgalactiae*
H	1 species:	- *Lactococcus lactis*
I	Mix 2 species:	- *Pediococcus claussenii* - *Lactococcus garvieae*
J	1 species:	- *Lactococcus garvieae*
K	1 species:	- *Lactococcus lactis*
L	1 species:	- *Lactococcus lactis*
M	Mix 2 species:	- *Pediococcus pentosaceus* - *Lactococcus garvieae*
N	Mix 2 species:	- *Macrococcus equipercicus* - *Lactococcus garvieae*
O	Mix 2 species:	- *Lactococcus garvieae* - *Vagococcus fessus*
P	Mix 3 species:	- *Streptococcus parauberis* - *Streptococcus dysgalactiae* - *Vagococcus carniphilus*
Q	Mix 2 species:	- *Streptococcus dysgalactiae* - *Vagococcus fluvialis*
R	Mix 3 species:	- *Streptococcus plurextorum* - *Streptococcus suis* - *Streptococcus criceti*
S	Mix 2 species:	- *Streptococcus phocae* - *Streptococcus parauberis*
T	Mix 2 species:	- *Streptococcus dysgalactiae* - *Streptococcus minor*
U	Mix 2 species:	- *Lactobacillus plantarum* - *Lactococcus garvieae*

The results of DGGE analysis showed that the bacterial species in both *nham* samples at the beginning of the fermentation (day 0) were diverse with at least 15 species from 14 DGGE bands. The bacteria identified at the beginning of the fermentation were 1 species from genus *Lactococcus*, 2 species from genus *Macrococcus*, 8 species from genus *Streptococcus*, and 4 species from genus *Vagococcus* (Table 2). The number of species was then reduced dramatically during the first 2 days of fermentation. In *nham* fermented naturally without starter culture added, two main species, i.e. *Lactococcus garvieae* and *Lactococcus lactis* were found at day 2 and remained throughout the test period of 15 days. In *nham* fermented with starter culture, the two main species occurred at day 2 to day 15 were the same as the naturally fermented *nham*, with an additional band of the *Lb. plantarum* (band U, Fig.1b) that was evident from the beginning to the end of the test period. However, in *nham* without starter culture, although no *Lb. plantarum* was added and no corresponding band appeared at the beginning of the fermentation, the band gradually appeared as faint band on day 3 and increased in intensity as fermentation progressed (band U, Fig.1a). Similar pattern was also observed on the band corresponding to *Pediococcus pentosaceus* (band M, Fig. 1a). This phenomenon suggested that *Lb. plantarum* and *P. pentosaceus* can be established successfully and may play an important role in *nham* fermentation system.

Four species, i.e. *Lc. garvieae*, *Lc. lactis*, *Lb. plantarum* and *P. pentosaceus* were found to be the dominant species in our *nham* samples, which were also described in other fermented meat products; for example, Vietnamese fermented sausage (7, 8). However, *Lactobacillus sakei*, that was usually found as dominant species in fermented meat products, i.e. Taiwanese naturally fermented ham (9), artisan Serbian sausage (10), Spanish dry-fermented sausage (11), was not found in our samples.

Notably, the result in Table 1 showed that on several occasions, different DNA fragments migrated to the same band position on DGGE gel or so-called co-migration. On the other hand, Table 2 showed another phenomenon in which fragments with the same DNA sequence present in different position on the DGGE gel. The co-migration of different DNA sequences is commonly known and can be resulted from similar melting behavior of different DNA fragments (12). While migration of the same DNA sequence to different positions on the DGGE gel has not been reported. This phenomenon should be further investigated. We speculated that it may be resulted from variation of the GC clamp primer which may caused by the error of repeated-nucleotide sequence synthesis. Another possible explanation is that, it may be resulted from the secondary structure that was randomly formed and affect the melting behavior of the same DNA sequence. Therefore, the number of DGGE bands could not be used to identify the number of bacterial species in the sample accurately.

Table 2. The species found in *nham* at the beginning of fermentation

No.	Species	Band location
1	*Lactococcus garvieae*	A, B, D, F, N, O
2	*Macrococcus brunensis*	A
3	*Macrococcus equipercicus*	A, E, F, N
4	*Streptococcus criceti*	R
5	*Streptococcus dysgalactiae*	B, C, E, F, G, P, Q, T
6	*Streptococcus minor*	G, T
7	*Streptococcus parauberis*	P, S
8	*Streptococcus phocae*	S
9	*Streptococcus plurextorum*	R
10	*Streptococcus porcinus*	E
11	*Streptococcus suis*	R
12	*Vagococcus carniphilus*	C, P
13	*Vagococcus fessus*	E, O
14	*Vagococcus fluvialis*	F, Q
15	*Vagococcus teuberi*	C

4. Acknowledgement

This study was supported by National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency (NSTDA), Thailand.

5. References

(1) Valyasevi R, Rolle RS. An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: scope for their improvement. Int J Food Microbiol. 2002;75(3):231-9.

(2) Tanasupawat S, Komagata K. Lactic acid bacteria in fermented foods in Thailand. World J Microbiol Biotechnol. 1995;11(3):253-6.

(3) Ercolini D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J Microbiol Methods. 2004;56(3):297-314.

(4) Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci USA. 1983;80(6):1579-83.

(5) Endo A, Okada S. Monitoring the lactic acid bacterial diversity during shochu fermentation by PCR-denaturing gradient gel electrophoresis. J Biosci Bioeng. 2005;99(3):216-21

(6) Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Applied and environmental microbiology. 2001;67(6):2578-85.

(7) Tran KTM, May BK, Smooker PM, Van TTH, Coloe PJ. Distribution and genetic diversity of lactic acid bacteria from traditional fermented sausage. Food Research International. 2011;44(1):338-44.

(8) Nguyen DTL, Van Hoorde K, Cnockaert M, De Brandt E, De Bruyne K, Le BT, et al. A culture-dependent and -independent approach for the identification of lactic acid bacteria associated with the production of nem chua, a Vietnamese fermented meat product. Food Research International. 2013;50(1):232-40.

(9) Tu R-J, Wu H-Y, Lock Y-S, Chen M-J. Evaluation of microbial dynamics during the ripening of a traditional Taiwanese naturally fermented ham. Food Microbiology. 2010;27(4):460-7.

(10) Danilovic B, Jokovic N, Petrovic L, Veljovic K, Tolinacki M, Savic D. The characterisation of lactic acid bacteria during the fermentation of an artisan Serbian sausage (Petrovska Klobasa). Meat Sci. 2011;88(4):668-74.

(11) Fonseca S, Cachaldora A, Gamez M, Franco I, Carballo J. Monitoring the bacterial population dynamics during the ripening of Galician chorizo, a traditional dry fermented Spanish sausage. Food Microbiology. 2013;33(1):77-84.

(12) Green SJ, Leigh MB, Neufeld JD. Denaturing Gradient Gel Electrophoresis (DGGE) for Microbial Community Analysis. In: Timmis K, editor. Handbook of Hydrocarbon and Lipid Microbiology: Springer Berlin Heidelberg; 2010. p. 4137-58.