MICROBIAL AND CHEMICAL STUDY OF CAMBODIAN TRADITIONAL FERMENTED FISH PRODUCTS

2014

MONY ROTH CHUON

LIST OF ABBREVIATIONS

%	Percentage
g kg^{-1}	Gram per kilogram
<	Less than
>	More than
Total-N	Total nitrogen
N-recovery	Nitrogen recovery
NaCl	Sodium cloride
ND	Not detected
ppm	Part per million
CFU	Colony Forming Unit
B. coagulans	*Bacillus coagulans*
B. megaterium	*Bacillus megaterium*
B. subtilis	*Bacillus subtilis*
M. colpogenes,	*Micrococcuscolpogenes*
M. varians	*Micrococcusvarians*
S. epidermidis	*Staphylococcusepidermidis*
S. saprophyticus	*Staphylococcussaprophyticus*
LAB	Lactic acid bacteria
PE	Prahok at early stage fermentation
PF_1	Prahok fermented for 1 month
PF_2	Prahok fermented for 3 months
KE	Kapiat early stage fermentation
KF_1	Kapifermented (final product)
KF_2	Kapifermented (final product)
TE	Toeuk trey at early stage fermentation
TF_1	Toeuk trey fermented for 6 month
TF_2	Toeuk trey fermented (final product)
TF_3	Toeuk trey fermented (final product)
TF_4	Toeuk trey fermented (final product)
TF_5	Toeuk trey fermented (final product)
TF_6	Toeuk trey fermented (final product)
FP_1	Fermented fish paste for 1 month
FP_2	Fermented fish paste for 2 months
FP_3	Fermented fish paste for 3 months
FP_4	Fermented fish paste for 4 months
FP_5	Fermented fish paste for 5 months
GC-MS	Gas chromatography mass spectrometry
TSA	Tryptone soy agar
MRS	Man, rogosa and sharpe
GAM	Gifu anaerobic medium
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

rDNA	Ribosomal DNA
BLAST	Basic local alignment search tool
RDP	Ribosomal Database Project
qPCR	Quantitative real-time PCR
TMAO	Trimethylamine N-oxide
DMA	Dimethylamine
TMA	Trimethylamine
H_2,	Hydrogen
CO_2	Carbone dioxide
V1-V2	Variable region 1 and 2

Contents

General Introduction

Fermented fish products in Asia

Fermentation is normally defined as the transformation of organic substances into simpler compounds by the action of enzymes or microorganisms.[1)] Fermentation of high protein food such as milk, soy bean, fish and meat involves the hydrolysis or breakdown of protein into free amino acids and peptides. In the most cases, the process of fermentation is carried out by microorganisms, and the extent proteolysis is a function of type of microorganism employed and the aging time. The characteristic taste and odor of fermented products usually depends upon the degree of proteolysis together with lipolysis and/ or carbohydrate fermentation. Salt is normally used to suppress the growth of undesirable microorganisms; hence spoilage by undesirable microorganisms is prevented. Fermentation is practiced as a mean of preserving and or altering the flavor of fish products more in the Orient than Europe or North and South America.[2)] In Southeast Asia, fermentation is still the most common method of fish preservation, although modern technologies such as chilling or freezing have been introduced.

There are three categories of fish fermentation according to the fermenting agents.[3)] 1) Traditional products fermented mainly by the action of endogenous enzyme in the presence of salt; the products of this category are, for example, fish sauce and fish paste. 2) Another traditional products, fermented by combined effects of both endogenous and microbial enzymes supplemented as a starter in addition to salt; these products are *mam-chao* (Cambodia), *pla-ra* (Thailand) and *funasushi* (Japan) etc. 3) Non-traditional products, produced by an accelerated process either by added enzymes or by chemical hydrolysis e.g., silage fish and soluble fish.

Subba Rao (1967)[4] also classified fermented fish products on the basis of the consistency of product into 3 types:

1. Products in which the fish retain much of their original form or in which large chunks are preserved and may be dried or partially dried, e.g. *trey prarma* (Cambodia), *pla-ra* (Thailand), and *buro* (Philippines).
2. Products in which the fish are reduced to paste e.g., *prahok* and *kapi* (Cambodia), *bagoong* (Philippines), *kapi* (Thaland), *belachan* (Malaysia).
3. Products in which the fish are degraded into liquid, e.g. fish sauce.

Fermented fish products were traditionally made from small pelagic fishes both marine and fresh water species. The major genera of fish used for fish sauce is not only *Stolephorus*, but also smaller *Scomber*, *Ristrelliger*, *Clupeoides*, *Crossocheiluss* and *Cirrhinus*.[5] Beddows et al. (1979)[6] reported that the most predominant species used were *Stolephorus*. *Hassett* was reported to be the best raw material for fish sauce production in Thailand because of its high fat content giving good flavor and aroma.[5,7] Freshwater fishes have been generally used especially Jullin's mudding carp or *Cirrhinus juttieni*, while Van Veen (1965)[8] reported that the products made from freshwater fish yielded inferior flavor and aroma. Chotiyarnwong and Chuapoehuk (1988)[9] reported that the products made from *Tilapia nil-otica Linnaeus* took at least 20 months until the end products were obtained. Thus, the products are made from various type of fishes, but the best fish sauce is believed to be anchovy sauce.[10]

Nowadays, there are many varieties of fermented fish products available in Southeast Asia. These includes liquid products such as fish sauce known as *toeuk trey* in Cambodia, *nampla* in Thailand, *budu* in Malaysia, *bakasang* in Indonesia, *patis* in Philippines, *nouc-mam* in Vietnam, *yu-lu* in China, *shottsuru* and *ishiru* in Japan, and *jeotkuk* in Korea. The paste products

such as fish and shrimp pastes are known as *prahok* and *kapi* in Cambodia, *pladek* and *kapi* in Thailand and Laos, *ngacha* and *ngapi* in Myanmar, *belacan* and *terasi* in Indonesia and Malaysia.

Production process of fish sauce in Cambodia

Fish sauce is an important fermented fish product in some parts of the world and particularly, well-known in Southeast Asia. It is a hydrolyzed product of fish protein basically consisting of water, salt and soluble nitrogen compounds. Fish sauce normally has been used as a condiment in Southeast Asia where rice is staple food. Fish sauce is important for Philippino, Thai, Cambodian and Vietnamese, as Japanese and Chinese mainly use soy sauce as a seasoning. Fish sauce alone can provide 7.5% of the daily nitrogen intake.[3] Fish sauce is also an important source of calcium in areas or countries where intake of this mineral from food stuff is insufficient.[11]

Many investigators[12,13] described many methods for fish sauce production. Many of fish sauces of Thailand and Cambodia are still manufactured by the traditional method.

In the traditional method, fresh, small and non-eviscerated fish were mixed with solar marine salt at varying ratios, i.e., from 3 (fish):1 (salt) by weight depending on the freshness of the fish. The salt and fish mixture was put in a wooden tank or an earthen jar. Nowadays, concrete containers covered with bamboo trays are also used for fish sauce production. After filling the air space with brine, the containers were kept undisturbed under the sun for a period of one year from 6 months or even longer without stirring. In some cases, the mixtures are left on a slightly sloping floor for a period up to 1 week to let the slime drain out and the mixture

partially dry up before it was placed in the fermentation tank.

The liquid obtained at this period yielded a slightly ammoniac flavor, with distinctively meaty and cheesy aroma. Certain concentration of brine is added to make up for the liquid lost during the draining period. Floating fish are prevented by weighting it down with bricks or hard timber placed on top of a bamboo screen. At the end of the fermentation period, the first fish extract constituting a special grade fish sauce is pumped or poured out. The filtrate is further aged under the sun for a period of one to three months, during this stage salt crystallized out. The residues which are filtered are leached twice or three times with brine to give the lower quality sauce.

They were usually blended with various amount of primary extract, coloring matter, and finally bottled. Three leaching steps are usually possible, and, finally, the residue is boiled to get the brine which is kept for later production. The fish bone was sold for its phosphate value to fertilizer producers. The flow diagram for traditional fish sauce production was shown in Fig.1.

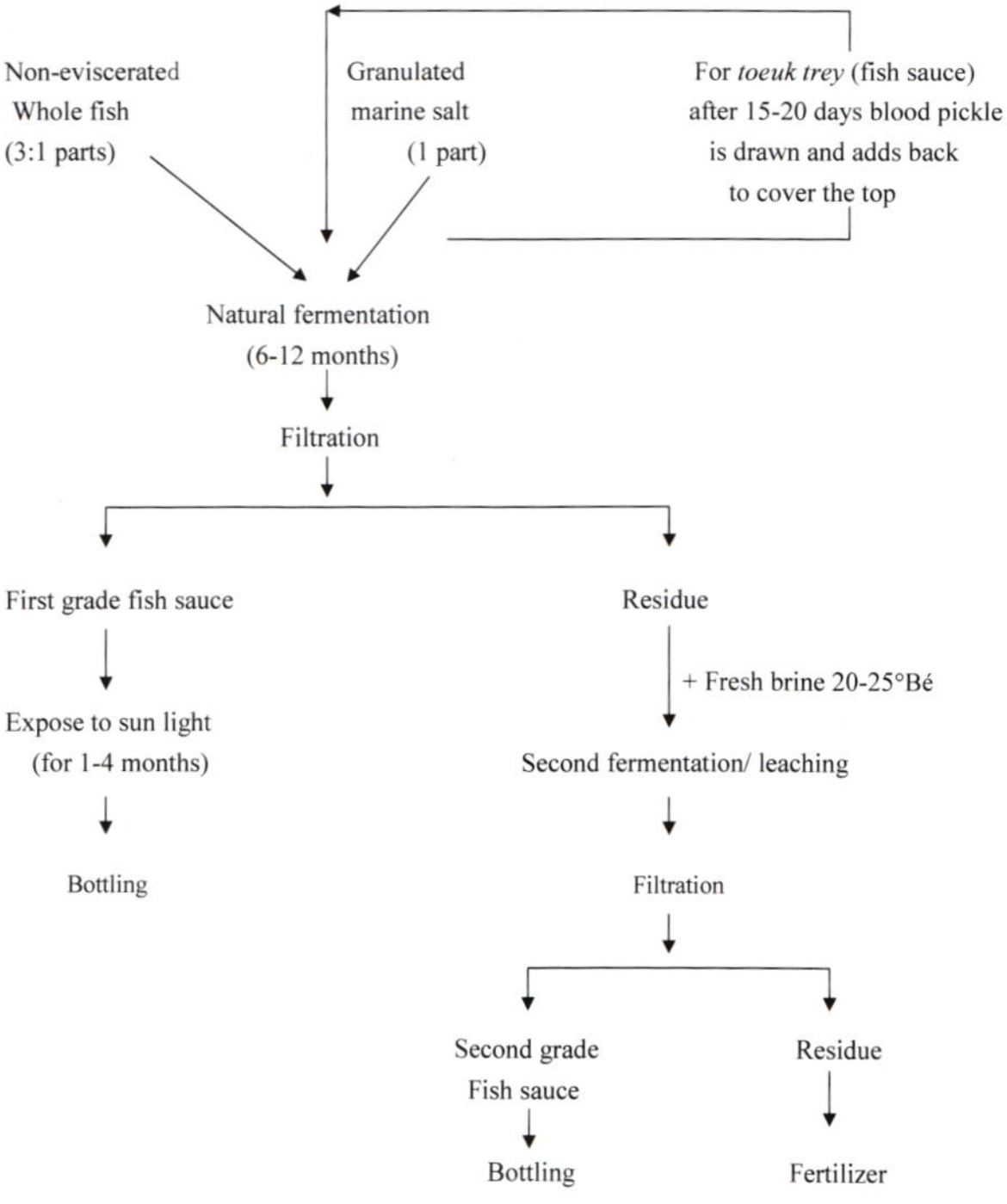


Fig.1 Flow diagram of the production of fish sauce *toeuk trey* in Cambodia

The blood pickle is allowed to flow out slowly over two week interval and then is poured back over the fish until the liquid becomes clear. After that, the liquid is transferred to the new tank, which is regarded as for the "first grade". Fresh brine (20-25 °Bé) is added to the residue in the tank or vat and the mixture is fermented for 2-4 months. The liquid drawn off is second grade fish sauce. The residue mass is normally used as fertilizer.

Production processes of fish paste and shrimp paste in Cambodia

Fermented fish pastes are common fermented products other than fish sauce. Fish pastes are used mainly as a flavoring agent or in condiment preparations. Moreover, almost all South and Southeast Asian countries prepare this product to preserve for future consumption. As an example of fermented fish pastes production, *ngari* is an ethnic fermented fish product of Manipur, India. It's made from *Puntius sophore*. The fish is rubbed with salt at 5:1 ratio and dried for 3-4 days, then pressed tightly in an earthen pot, sealed tightly, and fermented for 4–6 months.[14)]

Shrimp paste, generally is made from small salted shrimps, prepared by mixing with 10-15% of salt, or even higher to get a better product. The mixture is dried on straw mats to reduce the water content to about 50%, then minced or pounded into paste, and allowed to ferment for 7 days. At the end of fermentation, the mixture is broken and sun dried for 5-8 h, then minced again and formed into blocks or balls, and further fermented for about 1 month. This process can be repeated several times if needed.

General properties of fermented fish products

Physical properties

Fish sauce (Fig. 2A) is a clear brown liquid with a salty meaty and cheesy taste with distinct sensory characteristic. The specific gravity is around 1.2 and the pH was in the ranges of 5.0-6.5. Salt concentration is not less than 20% (w/w).[15)]

Fish paste (Fig. 2B) is known as a fermented fish paste with unique taste and flavor, grey to brown in color, made from tiny fish manually pounded into a mush with 15-20% salt before being preserved and fermented.

Shrimp paste (Fig. 2C) is fermented shrimp, dark brown and purple. It has a distinct taste and strong aroma. It is made from small salted shrimp, then dried and grounded into fine paste, and is allowed to be fermented for 2 days. Then, it is processed into ball shape in the presence of 10-20% of salt before fermentation for several months.

A. Fish sauce B. Fish paste C. Shrimp paste

Fig. 2 Traditional fermented fish products.

One example of chemical properties from seven countries of Southeast and East Asian fish sauce are shown in Table 1. Total nitrogen ranges from 0.35-2.59 g/ 100 ml. Such a big difference in the nitrogen content of fish sauce could be seen, it might be due to diverse use of raw materials and fermentation process of each country. Total nitrogen content (Total-N) and recovery ratio (N-recovery) were very low in fish sauce made in Myanmar and Laos.[16] Abe et al. (1999) reported that these were poor-quality fish sauces.[17] Conversely, total nitrogen was highest in fish sauce made in Vietnam followed by those of Japan and Thailand. Fish sauce is rich in amino acids contents, as shown in Table 2. The high concentration of lysine in fish sauce is believed to be important, as it could compensate for lysine deficiency in rice in people's lives.

Table 1 Nitrogen and NaCl compositions of fermented fish sauce

Fish sauce	NaCl[1] (g/ 100 ml)	Total-N[2] (g/ 100 ml)	N-recovery[3] (%)
Thailand (n=10)	21.4 ± 1.2^{ac}	1.68 ± 0.24^{b}	64.3 ± 5.5^{ac}
Vietnam (n= 20)	20.2 ± 1.1^{bc}	2.59 ± 0.51^{a}	61.6 ± 13.2^{bcd}
Myanmar (n= 7)	22.7 ± 1.9^{a}	0.97 ± 0.75^{cd}	45.6 ± 9.8^{c}
Laos (n= 2)	15.7 ± 1.9^{d}	$0.35 \pm 0.08b^{c}$	42.5 ± 6.6^{c}
China (n= 2)	22.0 ± 1.1^{ab}	1.49 ± 0.44^{d}	57.8 ± 17.7^{adc}
South Korea (n= 9)	22.2 ± 1.5^{a}	1.27 ± 0.20^{c}	68.2 ± 5.0^{ab}
Japan (n= 11)	18.0 ± 4.5^{d}	1.80 ± 0.31^{b}	70.4 ± 9.9^{a}
Mean ± S.D (n= 61)	20.5 ± 2.8	1.79 ± 0.77	61.8 ± 12.8

Mean values in the same column with different superscript letters are significantly different ($P<0.05$ or less).
[1]Values are expressed in g/100 ml for NaCl, [2]Total nitrogen (Total-NT), [3]Nitrogen recovery. Park et al. (2001).[17]

Table 2 Amino acid contents of fermented fish sauce

Amino acid	Quality (mg/ 100 ml)		
	Undigested[1]	Digested[2]	Fish flesh[3]
Taurine	167	211	-
Aspatic acid	1601	2009	1800
Threonine	819	873	834
Serine	683	861	924
Glutamic acid	1448	3280	2704
Glycine	552	1041	816
Alanine	1194	1272	1136
Valine	469	974	928
Metheonine	331	335	432
Isoleusine	372	379	800
Leusine	434	431	1472
Tyrosine	99	63	656
Phenylalanine	519	541	752
Histidine	707	649	576
Lysine	1767	1982	1692
Arginine	4	34	1120
Citrulline	1300	894	-

[1]Before preparation, [2]after fermentation (fish sauce), [3]mixture of extract (fish sauce) and fish paste. Saisithi et al. (1994).[12)]

Table 3 Bacterial species isolated from fermented fish products in other Asian countries

Species found in Myanmar fermented fish products		Species found in Thai fermented fish products		
Shrimp paste	Fish sauce	Shrimp paste	Fish paste	Fish sauce
Staphylococcus sp.	*Arachnia*sp.	*Bacillus* sp.	*Tetragenococcus halophilus*	*Tetragenococcus* sp.
Bacillus sp.	*Bacillus* sp.	*Clostridium perfringens*	*Bacillus* sp.	*Bacillus* sp.
Micrococcus sp.	*Micrococcus* sp.	*Tetragenococcus halophilus*	*Micrococcus* sp.	*Micrococcus* sp.
Nocardia madurae	*Nocardia madurae*	*Staphylococcus*sp.	*Staphylococcus* sp.	*Staphylococcus* sp.
Arachnia propionica	-	-	-	-
Corynebacterium sp.	-	-	-	-

Fish fermentation technology in Myanmar and Thailand in *Fish fermentation technology*; Lee C. H., Steinkraus, K. H., Alan Reilly, P. J, Eds.; United Nations University Press: Tokyo, Japan. 1993.[18)]

Microbial properties

Table 3 shows one report of detailed information on bacterial species isolated from fermented fish products in Asian countries except for Cambodia. Microflora plays an important role in the production of fermented fish. A high salt concentration of fermented fish sauce enables only halophilic and halotolerant bacteria to grow. The presence of microflora during fish fermentation enhances the degradation of fish proteins and develops flavor and aroma. The change of microflora during fermentation of the Southeast Asian fish sauce can be classified into proteolytic enzyme-producing bacteria and flavor and aroma-producing bacteria.[19,20)]

In purpose of this study, I made the first detailed analyses on the chemical and microbial composition of the traditional Cambodian fermented fish products, *prahok*, *kapi*, and *toeuk trey* to obtain a thorough scientific understanding of them. The data obtained in this study shed light on the fundamental signature of not only Cambodian but also whole Asian fermented fish products, benefiting the future improvement of manufacturing process including microbial control and quality stabilizing.

The thesis consists of four chapters;

(1) Chemical compositions in Cambodian traditional fermented fish products

(2) Volatile compounds in Cambodian traditional fermented fish products

(3) Microbial composition in Cambodian traditional fermented fish products using culture-dependent method

(4) Pyrosequencing analysis of microflora in Cambodian traditional fermented fish products.

CHAPTER I

Chemical Compositions in Cambodian Traditional Fermented Fish Products

1.1 Introduction

Cambodia has an extensive network of waterways, and freshwater fish comprise a major portion of the diet of most Cambodians, particularly in many products that are fermented and preserved. Daily fresh fish catches in the Mekong River, Bassac River, and the vast Tonlé Sap supply more than 50% of the protein intake for people living in Cambodia. Freshwater fish are most commonly used for fermented foods, such as fish paste (*prahok*) and fish sauce (*toeuk trey*), whereas marine products are also processed as shrimp paste (*kapi*) and fish sauce (*toeuk trey*).[21)]

Prahok is the representative fermented and salted fish in Cambodia, known for its unique taste and flavor. This product is gray to brown in color and is made by pounding freshwater fish (*Channastriata* or other tiny fishes) along 150–200 g kg^{-1} of salt before being preserved and fermented.[22)] *Toeuk trey* is a clear brown fish sauce produced by naturally fermenting small freshwater or marine fishes. This is mixed with salt at a ratio of 3:1 (fish:salt) and stored for several months up to a year. The liquid supernatant is then kept for more than three months, followed by several rounds of filtration before it is used or bottled. *Kapi* is a dark brown to purple-colored shrimp paste with distinct taste and strong aroma.[22)] It is made from small, salted planktonic shrimps that are sun dried for 2 days. These dried shrimps are then ground into a fine paste, allowed to ferment for two days, and processed into balls before fermenting for several more weeks. *Prahok*, *kapi*, and *toeuk trey* are widely used as seasonings and foods in Cambodia.

Studies on fermented fish products have attracted considerable attention due to an increasing demand for desirable biological functions and probiotic effects derived from the chemical constituents and microorganisms contained in these products. One report exhaustively described the oligopeptide composition of Vietnamese fish sauce *nuoc mam*.[23] Our and another group investigated volatile compounds in fish sauce in detail.[24,25] Several recent studies demonstrated varying antioxidant activities and phase-dependent changes in the chemical composition during the maturation of fermented fish products.[26-28] However, there is still insufficient knowledge to understand the complete nature of fermented fish products, as most studies only used limited chemical analyses[29-31] or microbial analyses that only provided viable counts on selective medium plates without phylogenetic identification.[32-35]

In this study, we obtained the first detailed analyses of the chemical composition of the traditional Cambodian fermented fish products, *prahok*, *kapi*, and *toeuk trey* to acquire a thorough scientific understanding of these products. The contribution of these chemical compositions to fermentation and maturation were discussed, including organic acids and amino acids. We particularly focused on acetic acid and lactic acid as indicators of the microbial fermentation. The data obtained in this study shed light on the fundamental signature of not only Cambodian but also whole Asian fermented fish products, benefiting the future improvement of manufacturing process including microbial control and quality stabilizing.

1.2 Materials and Methods

1.2.1 Samples used for analyses

A total of 13 samples of Cambodian traditional fish fermented products, *prahok*, *kapi*, and *toeuk trey*, were obtained from different plants and factories in Cambodia. These samples were directly procured from each factory at room temperature, packed into plastic containers and maintained at 4 °C until analyzed. Chemical analyses were immediately performed after the samples were brought to the laboratory (within several days). Fermentation periods, sampling location, and other information are provided in Table 1.1. The first samples of each fermented food (indicated as PE, KE, and TE) were those collected during the early fermentation stage of less than one month. Others samples (indicated as PF_1, PF_2, KF_1, KF_2, and TF_1–TF_6) were the final products that were ready for consumption and were obtained from different companies with fermentation periods of more than one month.

1.2.2 Proximate analyses

Solid samples (*prahok*, *kapi*, and *toeuk trey* TE) were ground prior to the particular analyses, while the liquid samples (*toeuk trey* $TF_{1\text{-}6}$) were directly used. The proximate compositions of these fish products were determined based on an official analytical method established in Japan.[36] Salinity was determined by the Mohr method and pH was determined with a pH meter (Sartorius, Goettingen, Germany). A Baume hydrometer was used to determine specific gravity of the liquid samples (*toeuk trey* $TF_{1\text{-}6}$).

Table 1.1 Cambodian traditional fish fermented products analyzed in this study

Group[a]	Fermentation period	Product code[b]	Sampling location[c]
prahok (fermented fish paste)	20 days	PE	Kompong Thom province
	1 month	PF_1	Seim Reap province
	3 months	PF_2	Seim Reap province
kapi (fermented shrimp paste)	5 days	KE	Kom pot province
	Final product	KF_1	KompongSom province
	Final product	KF_2	Kosh Kong province
toeuk trey (fish sauce)	2-3 months	TE	Phnom Penh city
	Pure grade 6 months	TF_1	Phnom Penh city
	Final product	TF_2	Phnom Penh city
	Final product	TF_3	Phnom Penh city
	Final product	TF_4	Kompot province
	Final product	TF_5	Keb province
	Final product	TF_6	Keb province

[a]*Prahok* was made from freshwater fishes, *kapi* was made from sea planktonic shrimps, *toeuk trey* TE, TF_1, TF_2, and TF_3 were made from freshwater fishes, and *toeuk trey* TF_4, TF_5, and TF_6 were made from seawater fishes.
[b]PE, KE, and TE, early fermentation stage samples, in which whole fish or shrimp were pre-fermented in the presence of low salt concentration (44–140 g kg^{-1}, see Table 1.2); PF_1, PF_2, KF_1, KF_2, and $TF_{1\text{-}6}$, final products obtained from different companies with long-term fermentation periods (more than 6 month), which are ready to be consumed.
[c]Geographical locations at which the indicated fermented foods were produced.

1.2.3 Amino acid and organic acid contents

One gram of *prahok* and *kapi* was suspended in 20 ml distilled water, the solid body was disrupted using homogenizer, and the mixture was centrifuged to obtain the supernatant. For *toeuk trey*, the liquid was diluted up to 400-fold using distilled water before analysis. Amino acids contained in these supernatants were analyzed with a model L-8000 amino acid analyzer equipped with an ion exchange #2622 SC column (4.6 × 60 mm) (Hitachi, Tokyo, Japan). The eluted amino acids were detected and quantified by a post-column ninhydrin labeling method.[37)]

Organic acids (lactic acid, acetic acid, citric acid, malic acid, formic acid, pyroglutamic acid, succinic acid, and propionic acid) were analyzed using a Dionex ion chromatography system, ICS-1500 with Ion Pac ICE-AS6 column (9 × 250 mm) (Dionex, Sunnyvale, CA, USA).

These compounds were detected and quantified by measuring the conductivity in the eluted fractions.

1.3 Results

1.3.1 Proximate compositions of Cambodian traditional fermented fish products

Table 1.2 summarizes the proximate compositions of three Cambodian traditional fermented fish products. The moisture contents of *prahok* and *kapi* were 503–594 g kg^{-1} and 460–510 g kg^{-1}, respectively, and those of *toeuk trey* ranged from 630 to 723 g kg^{-1}, except for the early fermentation stage sample (240 g kg^{-1}, *toeuk trey* TE).

Ash contents varied at around 200 g kg^{-1} (195–235 g kg^{-1} for *prahok* and 162–240 g kg^{-1} for *kapi*), while the range was slightly higher in *toeuk trey* (210–262 g kg^{-1}). All fermented products had crude fat contents no more than 11.7 g kg^{-1} (*kapi* KE), but the early fermentation stage samples of *prahok* PE and *toeuk trey* TE had extremely higher values (151 and 234 g kg^{-1}, respectively). Salt concentrations ranged from 170 to 270 g kg^{-1} for final products (*prahok* PF_1, PF_2, *kapi* KF_1, KF_2, and *toeuk trey* $TF_{1\text{-}6}$), whereas three early stage samples had low concentrations; 44 g kg^{-1}, *prahok* PE, 73 g kg^{-1} for *kapi* KE and *toeuk trey* TE (140 g kg^{-1}).

Prahok and *Toeuk trey* had acidic pH values of 6.30–6.60 and 5.20–6.80, respectively, whereas *kapi* had a slightly alkaline pH ranging from 7.40 to 7.90. The specific gravity was 1.16–1.19 for liquid *toeuk trey* samples.

1.3.2 Amino acid and organic acid contents

The amino acid and organic acid compositions of *prahok*, *kapi,* and *toeuk trey* are summarized in Table 1.3. The predominant amino acids in *prahok* were glutamic acid (1.49–2.93 g kg^{-1}), alanine (1.78–2.64 g kg^{-1}), valine (1.23–1.83 g kg^{-1}), leucine (2.27–3.40 g kg^{-1}), and lysine (1.13–3.67 g kg^{-1}) (Table 1.3). Interestingly, in *kapi*, the asparagine and leucine contents were highly product dependent (asparagine, ND–3.54 g kg^{-1}; leucine, ND–6.62 g kg^{-1}). The lysine concentration was high in all *kapi* products (4.53–14.40 g kg^{-1}). Aspartic acid, threonine, glycine, alanine, valine, isoleucine, and leucine were detected in all *toeuk trey* samples, but other amino acids were occasionally absent.

In ten out of thirteen samples, the organic acid detected at the highest concentration was acetic acid: 2.29–7.24 g kg^{-1} in *prahok*, 6.61–26.60 g kg^{-1} in *kapi*, and 1.90–11.30 g kg^{-1} in *toeuk trey*. However, in a few samples, the highest concentrations were found for either lactic acid (*kapi* KF_1, 6.83 g kg^{-1}), pyroglutamic acid (*kapi* KF_2, 16.10 g kg^{-1}), or propionic acid (*toeuk trey* TF_2, 2.72 g kg^{-1}). Lactic acid concentrations were 0.39–1.14 g kg^{-1} for *prahok*, 3.78–12.90 g kg^{-1} for *kapi,* and 0.70–3.55 g kg^{-1} for *toeuk trey*; thus, considerable amounts of lactic acid were detected. Succinic acid was generally found at concentrations of 0.30–1.26 g kg^{-1} in *prahok*, 2.50–7.96 g kg^{-1} in *kapi*, and 0.42–3.21 g kg^{-1} in *toeuk trey*. Malic acid and formic acid were

Table 1.2 Proximate compositions of Cambodian traditional fermented fish products

Parameter[a]	*prahok* (fish paste)				*kapi* (shrimp paste)			
	PE	PF_1	PF_2	Mean ± SD	KE	KF_1	KF_2	Mean ± SD
Moisture (g kg^{-1})	503	594	560	552 ± 46	488	460	510	486 ± 25
Ash (g kg^{-1})	195	206	235	212 ± 21	162	240	240	214 ± 45
Crude fat (g kg^{-1})	151	1.7	10.7	55 ± 84	11.7	3.2	5.0	6.6 ± 4.5
Protein (g kg^{-1})	334	249	248	277 ± 49	343	262	213	273 ± 66
Salt (NaCl) (g kg^{-1})	44	170	180	131 ± 76	73	210	200	161 ± 76
pH	6.40	6.30	6.60	6.43 ± 0.15	7.80	7.40	7.90	7.7 ± 0.27

Parameter	*toeuk trey* (fish sauce)							
	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Mean ± SD
Moisture (g kg^{-1})	240	723	666	703	630	702	643	615 ± 169
Ash (g kg^{-1})	210	256	233	262	230	220	236	235 ± 19
Crude fat (g kg^{-1})	234	0.6	5.5	1.3	1.8	4.2	3.1	36 ± 87
Total nitrogen (g kg^{-1})	23.0	15.6	16.9	13.1	18.0	26.9	26.2	20 ± 5.4
Salt (NaCl) (g kg^{-1})	140	270	250	250	230	240	240	231 ± 42
pH	6.80	5.30	6.00	5.20	5.90	5.90	5.70	5.83 ± 0.53
Specific gravity	–[b]	1.18	1.16	1.19	1.18	1.19	1.17	1.18 ± 0.01

[a]each value indicates mean of at least three measurement.

[b]–, not determined

commonly found in *kapi* (0.31–0.36 and 0.78–2.46 g kg^{-1}, respectively), but in *prahok* these were only detected in sample PF_1 (0.15 and 0.43 g kg^{-1}, respectively). Malic acid was not detected in *toeuk trey*, although formic acid was detected in the early processing stage (*toeuk trey* TE, 0.73 g kg^{-1}). Citric acid was not detected in any samples of *prahok*, *kapi*, and *toeuk trey*.

Table 1.3 Amino acid and organic acid contents of Cambodian traditional fermented fish products

Amino acid or organic acid ($g\ kg^{-1}$)[a]	*prahok* (fish paste)				*kapi* (shrimp paste)			
	PE	PF_1	PF_2	Mean ± SD	KE	KF_1	KF_2	Mean ± SD
Amino acid								
Asp	0.91	1.31	0.84	1.02 ± 0.25	3.05	4.74	4.92	4.24 ± 1.03
Thr	0.66	1.03	1.21	0.96 ± 0.28	1.09	2.90	3.16	2.38 ± 1.13
Ser	0.40	1.02	1.07	0.83 ± 0.37	0.83	1.99	2.69	1.83 ± 0.94
$AspNH_2$	0.30	ND[b]	ND	0.10 ± 0.17	3.54	ND	0.94	1.49 ± 1.83
Glu	1.49	2.52	2.93	2.31 ± 0.74	7.39	4.95	2.49	4.94 ± 2.45
$GluNH_2$	0.54	ND	ND	0.18 ± 0.31	7.60	4.06	5.32	5.66 ± 1.79
Gly	0.35	0.63	0.87	0.62 ± 0.26	9.66	3.77	3.96	5.80 ± 3.35
Ala	1.78	2.06	2.64	2.16 ± 0.44	7.12	5.88	5.86	6.29 ± 0.72
Val	1.23	1.36	1.83	1.47 ± 0.31	7.04	3.34	4.04	4.81 ± 1.97
Cys	0.08	0.23	0.07	0.13 ± 0.09	0.57	0.51	0.40	0.49 ± 0.09
Met	0.42	1.02	1.22	0.89 ± 0.42	4.89	1.73	2.13	2.92 ± 1.72
Ile	1.07	0.96	1.33	1.12 ± 0.19	7.53	3.26	3.58	4.79 ± 2.38
Leu	2.87	3.40	2.27	2.85 ± 0.57	ND	5.98	6.62	4.20 ± 3.65
Tyr	0.81	1.01	1.00	0.94 ± 0.12	7.01	3.40	3.11	4.51 ± 2.17
Phe	0.81	0.87	0.98	0.89 ± 0.09	6.15	3.21	3.51	4.29 ± 1.62
Trp	0.34	0.32	0.44	0.37 ± 0.07	3.81	1.23	1.36	2.14 ± 1.45
Lys	1.13	3.13	3.67	2.64 ± 1.30	14.40	4.53	6.52	8.49 ± 5.23
His	0.47	0.19	0.26	0.30 ± 0.15	2.59	0.94	1.11	1.55 ± 0.91
Arg	0.34	0.12	0.05	0.17 ± 0.15	2.18	1.13	1.17	1.49 ± 0.60
Pro	0.14	0.39	0.44	0.32 ± 0.16	6.26	1.97	1.76	3.33 ± 2.54
Citric acid	ND	ND	ND	ND	ND	ND	ND	ND
Malic acid	ND	0.15	ND	0.05 ± 0.09	0.36	0.31	0.31	0.32 ± 0.03
Formic acid	ND	0.43	ND	0.14 ± 0.25	2.46	0.78	1.11	1.45 ± 0.90
Lactic acid	1.14	0.39	0.75	0.76 ± 0.38	12.90	6.83	3.78	7.83 ± 4.62
Pyroglutamic acid	0.43	0.87	0.53	0.61 ± 0.23	4.95	3.94	16.10	8.31 ± 6.72
Acetic acid	4.00	2.29	7.24	4.51 ± 2.52	26.60	6.61	6.70	13.30 ± 11.5
Succinic acid	1.26	0.59	0.30	0.72 ± 0.49	7.96	3.16	2.50	4.54 ± 2.98
Propionic acid	0.96	0.61	3.32	1.63 ± 1.47	0.63	0.38	0.53	0.51 ± 0.12

[a]each value indicates mean of at least three measurement.

[b]ND, not detected

Table 1.3 Amino acid and organic acid contents of Cambodian traditional fermented fish products (Continue)

Amino acid or organic acid ($g\ kg^{-1}$)[a]	*Toeuk trey* (fish sauce)							
	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Mean ± SD
Amino acid								
Asp	1.58	1.01	3.96	2.58	8.38	10.70	6.47	4.96 ± 3.68
Thr	0.73	0.50	1.87	1.01	6.26	7.55	4.00	3.13 ± 2.85
Ser	ND[b]	ND	1.10	0.52	2.94	4.72	1.72	1.57 ± 1.73
$AspNH_2$	0.63	ND	ND	ND	ND	ND	ND	0.09 ± 0.24
Glu	ND	1.44	6.32	7.26	12.00	14.30	10.10	7.33 ± 5.27
$GluNH_2$	0.21	ND	ND	ND	ND	ND	ND	0.03 ± 0.08
Gly	2.59	0.64	3.01	2.02	6.38	6.73	4.73	3.73 ± 2.28
Ala	3.19	1.52	6.88	4.82	11.20	13.40	9.66	7.23 ± 4.36
Val	10.50	1.05	4.56	3.28	7.96	9.43	6.61	6.19 ± 3.41
Cys	1.31	ND	0.57	0.60	0.68	0.73	0.64	0.65 ± 0.38
Met	ND	0.44	1.86	1.97	3.28	4.08	3.29	2.13 ± 1.53
Ile	3.75	1.12	3.71	2.84	4.63	5.68	4.17	3.70 ± 1.44
Leu	1.56	1.91	5.20	4.21	5.40	6.94	5.39	4.37 ± 1.97
Tyr	1.20	ND	1.25	1.14	1.16	1.37	1.14	1.04 ± 0.46
Phe	0.12	ND	1.38	1.67	4.51	5.58	4.06	2.47 ± 2.23
Trp	0.08	ND	ND	ND	2.14	1.75	ND	0.57 ± 0.95
Lys	ND	1.66	6.16	4.52	13.20	16.10	11.50	7.59 ± 6.10
His	ND	ND	0.52	0.73	1.79	2.64	1.57	1.03 ± 0.99
Arg	ND	ND	0.24	ND	ND	ND	ND	0.03 ± 0.09
Pro	ND	ND	ND	ND	3.02	3.41	1.75	1.17 ± 1.54
Organic acid								
Citric acid	ND	ND	ND	ND	ND	ND	ND	ND
Malic acid	ND	ND	ND	ND	ND	ND	ND	ND
Formic acid	0.73	ND	ND	ND	ND	ND	ND	0.10 ± 0.28
Lactic acid	1.00	3.39	0.70	1.65	3.25	2.82	3.55	2.34 ± 1.20
Pyroglutamic acid	0.35	1.65	0.35	0.71	2.49	2.74	1.94	1.46 ± 1.00
Acetic acid	3.57	11.30	1.90	4.38	6.00	4.10	5.99	5.32 ± 3.00
Succinic acid	ND	3.21	0.48	1.18	2.29	1.51	1.80	1.49 ± 1.09
Propionic acid	ND	2.04	2.72	0.74	1.33	1.03	1.33	1.31 ± 0.88

[a]each value indicates mean of at least three measurement.

[b]ND, not detected

1.4 Discussion

During manufacture, Cambodian fermented fish products go through primary processing phases, referred to as early fermentation stage (samples PE, KE, and TE). During these phases, fishes were incubated in the presence of relatively low concentrations of salt (44, 73, and 140 g kg^{-1}, respectively, Table 1.2), and pre-maturated prior to the main fermentation, which is

performed in the presence of higher concentrations of sodium chloride (170–180 g kg^{-1} for *prahok*, 200–210 g kg^{-1} for *kapi*, and 230–270 g kg^{-1} for *toeuk trey*). *Toeuk trey* TE was an early stage sample before extracting liquid fish sauce; thus, it had a markedly low moisture content (240 g kg^{-1}) (Table 1.2). At this stage, the fish materials in *prahok* and *toeuk trey* contained the entire gut, which was removed later.

High crude fat contents were found, possibly due to these manufacturing procedures (151 g kg^{-1} for *prahok* PE and 234 g kg^{-1} for *toeuk trey* TE). In contrast, because of the removal of guts, the final products of *prahok* and *toeuk trey* had considerably low fat contents (1.7–10.7 g kg^{-1} for *prahok*, and 0.6–5.5 g kg^{-1} for *toeuk trey*). Accordingly, two fish pastes produced in other countries, *lona ilish* in India and *adjuevan* in Cote d'Ivoire that are fermented using whole fish bodies, including the guts, have high fat contents (94 g and 123 g kg^{-1}, respectively).[38-40)]

Interestingly, more than half of the samples (ten out of thirteen samples) contained acetic acid as the predominant organic acid (Table 1.3). The other three samples (*kapi* KF_1 and KF_2, *toeuk trey* TF_2) contained lactic acid, pyroglutamic acid, and propionic acid as the predominant constituents, although these products also had considerable amounts of acetic acid (Table 1.3). The presence of these organic acids could be attributable to the presence of many gram-positive rods and cocci identified in all three fermented foods, as described later (Fig. 3.5, chapter III).

However, the basis of preservation effects was probably primarily due to a high salt concentration and not due to the bacteriocidal effect of the organic acids, as these acids cannot be sufficiently protonated to diffuse across the plasma membrane of bacterial cells within the mildly acidic and alkaline pH ranges observed for *prahok*, *kapi*, and *toeuk trey* (pH values >6; Table

1.2).[41] The salt concentrations of Cambodian fermented fish products (170–270 g kg^{-1}) were comparable to the salt-cured fish sauces in other Asian countries (160–230 g kg^{-1}).[17]

Free amino acids are substances naturally present in food and they determine the taste, flavor and quality of various foodstuffs.[42-45] In *toeuk trey*, especially in TF_4, TF_5 and TF_6, high concentrations of aspartic acid and glutamic acid might contribute to umami taste, and alanine and lysine might be related to sweetness and bitterness, respectively.[46] Also in *prahok* and *kapi*, these four amino acids were detect at high concentrations, excepting aspartic acid in *prahok*. High level of leucine, which is also bitter taste, was commonly found in *prahok* and *kapi*. These compounds have been revealed to be taste-determining amino acids in Italian fish sauce and Asian fish sauces, produced by autolysis and microbial action during fermentation.[42,47-49] Raw materials and salt content may also determine the composition of above substances. Other reports described that the total essential amino acids in fish sauce ranged between 42.36 and 46.65 mg/mL, which suggested that fish sauce will contribute significantly to the supply of essential amino acids such as valine, isoleucine, phenylalanine and lysine in the diet.[50]

CHAPTER II

Volatile Compounds in Cambodian Traditional Fermented Fish Products

2.1 Introduction

Based on the studies described chapter I on chemical compositions, we obtained the first detailed results of the proximate composition, organic acids and amino acids in the Cambodian traditional fermented fish products, *prahok*, *kapi*, and *toeuk trey*.

Recently, Nattida (2012)[45] reported that glutamic acid is the most abundant amino acid both in total amino acids of *plara* and free amino acids of *prahok*, two fermented fish pastes. These suggest that preferred typical taste of these fermented fish products are also related to umami substance as same as in *nampla* and *budu*, fish sauce.[51] *Prahok* had greater content of calcium and phosphorus than fresh fish since of decomposition process of fish bone and another structures by microorganisms during fermentation process.[27,52-54] It was reported that the protein hydrolysis of shrimp paste *kapi* is mediated by microbial or indigenous proteases.[55] Consequently, short chain peptides and free amino acids are released, resulting in the formation of typical flavor and taste.[28] It should be note that glutamic acid was also determined to be the most abundant amino acid in *kapi*, in both total amino acids and free amino acids.[45] These compounds are a good source of flavor, together with natural antioxidants, which provide health benefits.[56] Giri et al. (2010)[57] reported that enzymes present in fish together with some halotolerant and halophilic microorganisms induce the hydrolysis of the fish proteins and then

produces free amino acids, peptides and ammonia, supporting the above reports for formation of variety of volatile flavor compounds.

Many researches have been studied for volatile compounds in fish sauce from different countries by using different methods and sample preparations.[25,58-63] But so far, no detailed research have been done scientifically for Cambodian traditional fermented foods. Particularly, there is no information on volatile compounds of fermented foods studied in this thesis, *prahok*, *kapi* and *toeuk trey*. This chapter aims to identify the volatile compounds of Cambodian traditional fermented fish products by using Gas Chromatography-Mass spectrometry (GC-MS).

2.2 Materials and Methods

2.2.1 Sample information

A total of 12 samples used in this chapter are summarized in chapter I, (Table 1.1). Excluding sample TE, which is not used in this studied.

Toeuk trey TF_1 is pure grade which was first extract after 2-3 months fermentation. TF_{2-6} is final products. TF_{1-3} are fish sauces made from mix of small freshwater fish and TF_{4-6} is made from sea fish.

2.2.2 Volatile compounds analyses

The identification of volatile compound from *prahok*, *kapi* and *toeuk trey* was carried out by modified methods of Michihata et al. (2002) and Shimoda et al. (1996).[25,61] A 5 ml or 5 g of sample was used, then 100 µl of 50 ppm cyclohexanol was added as the internal standard. Thereafter, it was placed into glass vessel at 45 °C and purged with a flow rate of 50 ml/ min of N_2 gas, passed through the vessel for 5 min. The volatile compounds were introduced and trapped in a TENAX TA column (20/35 mesh, 100 mg) (Gestel Inc.) 60 mm x 4 mm i.d. for 60 min. Then dry N_2 was passed through the TENAX TA column for 5 min to remove water adsorption. The column was placed in a heating block of the thermal desorption unit, TDU (Gestel Inc.), and heated to 230 °C volatilization, which were then cryofocused at -120 °C and injected into the gas chromatography (GC) column. The gas chromatography mass spectrometry (GC-MS) system was used for identification of the volatile compounds was GC-MS C8790A/5375C (Agilent Technologies Inc.); gas chromatography column, DB-Wax 60m x 0.25 mm x 0.25 um (Length x Diameter x film) (Agilent), with split ratio of 10. The column was kept at 40 °C, and held at 40 °C for 10 min, then heated up to 230 °C at a program rate of 4 °C/ min and held at 230 °C for 12 min. The values of identified volatile compounds were calculated from the peak area by referring to retention time of each compound; it was carried out in relation to the internal standard corresponding to 0.5 ppm. Results were means of triplicate samples.

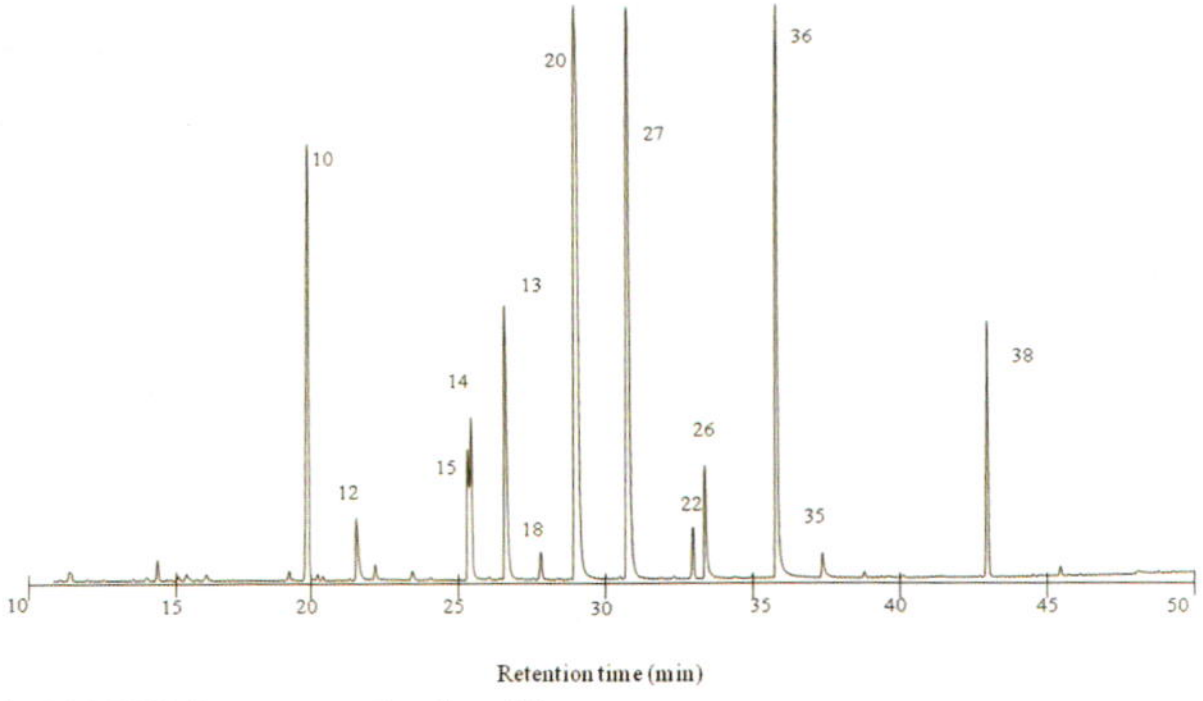


Fig. 2.1 GC-MS chromatogram of *toeuk trey* TF_1

Chromatogram of volatile compounds identified for *toeuk trey* obtained by Gas Chromatography system. The numbers correspond to the compounds indicated in Table 2.1

2.3 Results

Table 2.1 showed the results of volatile compounds identified for six *toeuk trey* samples from the GC-MS analyses. A total of 39 volatile compounds were detected. They included various acids, alcohols, aldehydes, ketones, nitrogen-containing, sulfur-containing, and other compounds. The concentration of each compound was determined by comparing the peak area with an internal standard, cyclohexanol.

In the group of acid compounds, butanoic acid was identified as major acid in all *toeuk trey* samples. Meanwhile, 4-methylpentanoic acid, 2-methylpropanoic acid and acetic acid were frequently detected in *toeuk trey* samples. Propanoic acid was detected in all *toeuk trey* samples. Other acids were frequently detected, product dependent.

Large amount of butanoic acid and 2-methylbutanoic acid were detected in *prahok* PF_1 (Table 2.1). Interestingly, acid contents of *kapi* KE, KF_1 and KF_2 were quite minor. As for alcohols, 1-butanol 3-methyl was largely detected in *prahok* PF_1. Besides that, 2-propanol was predominantly found in PE, whereas other samples were found to be lower level. Notably, a large content of aldehydes and esters were detected only in PE, although butanal, propanal, 2-methylbutanal, 3-methylbutanal and benzaldehyde were slightly found in KF_1 and KF_2.

Only 2-butanone was detected in all *prahok* and *kapi* samples. 2-heptanone was found only in *kapi* samples. A large amount of nitrogens were detected in almost all samples of *prahok* and *kapi*, excepting *prahok* PE. Meanwhile, sulfurs containing N,N-dimethylmethanamine was detected in PE and KE, and thiobismethane was slightly found in KE and KF_2. Dimethyl disulfide and trisulfidedemethyl were detected in KF_1 and KF_2.

Table 2.1 Volatile compounds identified in *toeuk trey*

Peak No.[1]	RT	Compound name	*Toeuk trey* (fish sauce)					
			TF_1	TF_2	TF_3	TF_4	TF_5	TF_6
		Acids						
22	30.91	Acetic	0.23±0.132	0.23±0.206	0.77±0.156	1.05±0.187	1.39±1.627	0.35±0.084
26	33.22	Propanoic	0.20±0.061	0.48±0.430	0.69±0.138	0.67±0.063	1.18±0.198	0.64±0.138
27	33.98	Propanoic, 2-methyl	0.74±0.043	0.66±0.201	0.74±0.069	0.57±0.067	0.33±0.035	0.21±0.50
29	35.42	Butanoic	2.31±0.220	2.31±0.105	5.17±0.56	2.19±0.321	1.66±0.450	1.34±0.465
32	36.46	Butanoic, 3-methyl	ND[2]	ND	ND	1.58±0.258	ND	ND
33	36.51	Butanoic, 2-methyl	1.87±0.042	ND	2.44±0.133	ND	ND	ND
35	38.11	Pentanoic	0.24±0.022	1.92±0.883	0.19±0.027	ND	ND	0.84±0.249
36	39.58	Pentanoic, 4-methyl	1.35±0.051	0.67±0.511	1.57±0.050	0.19±0.180	0.13±0.070	0.36±0.230
37	40.53	Hexanoic	0.07±0.013	ND	0.13±0.010	ND	ND	ND
		Alcohols						
7	15.65	2,2-dimethyl propanol	0.03±0.003	ND	0.07±0.010	ND	0.08±0.002	ND
8	16.20	2-Butanol	0.02±0.001	0.06±0.051	0.05±0.004	ND	ND	ND
10	19.26	1-Propanol, 2-methyl	0.10±0.012	0.13±0.10	0.16±0.025	0.15±0.008	0.17±0.010	ND
11	20.39	2-Pentanol	0.01±0.00	ND	ND	ND	ND	ND
12	21.30	1-Butanol	0.11±0.006	ND	0.11±0.003	ND	ND	ND
13	21.81	1-Penten-3-ol	0.04±0.001	ND	0.04±0.002	ND	ND	ND
14	23.57	1-Butanol, 3-methyl	0.06±0.001	ND	ND	0.11±0.04	0.21±0.011	0.34±0.006
15	24.99	3-Buten-1-ol, 3-methyl	0.03±0.001	ND	ND	ND	ND	ND
20	29.92	Cyclohexanol	1.00±0.000	1.00±0.000	1.00±0.000	1.00±0.000	1.00±0.000	1.00±0.000
24	32.07	1-Hexanol, 2-ethyl	0.02±0.004	0.03±0.018	ND	0.04±0.016	ND	0.05±0.047
28	34.72	1,2-Propanediol	0.04±0.012	ND	ND	0.05±0.000	ND	ND
30	35.54	Ethanol,2-(2-ethoxyethoxy)	ND	0.08±0.000	ND	ND	ND	ND
34	37.86	1-Propanol, 3-(methylthio)	0.07±0.038	0.13±0.039	0.05±0.011	ND	0.06±0.004	0.02±0.034
38	43.94	Phenol	0.49±0.013	0.30±0.025	0.19±0.001	ND	0.17±0.010	0.27±0.034
39	45.45	Benzenemethanol	0.02±0.000	ND	ND	ND	ND	ND
		Aldehydes						
1	7.63	Butanal	ND	ND	0.09±0.075	ND	ND	ND
4	10.88	Butanal, 2-methyl	0.05±0.010	0.20±0.16	0.08±0.011	0.48±0.047	0.25±0.055	0.24±0.128
5	11.07	Butanal, 3-methyl	0.15±0.017	0.14±0.051	0.19±0.030	0.58±0.052	0.18±0.035	0.15±0.071
19	29.53	Nonanal	0.02±0.003	ND	ND	0.04±0.002	ND	0.12±0.054
23	31.31	Propanal, 3-(methylthio)	0.03±0.005	ND	ND	ND	ND	ND
25	33.28	Benzaldehyde	0.35±0.015	ND	ND	ND	0.69±0.183	ND
31	35.55	2-Butenal, 2 methyl	ND	0.12±0.014	ND	ND	ND	ND
		Ketones						
2	7.71	2-Propanone	ND	ND	ND	ND	ND	1.08±0.391
3	10.37	2-Butanone	0.04±0.002	0.42±0.223	0.07±0.017	0.68±0.077	0.14±0.150	1.49±0.123
6	13.78	2-Butanone, 3-methyl	0.03±0.001	0.06±0.013	ND	ND	ND	0.12±0.020
		Nitrogens						
16	25.63	Pyrazine, methyl	ND	ND	ND	ND	ND	0.05±0.002
17	27.50	Pyrazine, 2,5-dimethyl	ND	ND	ND	ND	0.09±0.002	0.10±0.014
18	27.82	Pyrazine, 2,6-dimethyl	0.02±0.001	ND	ND	ND	0.10±0.004	0.05±0.006
21	30.11	Pyrazine, trimethyl	0.01±0.000	ND	ND	ND	ND	ND
		Sulfur-containing						
9	18.03	Disulfide, dimethyl	ND	ND	ND	ND	0.10±0.017	0.03±0.043

[1]Peak number refers to peaks in Fig. 2.1; [2]ND, not detected; Values are average ± standard deviation. The amounts of volatile compounds are expressed in part per million (ppm).

Table 2.2 Volatile compounds identified in *prahok* and *kapi*

Peak No.[1]	RT	Compound name	*Prahok* (fish paste)			*Kapi* (shrimp paste)		
			PE	PF_1	PF_2	KE	KF_1	KF_2
		Acids						
44	30.85	Acetic acid	1.82±1.361	1.55±1.98	0.15±0.066	0.20±0.071	ND	0.04±0.010
50	33.15	Propanoic acid	1.35±0.331	5.41±4.54	ND[2]	ND	ND	ND
53	33.95	Propanoic acid, 2-methyl	0.74±0.169	8.79±8.162	0.15±0.097	ND	ND	ND
54	35.38	Butanoic acid	12.24±1.802	33.96±29.386	0.46±0.038	0.13±0.090	ND	ND
55	36.46	Pentanoic acid	0.17±0.000	0.39±0.484	ND	ND	ND	ND
56	36.47	Butanoic acid, 2-methyl	1.23±1.628	33.71±28.685	ND	0.30±0.240	ND	ND
57	39.54	Pentanoic acid, 4-methyl	0.09±0.075	1.77±1.460	0.33±0.067	0.20±0.105	ND	ND
63	51.15	Hexadecanoic acid	ND	3.78±5.693	0.26±0.003	ND	ND	ND
65	53.71	Hexadecenoic acid, Z-11	ND	ND	0.07±0.044	ND	ND	ND
		Alcohols						
11	11.80	Ethanol	ND	ND	ND	ND	0.30±0.087	1.52±1.380
12	11.84	2-Propanol	2.60±3.471	ND	ND	ND	ND	ND
16	16.15	Ethanol, 2-trimethylsilyl	ND	ND	ND	0.19±0.007	ND	ND
18	16.72	1-Propanol	ND	ND	0.07±0.030	0.13±0.043	ND	ND
23	19.14	1-Propanol, 2-methyl	0.50±0.544	1.12±0.924	0.05±0.032	0.10±0.034	ND	0.20±0.045
24	21.23	1-Butanol	2.09±2.314	2.62±2.183	0.07±0.020	ND	ND	ND
26	21.78	1-Penten-3-ol	2.54±2.757	8.07±6.720	0.18±0.105	0.42±0.135	0.54±0.192	1.20±0.160
30	23.51	1-Butanol, 3-methyl	ND	12.10±10.089	0.48±0.208	1.66±0.161	0.54±0.186	1.20±0.141
32	24.89	2-Buten-1-ol, 2-methyl	0.49±0.646	ND	ND	ND	ND	ND
33	24.98	1-Pentanol	0.77±0.050	1.93±1.604	0.27±0.370	ND	ND	ND
36	27.19	2-Penten-1-ol, (Z)	0.46±.113	ND	ND	ND	ND	ND
38	28.06	3-Pentanol, 2-methyl	1.57±2.356	ND	ND	ND	ND	ND
39	28.21	1-Hexanol	1.09±1.712	0.92±0.770	0.06±0.014	ND	ND	ND
45	30.92	1-Octen-3-ol	0.85±0.283	5.10±4.430	0.17±0.018	ND	ND	ND
47	32.02	1-Hexanol, 2-ethyl	ND	ND	0.06±0.012	0.08±0.022	ND	ND
58	42.15	Benzeneethanol	ND	ND	ND	0.05±0.005	ND	0.70±0.823
59	43.89	Phenol	0.16±0.004	ND	ND	0.09±0.014	0.50±0.312	0.75±0.524
60	45.38	Phenol, 4-methyl	ND	ND	0.05±0.022	ND	ND	ND
61	45.39	Benzenemethanol	0.45±0.005	2.38±2.179	ND	ND	ND	ND
62	49.48	Phenol, 2,4-bis(1,1 -dimethylethyl)	ND	2.11±2.318	0.06±0.000	ND	ND	ND
51	33.17	2,3-Butanediol [R-(R*, R*)]	ND	ND	ND	ND	0.30±0.056	0.60±0.384
		Aldehydes						
4	7.10	Propanal	ND	ND	ND	ND	0.10±0.109	0.15±0.029
7	9.39	Butanal	1.14±1.586	ND	ND	ND	0.04±0.013	0.08±0.018
9	10.85	Butanal, 2-methyl	1.90±2.786	ND	ND	ND	0.23±0.094	0.45±0.115
10	11.04	Butanal, 3-methyl	ND	ND	ND	ND	0.28±0.087	0.70±0.180
14	13.81	Butanal, 3-methyl-Isovaleraldehyde	1.31±0.694	ND	ND	ND	ND	ND
21	18.47	Hexanal	4.38±2.785	ND	ND	ND	ND	ND
22	18.96	2-Butenal, 2-methyl	0.49±0.381	ND	ND	ND	ND	ND
25	21.65	2-Pentenal, 2-methyl	0.45±0.034	ND	ND	ND	ND	ND
28	22.66	Heptanal	0.30±0.218	ND	ND	ND	ND	ND
35	26.95	Octanal	3.19±4.385	ND	ND	ND	ND	ND
41	29.50	Nonanal	0.63±0.970	1.49±1.249	0.04±0.025	ND	ND	ND
52	33.23	Benzaldehyde	0.45±0.005	ND	0.19±0.072	ND	0.15±0.043	0.19±0.052
		Esters						
15	14.17	Butanoic acid, methyl ester	0.23±0.048	ND	ND	ND	ND	ND
17	16.56	Butanoic acid, ethyl ester	0.06±0.057	ND	ND	ND	ND	ND
19	16.79	Isopropyl butanoate	6.36±7.341	ND	ND	ND	ND	ND
29	22.88	Pentanoic acid, 4-methyl-, ethyl ester	0.52±0.185	ND	ND	ND	ND	ND
31	23.88	Butanoic acid, butyl ester	0.36±0.317	ND	ND	ND	ND	ND
66	55.77	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	ND	2.54±3.184	0.21±0.016	ND	ND	ND
		Furan						
13	12.65	Furan, 2-ethyl	0.74±0.797	ND	ND	ND	ND	ND

Table 2.2 (Continued)

Peak No.[1]	RT	Compound name	*Prahok* (fish paste)			*Kapi* (shrimp paste)		
			PE	PF_1	PF_2	KE	KF_1	KF_2
		Ketones						
5	7.60	Acetone	ND	ND	ND	ND	7.44±6.705	ND
6	7.75	2-Propanone	ND	0.54±0.736	0.03±0.002	ND	ND	2.42±1.372
8	10.33	2-Butanone	2.63±4.166	1.77±1.484	0.07±0.012	0.11±0.046	0.30±0.112	0.30±0.092
27	22.54	2-Heptanone	ND	ND	ND	0.95±1.54	0.54±0.186	0.23±0.048
48	32.97	3,5-octadiene-2-one	0.19±0.001	ND	ND	ND	ND	ND
49	32.99	3,5-octadiene-2-one	0.28±0.018	ND	ND	ND	ND	ND
		Nitrogens						
3	6.70	Trimethylamine	ND	2.23±3.143	0.01±0.009	0.08±0.000	0.35±0.310	4.10±1.283
34	25.63	Pyrazine 2,5-dimethyl	ND	15.96±13.304	0.26±0.217	3.97±1.126	0.13±0.039	0.51±0.045
37	27.69	Pyrazine, 2,6-dimethyl	ND	1.38±1.151	ND	0.18±0.052	0.50±0.150	0.56±0.228
42	29.60	Pyrazine,2-ethyl-6-methyl	ND	ND	ND	0.06±0.017	ND	ND
43	29.98	Pyrazine, trimethyl	ND	29.21±26.824	0.05±0.007	0.52±0.131	0.11±0.018	ND
46	31.09	Pyrazine, 3-ethyl-2,5-dimethyl	ND	2.45±2.045	0.06±0.009	0.60±0.155	ND	ND
64	53.07	Indole	ND	1.64±1.733	0.06±0.000	0.06±0.003	ND	ND
		Sulfurs						
1	5.48	Methanamine, N,N-dimethyl	2.23±3.143	ND	ND	1.28±0.114	ND	ND
2	6.60	Methane, thiobis	ND	ND	ND	0.09 ±0.063	ND	0.21±0.143
20	17.70	Dimethyldisulfide	ND	ND	ND	ND	1.41±0.541	0.50±0.188
40	29.08	Trisulfide dimethyl	ND	ND	ND	ND	0.80±0.233	0.70±0.175

[1]Peak number refers to peaks in figure 2; [2]ND, not detected; Values are average ± standard deviation. Volatile compounds are expressed part per million (ppm).

2.4 Discussion

Several reports showed specific acid compounds dominantly contained in fish sauces. Among them, acetic acid was the most abundant acid in Malaysian fish sauce *(Budu)*[56] and Thai fish sauce (*nampla*),[62] which might contribute to vinegar-like pungent aromas of fish sauce as reported by Mohamed et al. (2012).[56] On the other hand, 2-methylpropanoic acid and 2,2-dimethylpropanoic acid were found to be abundant in Taiwanese and Philippine fish sauce (*patis*),[63] and 3-methylbutanoic acid was the most abundant acid in Japanese fish sauce (*shottsuru*).[25,62] Some of these reports correspond to our results that all *toeuk trey* samples contained acetic acid and 2-methylopropanoic acids (Table 2.1). In *toeuk trey*, the most representative volatile compound was found to be butanoic acid. Butanoic acid was also identified as one of the major acids in *patis*, although the production process is greatly different (*patis* is treated by distillation under reduced pressure).[63,64] Butanoic acid was thus implicated in the characteristic of *toeuk trey* aroma and flavor.

A large number of normal and branched alcohols were detected in *toeuk trey* (Table2.1). The long straight chain alcohol, such as 1-pentanol, might slightly contributed to aroma of *toeuk trey* referring to the lower threshold values of this acid.[56] Moreover, branched chain alcohols such as 1-propanol 2-methyl, 1-penten-3-ol and 1-butanol 3-methyl were detected as significant flavor contributor toward *toeuk trey*.[24,25,65] Aldehyde and ketone compounds, 2-methylbutanal, 3-methylbutanal, and 2-butanone, were detected in all *toeuk trey* samples. These were similar to aldehydes and ketones found in other fish sauce by the same technique.[62]

Aldehydes were possibly derived from the lipid oxidation during fermentation in the ripening period, and branched short-chain aldehydes or aromatic aldehydes might have been generated from the amino acid metabolism.[25,56,66] These aldehydes were generally considered to cause un-pleasant oxidation flavor in foods,[25,59] and their odor threshold value are low.[61] However, Mohamed et al. (2012)[56] reported that such aldehydes are very important aroma for fish sauce, therefore aldehydes content might be important characteristic in *toeuk trey* odor.

Ketones are responsible for the cheesy note in fish odor,[25,56,64] but it is unclear whether these compounds had impact on *toeuk trey* flavor because of their high threshold value.[25]

Small amount of nitrogen-containing pyrazine and its derivatives were also found in TF_1, TF_5 and TF_6. Pyrazine could be responsible for the burnt and sweet odor,[59,64] and have been characterized as unique flavor and aroma associated with the roasting and toasting of numerous foods.[56,65] Sanceda et al. (1986)[62] reported that pyrazine is cause of the fragrant smell of fish sauce, and also in the *toeuk trey* seems to affect the aroma. Single sulfur-containing compound, dimethyldisulfide, was detected in *toeuk trey* TF_5 and TF_6. This compound normally originates from the raw fish or forms during the fermentation process,[56,67] and is especially the un-pleasant odor.[25,59] Noteworthy, TF_1 had large number of volatile compounds due to the process of fermentation, possibly by the degradation of fish material at relatively low salt concentration (Table 1.2). From the above consideration, the smell of *toeuk trey* includes numerous aroma components, but among which butanoic acid mainly contributes to exude peculiar smells.

As discussed above for fish sauce, a lot of volatile compounds found in *prahok* and *kapi* might contribute to distinctive aroma and flavor of the products. Among the components contained in these two type of foods, esters were essentially absent in *prahok* PF_1 and PF_2, and all *kapi* samples, excepting 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester (Table 2.3). It is interesting to speculate that microbes inhabiting fermentation medium consumed these esters during maturation process of *prahok*, although the compounds could be produced by esterification of alcohol with carboxylic acid that are also formed by microbial and enzymatic decomposition of lipids.[60,68,69)]

Similar to esters, it is noteworthy that *prahok* PE included varieties of aldehydes. Hence, these ester and aldehyde compounds exude peculiar smells of PE, which was different from other samples, PF_1 and PF_2. This is because PE was prepared from tiny fresh water fish with low salt concentration (4.5%) and fermented for short period (20 days) as a primary processing sample, probably affecting the release of metabolite compounds in fermentation medium as compared to maturated products. *Kapi* is relatively rich in sulfur-containing compounds, representing the specific flavor of the products that were clearly different from fish made *prahok* and *toeuk trey*.

CHAPTER III

Microbial Composition in Cambodian Traditional Fermented Fish Products using culture-dependent method

3.1 Introduction

Fermentation is one of the method used to produce and preserve foods and has been practiced for many years in many part of the world. It provides ways to preserve food products, enhance nutritive value, destroy undesirable factors, improve appearance and taste of the foods.

In fermentation, raw materials are converted into products through activities of endogenous enzymes or microorganisms (bacteria, yeasts, and molds). Various microorganisms are involved in common food fermentation processes. In particular, lactic acid bacteria (LAB) in food are a type of bio-preservation system. Accordingly, LAB are able to control pathogenic and spoilage microorganisms through production of peroxidases, organic acids, bacteriocins, and so on.

Besides, fermentation of fish products, in itself, will not do much in preserving them, as it will degrade fish muscle proteins into smaller peptides and amino acids that are nutrients for microorganisms. Therefore, fermentation is often combined with the addition of salt or drying to reduce water activity and eliminate proteolytic and putrefying microorganisms. The process can be partial and last for several hours to several weeks, such as in fermentation of fish foods in

Asian countries, or extensive and last for several months such as in fish paste and fish sauce preparation.

Although, there are many types of fermented fish products in Asia and around the world, it is continues to use up to now. Particularly, fermented fish products in Cambodia are still the main part of Cambodian culture since these are intimately entwined with the life of local people. The fermentation processes employed in the production of these indigenous products often rely entirely on natural microflora of the raw material and the surrounding environment.

The procedures are handed down from one generation to the next as a village-art process. The traditional fermentation food industries are commonly home-based and highly reliant on indigenous materials without the benefit of using commercial starter cultures; microbial assemblages are unique and highly variable per product and per region. Moreover, studies on fermented fish products have attracted considerable attention due to an increasing demand for desirable biological functions and probiotic effects derived from the chemical constituents and microorganisms contained in these products.

In this chapter, we obtained the first detailed analyses of the microbial composition of the traditional Cambodian fermented fish products, *prahok*, *kapi*, and *toeuk trey* to acquire a thorough scientific understanding of these products. Isolated microbes were taxonomically classified by using 16S ribosomal RNA gene (rDNA)-dependent phylogenetic analysis.

3.2 Materials

3.2.1 Samples used for analyses

The samples used in this chapter are summarized in chapter 1 (Table 1.1).

3.2.2 Microbial analyses

3.2.3 Physiological and morphological characteristics

A 0.5 g sample that was stored in refrigerator was homogenized with 4.5 ml of saline containing 8.5 g kg^{-1} NaCl. Ten-fold dilutions of homogenates (100 µl) were spread on tryptone soy agar (TSA; Oxoid, Basingstoke, UK), Gifu anaerobic medium (GAM) agar (Nissui, Tokyo, Japan), de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Basingstoke, UK), and potato dextrose agar (PDA; Nissui, Tokyo, Japan) containing 0.1 g kg^{-1} chloramphenicol. To detect halophilic or halotolerant bacteria, agar plates containing 100 g kg^{-1}NaCl were used. TSA and PDA plates were aerobically incubated at 30°C for 2–7 days, and MRS and GAM agar plates were anaerobically incubated at 30 °C using an Anaero Pack system (Mitsubishi Gas Chemical, Tokyo, Japan) for 5–7 days. Viable counts (colony-forming units;cfu) were determined separately based on colony colors and shapes. Representative colonies (up to 4 isolates from each group) were evaluated for their morphological properties, including Gram staining (Fig 3.1), catalase test, and spore formation.[70)]

The 181 strains which isolated were streaked twice (Fig. 3.1B), and single colony was picked up for making 5 ml liquid culture, then making freeze stocks containing 15-20 % glycerol to be stored at -80 °C.

A. Bacterial strain cultured

B. Isolated bacteria

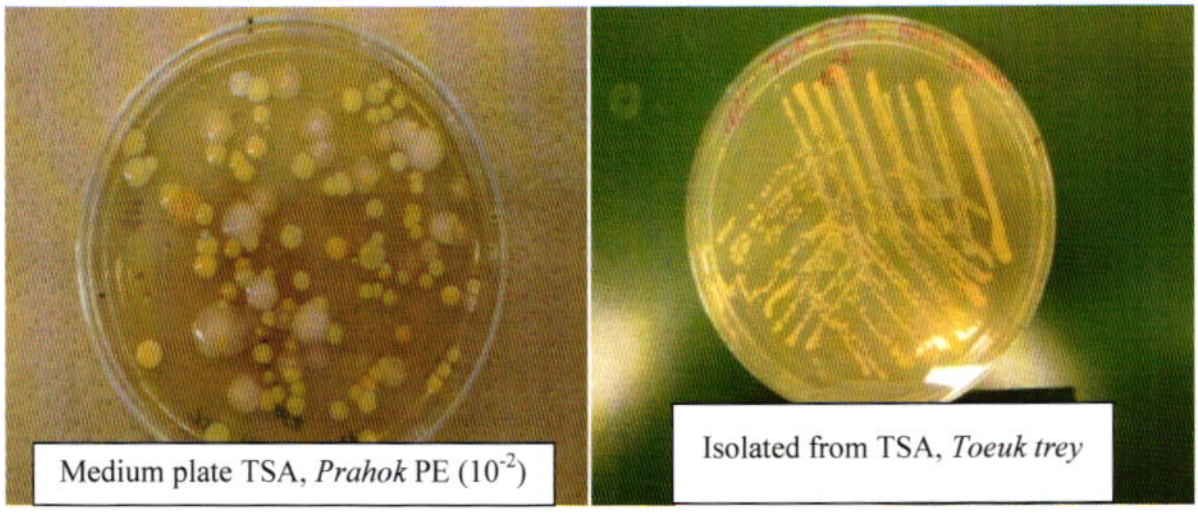


Fig. 3.1 Isolation and gram staining of bacterial cells

C. Microscope image of microorganisms

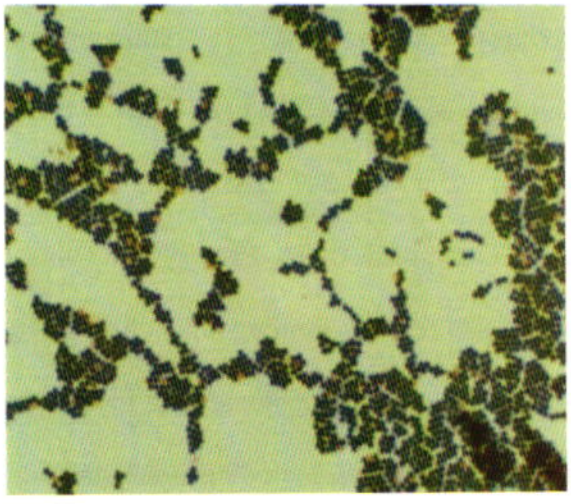


Fig. 3.1 Isolation and gram staining of bacterial cells (continued)

3.2.4 Sequence analyses of 16S ribosomal RNA gene

The stock cultures of 181 strains were inoculated into the media corresponding to that used in isolation. Genomic DNA was extracted using a Wizard Genomic DNA Extraction Kit (Promega, Fitchburg, WI, USA), entire 16S rDNA regions were amplified using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) and the oligonucleotides 7F (5′ AGAGTTTGATYMTGG CTCAG-3′) and 1510R (5′-ACGGYTACCTTGTTACGACTT-3′) as the primer pair (Fig. 3.2).[71]

Amplified fragments were purified with a QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands) and then directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) (Fig. 3.3). Polymerase chain reaction was performed with 25 cycles of denaturation at 96°C for 20 s, annealing at 50 °C for 20 s, and extension at 72 °C for 1 min 30 s. At least 600 bp covering plural hyper-variable regions were analyzed to determine closely-related genus, within the range of >97% sequence identity. The resulting sequences were compared with known sequences using the basic local alignment search tool (BLAST); (http://www.ncbi.nlm.nih.gov)

Taxonomic classifications were confirmed from both the morphological and 16S rDNA sequence data. The sums of the assigned genera were calculated for respective media plates, and average values were obtained from multiple experiments at least three times. When the counts for specific genera differed between the media plates, the data from the plates with the maximum counts were regarded as the final values. The total cell counts from the different fermented food samples were determined by summing the final counts obtained for each genus.

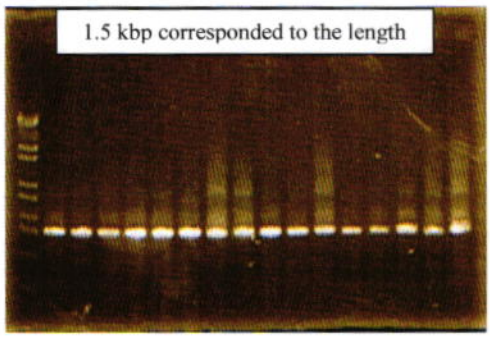
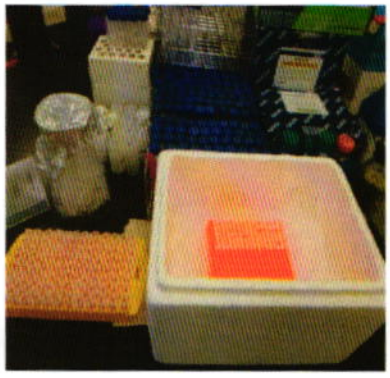


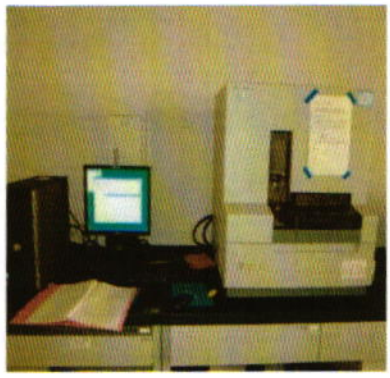
Fig. 3.2 16S ribosomal DNA amplification with PCR

A. Sequencing reaction



B. 96-micro plate for genetic analyzer

C. Sequencing apparatus (Genetic analyzer)



D. Image of sequence analysis

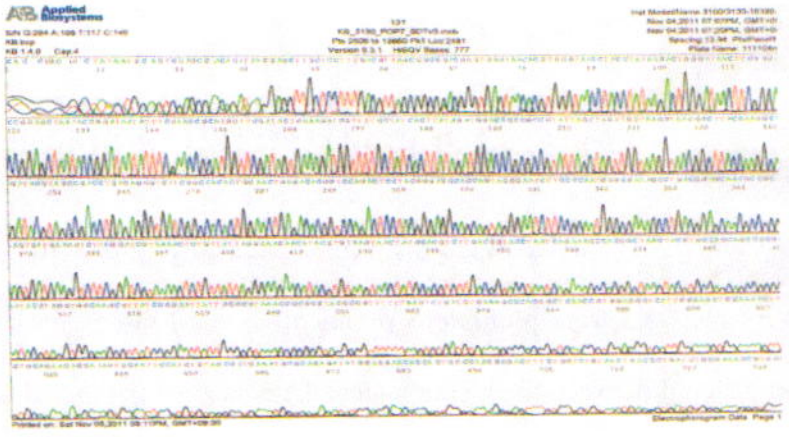


Fig. 3.3 Preparations of sequence reaction and analysis for their species identification

3.3 Results

The data for the colony counts are shown in Table 3.2, and microbiota at genus level found in each product are shown in Fig. 3.4. Among the fermented food samples, the bacteria were primarily gram-positive cocci or rods. Total cell counts were calculated by summing the counts for the respective identified genera; 10^5–10^7 cfu g^{-1} for *prahok*, and 10^5–10^8 cfu g^{-1} for *kapi*, and 10^2–10^6 cfu g^{-1} for *toeuk trey*. In particular, halophilic and halotolerant bacterial genera, *Staphylococcus* and *Tetragenococcus*, were frequently identified in *prahok* and *kapi*. By grouping the cell counts from colony morphologies, these bacteria were estimated to be at 10^5–10^6 cfu g^{-1} in *prahok* and 10^4–10^8 cfu g^{-1} in *kapi*. *Clostridium* was also detected as one of the major species in *prahok*, as observed for 1-month-fermented *prahok* PF_1 (10^7 cfu g^{-1}). This genus was also commonly found in *kapi* at 10^5–10^7cfu g^{-1}, but in *toeuk trey* only the early fermentation stage sample (TE) contained *Clostridium* (10^4 cfu g^{-1}).

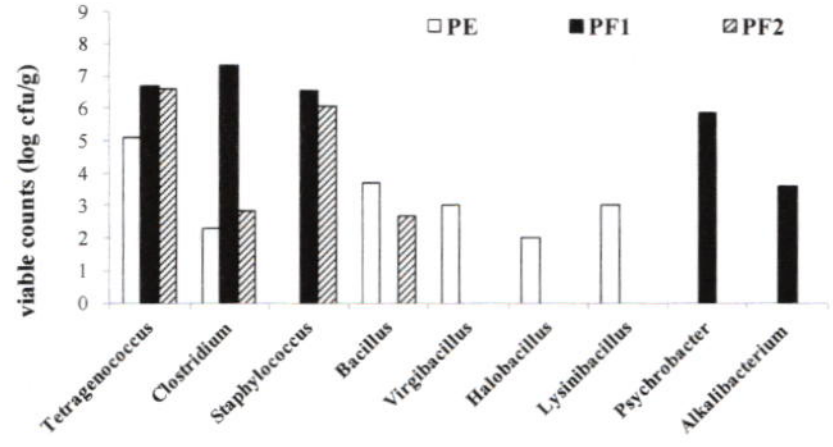
Interestingly, the normally non-halotolerant lactic acid bacteria *Lactobacillus* and *Weissella* were found in *kapi* KF_1 and KF_2, respectively. Aerobic spore-forming bacilli (*Bacillus*, *Virgibacillus*, *Halobacillus*, and *Lysinibacillus*) were occasionally found at 10^2–10^5 cfu g^{-1} in *prahok* and *kapi*. In fish sauce *toeuk trey*, the primary isolates were spore-forming bacilli (*Bacillus*, *Virgibacillus*, *Lentibacillus*, *Lysinibacillus*, and *Clostridium*) and other cocci (*Staphylococcus*, *Micrococcus*, and *Kocuria*). Gram-negative bacteria (*Psychrobacter*) were minor constituent detected only in *prahok* PF_1 and *toeuk trey* TF_6. Viable counts of fungi on the PDA plates were relatively low as compared with the bacterial counts, a maximum of 10^2 cfu g^{-1} was detected in *prahok* PF_1 (*Rhodotorula*) and PF_2 (*Candida*) (see data of PDA, Table 3.2).

Table 3.1 Colony counts of microorganisms isolated from Cambodian traditional fermented fish products (log cfu g^{-1})

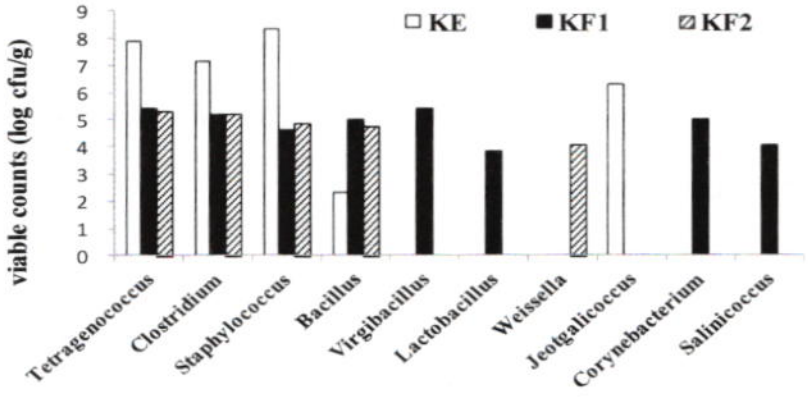
Media	*Prahok* (fish paste)			*Kapi*(shrimp paste)		
	PE	PF_1	PF_2	KE	KF_1	KF_2
halotolerant						
MRS	4.21	6.09	6.28	7.60	5.17	3.81
GAM	5.14	6.33	6.49	7.65	5.41	5.37
TSA	4.44	6.91	6.58	8.42	5.47	5.36
PDA	0	0	0	0	0	0
non-halotolerant						
MRS	0	3.83	3.91	2.70	0	4.10
GAM	4.17	7.41	6.10	7.56	5.18	5.21
TSA	3.79	5.93	5.72	8.06	5.23	5.10
PDA	0	2.00	2.00	0	0	0

Media	*Toeuk trey* (Fish sauce)						
	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6
halotolerant							
MRS	0	0	2.70	0	0	0	0
GAM	0	0	0	0	0	0	0
TSA	3.30	3.08	0	0	0	2.30	2.00
PDA	0	0	0	0	0	0	0
non-halotolerant							
MRS	0	0	0	0	0	0	0
GAM	4.19	0	0	0	0	0	6.06
TSA	3.00	2.48	2.48	4.60	3.36	2.60	3.04
PDA	0	0	0	0	0	0	0

A. *Prahok* (fish paste)



B. *Kapi* (shrimp paste)



C. *Toeuk trey* (fish sauce)

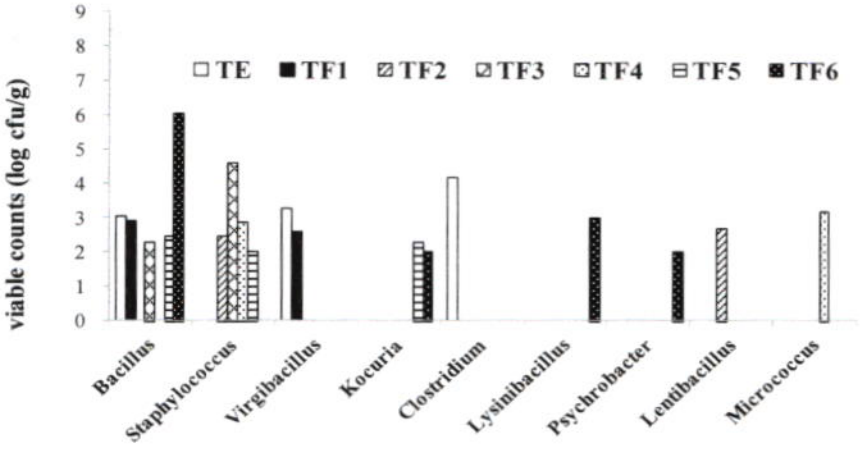


Fig. 3.4 Genus distribution in Cambodian traditional fermented fish products

3.4 Discussion

Many bacterial isolates from Cambodian fermented fish products were from non-LAB genera *Staphylococcus*, *Bacillus*, and *Clostridium*, and others (Fig. 3.4). In particular, *Clostridium* would be expected to have a degradative role when considering the strong proteolytic enzymes reported for this genus.[72] Fermentation of *prahok*, *kapi* and *toeuk trey* are conducted without air ventilation by stirring or shaking, before enough anaerobic condition for the growth of obligate anaerobic *Clostridium* could be achieved inside the fermentation barrels or tanks. Spore formation ability of this genus might also assist the survival in the presence of high concentration of sodium chloride. However, biological characteristics of *Clostridium* detected in the products should be carefully analyzed in the future studies, since some species belonging to this genus are regarded as pathogenic bacteria.

Staphylococcus and *Micrococcus* also have proteolytic and lipolytic activities that are necessary for fermentation, as noted by Yuen et al. (2009)[34] who found that *Staphylococcus* was present throughout the fermentation process of *budu*, a Malaysian fish sauce. Indian fermented sun-dried fish *ngari* contained *Bacillus*, *Staphylococcus*, and *Micrococcus* as the main genera observed with counts of approximately 10^6 cells.[33] Itoh et al. (1993)[73] thoroughly examined microflora changes during the fermentation of old *patis* (fish sauce) from Philippines, and found that *Bacillus* species (*B. coagulans*, *B. megaterium*, and *B. subtilis*) played a primary role during the early fermentation stage, while other *Micrococcus* and *Staphylococcus* species (*M. colpogenes*, *M. varians*, *S. epidermidis*, and *S. saprophyticus*) thrived at later stages. Other studies described isolating the non-LAB genera from fish sauces made from anchovy and Pacific

whiting.[74-77] These microorganisms might be related to the formation of volatile compounds or oligopeptide as reported previously,[23-25,43] through the degradation of fish-containing materials. It is also interesting to speculate that these bacteria might suppress growth of other putrefactive microbes in *prahok*, *kapi*, and *toeuk trey*, as gram-negative bacteria including coliforms were rarely detected in our samples (Fig. 3.5). Thus, the considerable amounts of acetic acid found in *prahok* and *kapi* could possibly have been produced by these bacteria. In particular, *Clostridium* could be a candidate to produce acetic acid, as some member of this genus are regarded as strong producers of this acid.[41] Also bacterial species that belong to *Tetragenococcus* are known to be major lactic acid producers in high-salt-containing fermented foods, such as fish sauce and soy sauce.[46,47,78,79]

Next study should address whether the suppressive effect on the growth of gram-negative bacteria is present or not in the gram-positive constituents isolated in this study. It is noteworthy that, even in the same type of food, the microbial compositions varied depending on the products. Some genera were commonly found, whereas others were occasionally absent; thus, these microbiotas were unstable (Fig. 3.4). The production procedures for Cambodian fermented fish products do not include starter inoculation. Therefore, the microbial compositions could readily be affected by bacteria originally present in the raw materials. Many investigators have isolated LAB such as *Tetragenococcus* from fermented fish products for use as starters to process fermented foods.[33,51,53,70,80-84] If the salt-tolerant *Tetragenococcus* strains isolated in this study could be used as starters, the quality of *prahok*, *kapi*, and *toeuk trey* could be controlled or further improved by stabilizing the chemical and microbiota compositions by enriching for

beneficial bacteria. This could result in optimal processing methodologies, high preservation efficiencies, avoiding of contamination risks, and providing health benefits to the consumers.

Although many aspects remain to be elucidated, the insights on Cambodian fermented fish products acquired in this chapter will help to both establish basic scientific knowledge of these products and aid in the future development of their manufacturing.

CHAPTER IV

Pyrosequencing Analysis of Microflora in Cambodian Traditional Fermented Fish Products

4.1 Introduction

The chapter III showed the detailed analyses of the microbial composition of the traditional Cambodian fermented fish products, *prahok*, *kapi*, and *toeuk trey*. The 16S ribosomal RNA gene-dependent phylogenetic analysis indicated that gram-positive cocci and rods, such as *Bacillus*, *Clostridium*, *Staphylococcus* and *Tetragenococcus* were the major microbial populations. The other bacterial genera such as *Psychrobacter*, *Virgibacillus*, *Lysinibacillus*, *Kocuria*, *Micrococcus*, *Lentibacillus*, and *Jeotgalicoccus* were occasionally found at 10^2–10^5 cfu/g and low numbers of fungi *Rhodotorula* and *Candida* were also seen (see Chapter III; Fig. 3.5). High sodium chloride concentrations detected in these products (170–270 g kg^{-1}) could be responsible for inhibition of the growth of gram-negative putrefactive microorganisms (see chapter I). As described in the Discussion section of chapter III, the non-LAB genera such as *Bacillus*, *Staphylococcus*, and *Micrococcus* have been frequently identified in fermented fish products by other researchers,[66,70] which was similarly observed in this study.

In the result of chapter III, microbiota revealed was mainly comprised of living microorganisms. Moreover, many studies were reported primarily focusing on culture-dependent approaches in order to obtain an understanding of microbial communities in fermenting

food.[53,54,70,85-88] During the last decade, a few scientific papers have described the microbial diversity of fermented food investigated through the application of culture-independent methods.[89-100] The culture-independent methods can identify the whole microorganisms including living and non-living organisms, even the microbes difficult to cultivate. In this chapter, to get more details of microbial population and its change in Cambodian traditional fermented fish products during fermentation process, we employed the pyrosequencing methodology, which includes direct DNA extraction from food samples.

4.2 Materials and methods

4.2.1 Samples used

Prahok was prepared by Ms. Chuon Chanseiha (Takeo, Cambodia) by using freshwater fish *Channa triata*. Fishes were scaled, excrement and gut inside of the fishes were removed, and then they were washed thoroughly in clean water. Fishes were soaked for overnight, dried for a day, mixed with 10% of salt, let fermented for five to seven days, and then liquid was drained. Afterward, salt was added in the ratio of 15-20% (w/v). Fermentation was carried out for five months at ambient temperature. The sample were taken at every month after the start of fermentation and kept at -20 °C. Moreover, seven types of *toeuk trey* samples were directly obtained from different plants and factories in target provinces of Cambodia. These samples were brought directly to Japan and kept at -20 °C for DNA extraction. The specific of products, codes and company names are listed in Table 4.1. *Toeuk trey* samples used in this chapter are summarized in chapter I, table 1.1.

4.2.2 DNA extraction from *prahok* and *toeuk trey*

One gram/1 ml of each sample was suspended in 10 ml of 10% sodium chloride aqueous solution, and total DNA was extracted from 400 μl of these suspended samples using EZ-Extract for DNA (AMR Inc., Gifu, Japan) and Fast Pure DNA kit (Takara Bio Inc., Otsu, Japan). The extracted DNA was used as template for pyrosequencing or quantitative real-time PCR analyses.

4.2.3 Pyrosequencing

Pyrosequencing was targeted to the variable regions of V1 and V2 in bacterial 16S rDNA, since these regions are reported to be useful for the classification of the Lactic acid bacteria (LAB) genera and species.[89,92,93)]

The primers used for PCR-amplification were F8 (5′-AGAGTTTGATCATGGCTCAG-3′ forward) and R357 (5′-CTGCTGCCTYCCGTA-3′, reverse); their 5′-ends were flanked by specific adaptors (5′CGTATCGCCTCCCTCGCGCCATCAG-3′ [forward] and 5′-CTATGCGC CTTGCCAGCCCGCTCAG-3′ [reverse]) that is applicable to the pyrosequencing equipment used, the Genome Sequencer FLX System (Roche). Between the adaptor and primer sequences, short tag sequences (ACAGT, ACGTA, AGACG, AGCAT, AGTGA) were inserted to allow reads from different fermentation periods of *prahok* to be sorted in the same run (FP_1, FP_2, FP_3, FP_4 and FP_5, respectively).

For *toeuk trey*, short tag sequences (CACGT, CATAG, CGATA, TGCAG, TGCTA, TACTG, CTATG) were similarly inserted to allow reads from different types of samples to be

sorted in same run (TE, TF_1, TF_2, TF_3, TF_4, TF_5, and TF_6, respectively). The reaction was performed using PrimeSTAR GXL DNA polymerase (Takara) and MightyAmp DNA polymerase Ver.2 (Takara) with 30 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec) and extension (68°C, 1 min). The amplified fragments were then used in pyrosequencing analysis.

4.2.4 16S rDNA-based taxonomical and statistical analysis of sequence reads

Analyses of sequence reads were performed manually by using Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). Reads obtained in the FASTA format were assigned to the genus levels with an 80% confidence threshold.[86] For species determination, the Basic Local Alignment Search Tool (BLAST) was used. Calculation of Shannon-Wiener index was carried out using the FastGroupII program.[93,101] The reads shorter than 300 bp were excluded.

4.2.5 Real-time quantitative PCR

Real-time quantitative PCR assays (qPCR) were performed to determine the total bacterial population. The DNA samples prepared for pyrosequencing as above were reused as qPCR templates. The 16S rDNA V3-V4 regions were amplified using 331-f (5′-TCCTACGGGA GGCAGCAGT-3′) and 797-r (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) as forward and reverse primers, respectively.[89,92,93] (5′-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3′) was used as a TaqMan probe. The reaction mixtures were prepared using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA), and run on a StepOnePlus Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions.

Table 4.1 Cambodian traditional fish fermented products analyzed in this chapter

Group[a]	Fermentation period	Product code[b]	Sampling location[c]
Prahok (fermented fish paste)	1 month	FP_1	Takeo province
	2 months	FP_2	
	3 months	FP_3	
	4 months	FP_4	
	5 months	FP_5	
Toeuk trey (fish sauce)	2-3 months	TE	Phnom Penh city
	Pure grade 6 months	TF_1	Phnom Penh city
	Final product	TF_2	Phnom Penh city
	Final product	TF_3	Phnom Penh city
	Final product	TF_4	Kompot province
	Final product	TF_5	Keb province
	Final product	TF_6	Keb province

[a]*Prahok* was made from freshwater fish (*channa triata*) and all *toeuk trey* (TE, TF_{1-6}) were summarized in chapter I, Table 1.1.
[b]FP_1, FP_2, FP_3, FP_4 and FP_5, samples in each month of fermentation process.
[c]Geographical locations at which the indicated fermented foods were produced.

4.3 Results

4.3.1 Change of microbiota composition during fermentation of *prahok*

We conducted the pyrosequencing analysis of 16S rDNA V1-V2 regions amplified from Cambodian fermented fish products *prahok* (FP_1, FP_2, FP_3, FP_4, and FP_5) and *toeuk trey* (TE, TF_1, TF_2, TF_3, TF_4, TF_5, and TF_6).

Change of microbiota in each sample of *prahok*, which was sampled at different times during fermentation process, was shown in Table 4.2. A broad variety of genera from twenty five to sixty eight was identified for respective samples by using RDP classifier. The result was observed that eighty genera were found in total, and 76 genera represented less than 5% of the total population (Table 4.2). Noteworthy, number of reads with bootstrapping threshold values of <80% were cut off by RDP analysis (see materials and methods). The number of reads cut off did not exceed 23% of the total reads number in each sample, excepting sample FP_1 in which unclassified reads represented significant population (1,251 reads, 39%). Three reads in FP_2 were classified as belonging to archaea or chloroplasts. In the sample at the first month of fermentation process FP_1 (one month fermented), the microbiota was occupied by the genus *Clostridium* (1,647 reads, relative population 84.5%), and other genus minor was detected (301 reads, relative population 15.5%). However, in the sample at the second and third months of fermentation process (FP_2 and FP_3), *Halanaerobium* became the most abundant genus (16,095-19,581 reads, 52.5-61.6%), and *Clostridium* was changed to sub-dominated genus (8,769-6,604 reads), relative population was decreased to 28.6 and 20.8% during these fermentation periods, respectively. Thereafter, at the fourth and fifth months (FP_4 and FP_5) of fermentation process,

Halanaerobium was decreased dramatically (2,486 and 510 reads, 24.4 and 14.2%, respectively), while the relative population of *Clostridium* dramatically increased from 20.8 to 57.0-51.1% and stabilized at the maturity of fermentation process. The halophilic LAB *Tetragenococcus* represented up to 7.9%. In addition, to determine the diversity of bacterial population, Shannon-Wiener index of each *prahok* sample was assessed using FastGroup II program (Table 4.3). The Shannon-Wiener index was ranged from 3.58 to 4.92. It was higher compared to *narezushi* (Japanese fermented fish with rice), *Cobolabis saira- narezushi* and *ayu-narezushi* which were reported by Koyanagi et al. (2011) and Matsui et al. (2010) (Table 4.3).[93,99] Taken together, these results indicated that *Clostridium* and *Halanaerobium* play the leading role in *prahok* fermentation, and suppress the growth of other bacteria. Putrefactive LAB *Vagococcus* contaminated throughout the fermentation process, but its population was decreased at the endpoint of fermentation no more than 1.5% at total reads (57 reads, see Table 4.2).

4.3.2 Comparison of microbiota composition between the samples of *toeuk trey*

The *toeuk trey* microbiota was revealed by pyrosequencing analysis. *Toeuk trey* was sampled at different company/factory, which was produced by different procedure. The results are shown in Table 4.2 continued. Variety of genera was detected in each *toeuk trey* (184 genus in total). At the early stage of *toeuk trey* fermentation process TE, the microbiota was dominated by the genus *Closridium* (relative population 44.3%) and sub-dominated by *Escherichia/ Shigella* (39%). However, *Closridium* and *Escherichia/Shigella* were decreased in the final product (7 and 19%, see Fig. 4.2), respectively.

Noteworthy, *Tetragenococcus* was detected in *toeuk trey* made from fresh water fish (TE, TF_1, TF_2 and TF_3). It's seemed to be increased during fermentation from 4 to 16% of relative population regarding the same company samples (TE, TF_1, and TF_2 were produced by the same company, Table 4.1). In addition, different company's final product TF_3 had larger population. On the other hand, *Halanaerobium* was a major species in *toeuk trey* made from marine fish (TF_4, TF_5 and TF_6).

4.3.3 Quantitative real-time PCR analysis of 16S rDNA contained in *prahok* and *toeuk trey*

To estimate the approximate total bacterial population at each stage for *prahok* fermentation and for *toeuk trey* samples, the real-time quantitative PCR (qPCR) assay of 16S rDNA was taken in this study (Fig. 4.3 and 4.4). The relative bacterial population contained in *prahok* was increased during first three months and then after decreased to the end-point of fermentation (Fig. 4.3). At every stage of *prahok* fermentation, the significant amount of *Clostridium* was contained (Fig. 4.3). Particularly, *Halanaerobium* appeared in large amounts in *prahok* at the second (FP_2) and third (FP_3) months and then after drastically decreased at the end-point of fermentation process (FP_5) (Fig. 4.3). The final products of *toeuk trey* (TF_2, TF_3, TF_4, TF_5 and TF_6) contained lower amount of bacterial population when compared to early fermentation stage sample TE (Fig. 4.4). *Toeuk trey* TF_4, TF_5 and TF_6 showed the significant existing ratio of *Haloanaerobium*.

Table 4.2 Sequence read statistics of *prahok* and *toeuk trey* samples and their phylogenetic classification by RDP classifier

Datasets	*Prahok* (fish paste)					Total
	Fermentation period (time-course samples)					
	FP_1	FP_2	FP_3	FP_4	FP_5	
(I) Untrimmed reads	3,199	35,438	35,293	12,090	4,660	90,680
(II) Reads cut off with boot- strap threshold of 80%	1,251	4,763	3,486	1,908	1,075	12,491
(III) Reads for non-bacterial organisms	0	3	0	0	0	3
(IV) Trimmed reads [(I)-(II)-(III)]	1,948	30,672	31,808	10,182	3,585	78,195
Acinetobacter	6	593	704	97	68	1,468
Aeromonas	4	188	293	99	27	611
Anaerofilum	0	0	45	2	0	47
Anaerorhabdus	0	0	3	0	0	3
Anaerosphaera	0	19	0	7	0	26
Aquaspirillum	0	0	3	0	0	3
Arcobacter	0	0	24	2	0	26
Asticcacaulis	0	0	1	0	6	7
Atopobium	0	0	0	2	0	2
Bacillus	0	0	0	1	0	1
Bacteroides	2	34	30	28	0	94
Brevundimonas	0	14	21	0	5	40
Burkholderia	0	0	0	0	1	1
Cellulosilyticum	4	0	14	9	0	27
Cetobacterium	0	52	17	36	47	152
Chryseobacterium	0	0	7	0	0	7
Citrobacter	0	128	150	35	0	313
Clostridium	1,647	8,769	6,604	5,806	2,013	24,839
Comamonas	0	27	5	37	1	69
Corynebacterium	0	12	20	2	0	34
Delftia	0	1	2	2	13	18
Dermacoccus	0	7	0	0	0	7
Edwardsiella	3	140	34	24	14	215
Enhydrobacter	3	236	115	23	0	337
Enterobacter	0	0	6	2	3	11
Enterococcus	0	5	3	2	0	10
Erysipelothrix	0	56	63	9	0	128
Escherichia/Shigella	0	126	102	14	108	350
Gemella	0	0	1	0	0	1
Flectobacillus	0	0	0	0	20	20
Fusobacterium	0	0	0	2	0	2
Halanaerobium	18	16,095	19,581	2,486	510	38,690
Ignatzschineria	0	0	0	2	0	2
Janthinobacterium	0	10	5	0	0	15
Kocuria	0	1	0	0	0	1

Table 4.2 (Continued)

Datasets	FP_1	FP_2	FP_3	FP_4	FP_5	Total
Kurthia	4	217	197	19	0	437
Lactococcus	0	0	58	2	22	82
Lentibacillus	1	11	3	0	0	15
Leucobacter	0	0	0	1	0	1
Leuconostoc	0	0	0	1	0	1
Macrococcus	0	120	92	15	3	231
Massilia	0	1	6	0	3	10
Morganella	0	250	233	53	9	548
Myroides	0	20	0	9	0	29
Neisseria	0	0	3	1	0	4
Nocardioides	0	3	0	0	0	3
Ochrobactrum	0	13	0	0	5	18
Orbus	0	3	0	0	0	3
Paludibacter	0	1	0	0	0	1
Pantoea	0	15	0	0	0	15
Pedomicrobium	0	0	0	0	7	7
Peptostreptococcus	58	1,656	995	324	129	3,162
Plesiomonas	3	155	167	147	60	532
Propionibacterium	0	19	9	2	0	30
Proteiniclasticum	1	12	6	1	3	23
Proteocatella	3	21	0	28	0	52
Proteus	1	14	26	3	0	43
Providencia	0	76	54	32	18	180
Pseudomonas	27	10	0	129	0	166
Psychrobacillus	0	3	0	0	0	3
Psychrobacter	45	22	1	2	0	70
Raoultella	1	0	0	5	0	6
Rothia	0	0	15	0	0	16
Salicola	0	0	0	1	0	1
Serratia	0	0	0	1	1	2
Shewanella	0	4	7	2	0	13
Soonwooa	0	7	32	14	0	53
Sphingobacterium	0	0	1	0	11	12
Sphingomonas	0	0	8	0	25	33
Staphylococcus	0	16	51	8	46	121
Stenotrophomonas	0	0	0	1	0	1
Streptococcus	3	37	172	147	1	360
Streptophyta	0	0	1	8	0	9
Succinispira	0	0	1	1	0	2
Tetragenococcus	5	543	1,138	74	284	2,044
Vagococcus	96	577	395	281	57	1,406
Vitreoscilla	0	3	0	2	0	5
Wautersiella	0	7	57	1	3	68
Wohlfahrtiimonas	9	323	227	138	55	752
Yersinia	0	0	0	0	7	7

Table 4.2 (Continued)

Datasets	*Toeuk trey* (fish sauce)							Total
	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	
(I) Untrimmed reads	5,667	40,454	6,396	12,702	8,889	8,139	8,206	90,453
(II) Reads cut off with bootstrap threshold of 80%	2,270	19,692	1,904	672	1,528	3,418	833	30,317
(III) Reads for non- bacterial organisms	0	0	0	0	0	0	0	0
(IV) Trimmed reads [(I)-(II)-(III)]	3,397	20,762	4,492	12,030	7,361	4,721	7,373	60,136
Achromobacter	0	5	2	2	2	0	19	30
Acidovorax	0	2	1	0	0	0	0	3
Acinetobacter	2	249	9	15	3	114	5	397
Aeromicrobium	0	0	0	0	0	0	2	2
Aeromonas	0	18	104	3	0	0	0	125
Aggregatibacter	0	0	0	0	0	12	0	12
Alcanivorax	0	1	0	0	3	0	0	4
Algoriphagus	0	2	0	0	0	0	0	2
Alistipes	0	0	0	0	0	2	0	2
Alkalibacillus	0	1	0	0	1	0	8	10
Alkalibacterium	0	0	0	0	0	0	1	1
Alkaliphilus	0	1	0	0	2	0	0	3
Allomonas	0	24	0	0	0	0	0	24
Altererythrobacter	0	0	0	0	1	0	0	1
Anaerococcus	0	217	342	4,518	0	4	0	5,081
Anaerosinus	0	49	0	0	0	0	0	49
Anaerosphaera	7	330	282	155	16	0	0	790
Aquabacterium	0	2	2	0	0	1	0	5
Aurantimonas	0	0	2	0	0	0	0	2
Arcobacter	0	37	0	0	0	0	0	37
Azospirillum	0	2	0	0	0	0	0	2
Bacillus	3	23	6	1	0	0	20	53
Bacteroides	0	10	28	15	2	0	0	55
Beijerinckia	0	0	0	1	0	0	0	1
Brachybacterium	0	0	0	0	0	8	0	8
Bradyrhizobium	0	0	1	0	0	0	0	1
Brevibacterium	0	0	5	12	2	3	1	23
Brevundimonas	1	4	19	8	2	11	0	45
Burkholderia	0	0	1	0	0	0	0	1
Cetobacterium	8	10	148	34	669	527	344	1,740
Chelatococcus	0	0	0	2	0	0	0	2
Chromohalobacter	0	9	3	0	0	0	0	12
Chryseobacterium	0	0	3	6	0	0	0	9
Citrobacter	0	1	0	5	0	4	0	10
Cloacibacterium	1	1	287	0	2	0	4	295
Clostridium	1,506	56	310	138	77	240	31	2,358
Cobetia	0	5	0	0	0	0	0	5
Comamonas	0	18	16	3	4	0	0	41
Corynebacterium	12	21	1	16	9	29	0	88
Coxiella	0	4	0	0	0	0	0	4

Table 4.2 (Continued)

Datasets	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Total
Curvibacter	0	2	0	0	0	0	0	2
Cyanobacteriagems (GpIIa)	2	0	1	0	117	14	1	135
Dechloromonas	0	1	2	0	0	0	0	3
Deinococcus	0	0	0	3	3	0	0	6
Delftia	6	15	19	52	40	20	12	164
Dermacoccus	0	0	0	0	0	1	0	1
Desulfarculus	0	1	0	0	0	0	0	1
Desulfovibrio	0	1	0	0	0	0	0	1
Donghicola	0	1	0	0	0	0	0	1
Edwardsiella	0	1	1	0	0	0	0	2
Enhydrobacter	1	2	2	0	10	4	0	19
Enterobacter	0	0	0	0	1	0	0	1
Enterococcus	0	1	0	0	0	0	0	1
Enterovibrio	0	0	0	0	0	0	1	1
Erythrobacter	0	0	0	0	0	3	0	3
Erysipelothrix	0	1	1	0	0	0	0	2
Escherichia/Shigella	1,322	11,641	857	167	37	46	547	14,617
Exiguobacterium	0	0	8	0	0	14	0	22
Finegoldia	0	0	0	0	0	2	0	2
Flavobacterium	0	4	45	6	0	0	0	55
Fusibacter	0	1	0	0	0	0	0	1
Fusobacterium	0	16	0	0	3	1	0	20
Granulicatella	0	0	0	0	0	3	0	3
Haematobacter	0	0	4	0	0	0	0	4
Haemophilus	0	0	0	0	0	9	0	9
Halanaerobacter	0	19	0	0	1	0	0	20
Halanaerobaculum	0	38	1	0	7	0	0	46
Halanaerobium	10	4,739	221	1,856	4,818	2,543	6,108	20,295
Haliea	0	3	0	0	0	0	0	3
Halocella	0	1	0	0	0	0	0	1
Halolactibacillus	0	3	0	0	0	0	0	3
Halomonas	0	287	23	24	2	3	5	344
Halothiobacillus	0	3	0	0	0	0	0	3
Halovibrio	0	4	0	0	13	0	0	17
Hyphomicrobium	0	1	0	0	0	0	0	1
Ideonella	0	2	0	0	0	0	0	2
Idiomarina	0	40	0	0	5	0	0	45
Ilyobacter	0	1	0	0	0	0	0	1
Ignatzschineria	0	0	0	0	1	0	0	1
Janthinobacterium	0	0	0	0	0	1	0	1

Table 4.2 (Continued)

Datasets	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Total
Jeotgalicoccus	0	0	1	1	0	0	2	4
Kocuria	0	0	2	3	0	5	0	10
Kurthia	0	0	0	0	0	3	0	3
Lactobacillus	73	59	9	68	7	21	9	246
Legionella	0	3	0	0	0	0	0	3
Lentibacillus	3	9	4	3	2	29	8	58
Leptotrichia	0	0	2	0	0	0	0	2
Leucobacter	0	1	9	0	1	2	0	13
Listonella	0	3	0	0	3	3	0	9
Lysobacter	0	0	0	0	7	2	0	9
Marinicella	0	0	0	0	2	0	0	2
Marinilactibacillus	0	0	0	0	0	0	4	4
Marinobacter	0	76	1	0	4	2	0	83
Marinococcus	0	0	3	0	0	0	0	3
Martelella	0	0	0	0	2	0	0	2
Massilia	0	0	0	0	0	7	0	7
Methylobacterium	0	11	0	12	46	42	0	111
Methylocystis	1	4	0	0	0	0	0	5
Methylophilus	1	0	0	0	0	0	0	1
Microbacterium	9	0	0	0	4	2	0	15
Micrococcus	0	0	0	1	1	15	0	17
Morganella	0	2	21	2	0	10	0	35
Mucilaginibacter	0	0	0	1	0	0	0	1
Mycobacterium	1	2	0	0	0	0	0	3
Mycoplasma	0	1	0	0	0	0	0	1
Myroides	0	0	0	4	0	0	0	4
Neisseria	3	0	0	0	2	14	0	19
Nesterenkonia	0	0	0	0	0	4	0	4
Nosocomiicoccus	0	0	0	11	0	0	0	11
Oceanicola	0	3	0	0	0	0	0	3
Oceanimonas	0	14	41	6	2	0	0	63
Oceanospirillum	0	0	1	0	0	0	0	1
Ochrobactrum	0	0	2	0	3	0	0	5
Ornithinimicrobium	0	0	1	0	0	0	0	1
Opitutus	0	2	0	0	0	0	0	2
Paracoccus	0	0	1	0	8	22	0	31
Paraeggerthella	0	0	1	0	0	0	0	1
Pelagibaca	0	0	0	0	1	0	0	1
Pelomonas	2	8	2	9	6	3	2	32
Peptostreptococcus	9	5	251	12	41	12	5	335

Table 4.2 (Continued)

Datasets	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Total
Phascolarctobacterium	0	1	0	0	0	0	0	1
Phenylobacterium	0	0	2	0	0	0	0	2
Photobacterium	0	0	0	0	77	82	13	172
Pisciglobus	0	0	0	5	0	0	0	5
Planobacterium	0	0	3	0	0	0	0	3
Planococcus	0	0	0	0	0	38	2	40
Plantibacter	0	0	0	0	1	0	0	1
Plesiomonas	0	0	11	3	0	0	0	14
Polynucleobacter	0	10	0	3	0	0	0	13
Prevotella	2	1	0	1	0	1	0	5
Propionibacterium	23	35	25	19	44	56	7	209
Proteus	0	0	68	17	0	0	0	85
Proteiniclasticum	1	20	0	0	0	0	0	21
Providencia	0	1	93	19	0	0	0	113
Pseudoalteromonas	0	65	0	0	4	0	0	69
Pseudochrobactrum	0	0	0	0	2	0	0	2
Pseudomonas	132	114	346	15	293	291	84	1,275
Pseudoxanthomonas	0	0	0	4	0	1	0	5
Psychrobacillus	0	0	2	0	0	0	0	2
Psychrobacter	0	6	0	3	0	0	4	13
Puniceicoccus	0	1	0	0	0	0	0	1
Raoultella	3	0	11	0	21	27	5	67
Rheinheimera	0	3	15	0	0	0	0	18
Rhizobium	0	2	0	0	0	0	0	2
Rhodococcus	2	0	0	0	0	0	0	2
Rhodovibrio	0	13	1	7	0	0	0	21
Roseomonas	0	4	0	0	0	0	0	4
Rothia	0	1	0	2	4	1	0	8
Salicola	0	41	7	2	494	8	0	552
Salimicrobium	0	0	0	0	0	2	2	4
Salinibacter	0	5	0	0	0	0	0	5
Salinicoccus	0	2	0	0	0	0	0	2
Salinivibrio	0	77	9	53	13	4	0	156
Selenomonas	0	0	0	0	0	1	0	1
Serinicoccus	0	0	0	0	0	4	0	4
Serratia	0	0	1	0	0	3	0	4
Shewanella	0	671	0	3	15	3	0	692
Soonwooa	0	0	0	0	0	2	0	2
Sphingobacterium	0	0	3	3	4	5	0	15
Sphingobium	0	0	0	0	0	0	1	1

Table 4.2 (Continued)

Datasets	TE	TF_1	TF_2	TF_3	TF_4	TF_5	TF_6	Total
Sphingomonas	1	5	0	4	42	2	0	54
Sporohalobacter	0	0	0	0	1	0	0	1
Sporosalibacterium	0	10	0	0	0	0	0	10
Sporosarcina	0	0	0	0	0	0	2	2
Staphylococcus	123	171	41	697	12	66	35	1,145
Stenotrophomonas	0	0	0	0	14	0	0	14
Streptococcus	1	13	7	3	0	8	1	33
Streptophyta	0	10	0	0	5	13	0	28
Succinispira	0	6	0	0	0	0	0	6
Tessaracoccus	0	0	0	4	0	0	0	4
Tetragenococcus	125	1,113	703	3,828	32	88	8	5,897
Thermomonas	0	0	3	0	0	0	0	3
Thiohalorhabdus	0	8	0	0	1	0	0	9
Tissierella	0	0	3	0	0	3	0	6
Treponema	0	1	0	0	0	0	0	1
Turicibacter	0	0	0	0	0	4	0	4
Vagococcus	0	39	15	75	0	0	0	129
Vampirovibrio	0	2	0	0	0	0	0	2
Veillonella	2	5	0	0	0	0	0	7
Vibrio	0	151	0	8	287	188	68	702
Virgibacillus	0	13	6	0	0	0	2	21
Vogesella	0	0	6	5	0	0	0	11
Weissella	0	8	2	68	0	0	0	78
Wohlfahrtiimonas	0	0	0	4	0	3	0	7

Table 4.3 Sequence reads statistics for each *prahok* sample and comparison with *narezushi*

Sample	Total reads	Reads shorter than 300 bp	Reads analyzed	Shannon-Wiener index	Reference
FP_1	3,199	8	3,191	3.58	This study
FP_2	29,121	782	28,339	4.33	
FP_3	35,293	646	34,647	4.30	
FP_4	12,090	166	11,924	4.56	
FP_5	4,660	49	4,611	4.93	
narezushi product					
A	20,173	21	20,152	2.60	Koyanagi *et al.*, (2011)
B	21,672	85	21,587	2.04	
C	20,124	60	20,064	2.01	
D	24,007	88	23,919	1.69	
E	22,412	63	22,349	2.38	
F	19,625	762	18,863	2.42	

*Shannon-Wiener index indicates species diversity.

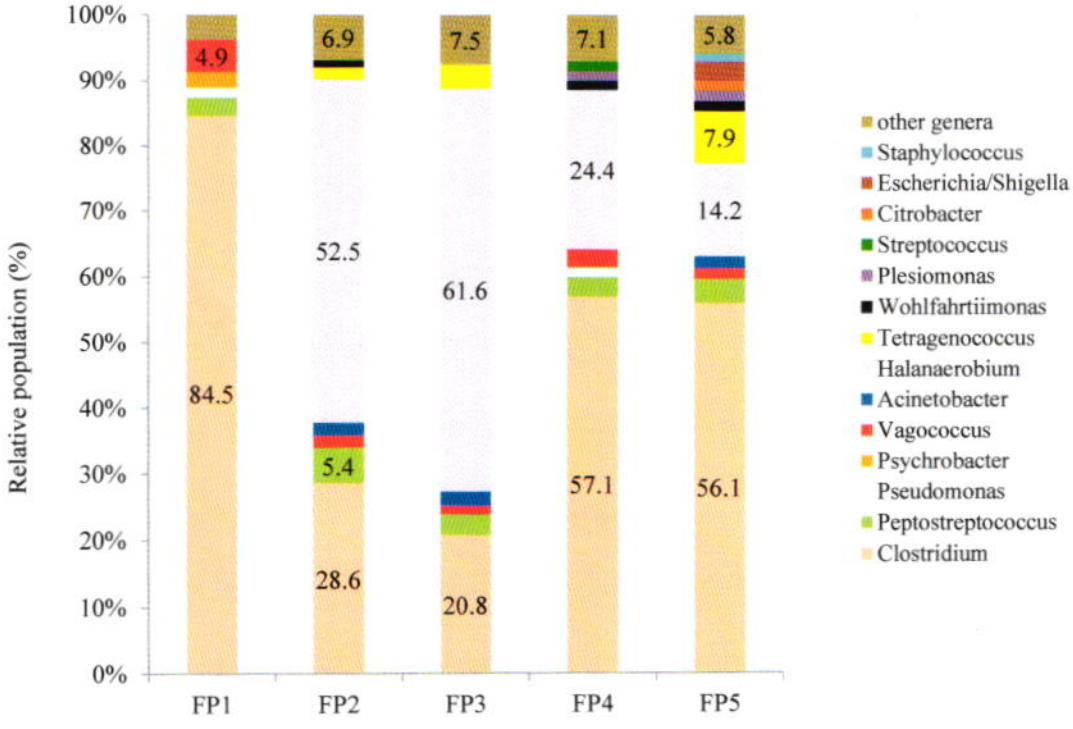


Fig. 4.1 Change of microbiota composition during fermentation of *prahok* (fish paste)

Table 4.4 Sequence reads statistics for each *toeuk trey* sample

Sample	Total reads	Reads shorter than 300 bp	Reads analyzed	Shannon-Wiener index	Reference
TE	5,667	10	5,657	3.08	This study
TF_1	29,818	66	29,752	3.87	
TF_2	6,396	181	6,215	4.59	
TF_3	12,702	31	12,671	4.63	
TF_4	8,889	46	8,843	4.08	
TF_5	8,139	51	8,088	4.39	
TF_6	8,206	26	8,180	2.59	

*Shannon-Wiener index indicates species diversity

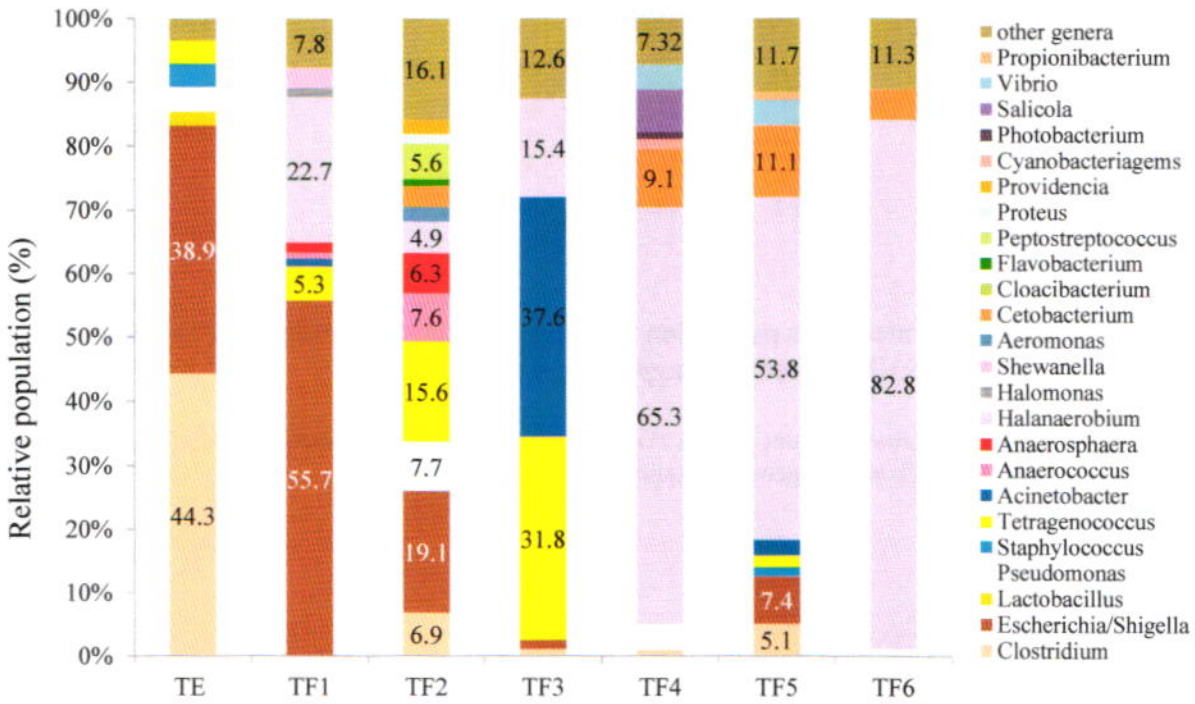


Fig. 4.2 Comparison of microbiota composition between the samples of *toeuk trey (*fish sauce)

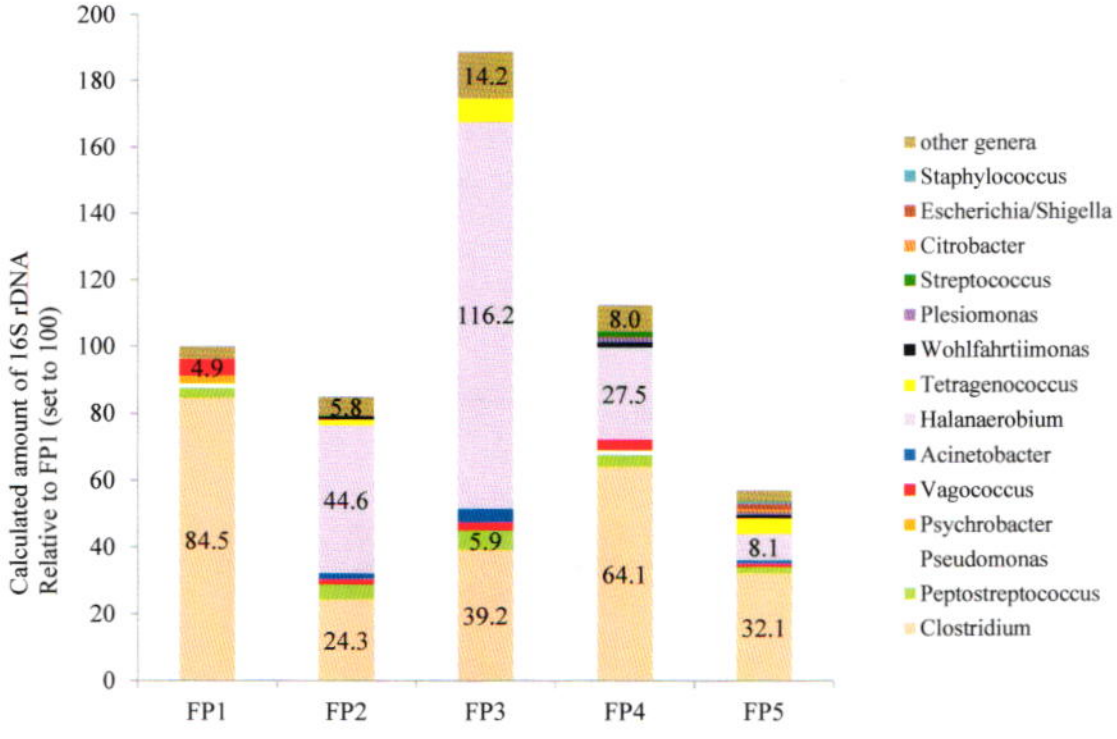


Fig. 4.3 Evaluation of microbiota population by using quantitative real-time PCR (qPCR) analysis of 16S rDNA contained in *prahok* sample

The bars indicate the relative amount of 16S rDNA when compared to control sample (FP_1), indicating the relative population of bacterial genera contained in respective samples.

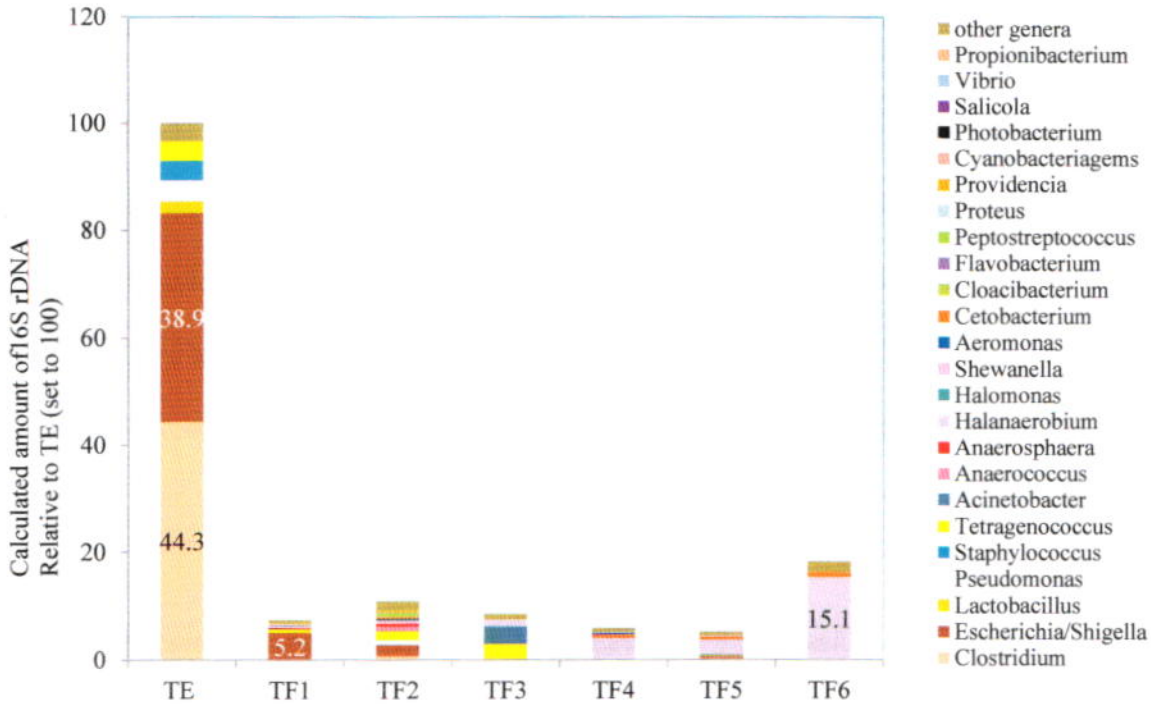


Fig. 4.4 Quantitative real-time PCR (qPCR) analysis of 16S rDNA contained in *toeuk trey* sample

The bars indicate the relative amount of 16S rDNA when compared to control sample (TE), indicating the relative population of bacterial genera contained in respective samples.

4.4 Discussion

In the present study, precious information have been obtained in detail of microbial composition insights into Cambodian traditional fermented fish products, *prahok* and *toeuk trey*. The pyrosequencing analysis revealed changes in bacterial population during fermentation of *prahok*, as well as for distinctive *toeuk trey* products. Pyrosequencing is thus useful in identifying bacterial groups having small populations as compared to classical methods as shown in chapter III and other report shown by Koyanagi et al. (2013).[89)]

In our study, we classified 80 genera for *prahok* and 184 genera for *toeuk trey* (Table 4.2). Particularly, the results of bacterial composition analysis demonstrated that genus *Halanaerobium* had in large population in *prahok* FP_3 (3 months fermentation) (Fig. 4.1 and Fig. 4.3). This genus decreased at the end-point *prahok* fermentation. *Toeuk trey* TF_4, TF_5 and TF_6, which were produced from marine fish (Table 1.1, chapter I), contained large number of genus *Halanaerobium* (Fig 4.2). *Halanaerobium* are known as obligately anaerobic halophiles capable of fermenting monosaccharides, amino acids, and glycerol, and also capable of producing butyrate, acetate, propionate, H_2, and CO_2 as the primarily fermentation products.[102)] Other study showed that *Halanaerobium* were clearly associated with the metabolism of glucose, glycerol, and trimethylamine N-oxide (TMAO) and the production of acetate, butyrate, trimethylamine (TMA) and dimethylamine (DMA).[102,103)] Although, Timm and Jorgensen (2002)[104)] reported that butyrate, acetate, TMA, and DMA caused off-flavors and taste changes at high concentrations, and their absence in the production of *saeu-jeot* (Korean fermented salted seafood with 20-25% of salt) was very important in view of *saeu-jeot* quality. Recently, Jung et

al. (2013)[83] showed that the members of genus *Halanaerobium* appeared as one of dominant populations after 49 days (1.5 months) of fermentation and eventually they predominated in the *saeu-jeot* microbial community after 66 days (2 mouths), and then decreased to minor populations again as the fermentation progressed. This information is consistent with our result that *Halanaerobium* increased at 3 months and thereafter drastically reduced its population (Fig. 4.3).

Jung et al. (2013)[90] also reported that *Halanaerobium* produced acetate, butyrate, and methylamines within only 15-49 days of fermentation and when the temperature was between 20 and 25 °C. At more than 25 °C, the production ability was decreased. Based on this result, they suggested that, to produce good quality of saeu-jeot, fermentation should be stopped within 15-49 days and below 20 °C. In more recent study, Lee et al. (2014)[105] described that the genus *Halanaerobium* predominated during the saeu-jeot maturation stage, which was in accordance with previous results of Jung et al. (2013).[90] They reported that *Halanaerobium* and their metabolic products could be potential indicators to determine abnormal fermentation or spoilage of *saeu-jeot* because these species are primarily responsible for the production of acetate, butyrate, and methylamines.

However, our results suggested that large numbers of *Halanaerobium* presented in *prahok* FP_3 and in *toeuk trey* TF_4, TF_5 and TF_6 might not be related to those problematic effects mentioned before, since Cambodian traditional fermented fish products were taken for more than 3 months of fermentation; such long maturating period could omit the undesirable effects of *Halanaerobium* which inhabits in middle stage (2-3 months). Actually, the smell and taste of

prahok and *toeuk trey* is not good in the middle stage, but it gradually becomes better in the late stage. In addition, *prahok* and *toeuk trey* were produced under condition of higher temperatures more than 25 °C. Hence, the ability of *Halanaerobium* to produce acetate, butyrate, trimethylamine and dimethylamine could be not so high under such condition. Based on the above discussion, we suggest that the metabolites causing undesirable tastes and flavor had been decreased after middle stage, and only the desirable compounds such as amino acids including umami taste were remaining in the final products (Chapter I and Nattida, 2012).[45)]

The genera other than *Halanaerobium*, *Clostridium* and *Acinetobacter* did not count large population (less than 20%), indicating that these three genera are possible candidate for fermentation of the products. One harmful bacterial genera *Escherichia/Shigella* were detected at high population in TE and TF_1 (not maturated samples), but they dropped to lower population in the final product TF_2 (Fig. 4.2). High salt concentrations of these products might prevent such bacteria.

CHARPTER V

Conclusion

In this study, we obtained the first detailed insights into the chemical and microbial properties of the Cambodian traditional fermented fish products *prahok*, *kapi*, and *toeuk trey*. Acetic acid was the most common organic acid with highest concentration in 10/13 samples, and lactic acid was also found at high concentrations.

As for volatile compounds, various acids, alcohols, aldehydes, ketones, nitrogen-containing, sulfur-containing, and others compounds were detected. Butanoic acid was identified as major acid in *toeuk trey* samples, indicating its contribution to specific flavor. Final products of *prahok* and *kapi* contained less amounts of ester compounds, and *kapi* was relatively rich in sulfur-containing compounds.

Culture-dependent microbiota analysis revealed that gram-positive cocci or rods were main constituents of Cambodian fermented fish products. Among them, *Bacillus*, *Clostridium*, and *Staphylococcus* were frequently identified at up to 10^8 cfu/g. Halotolerant and halophilic lactic acid bacteria *Tetragenococcus* were present as another major genus in *prahok* and *kapi*. Other bacterial genera such as *Psychrobacter*, *Virgibacillus*, *Lysinibacillus*, *Kocuria*, *Micrococcus*, *Lentibacillus*, and *Jeotgalicoccus* were occasionally found at 10^2–10^5 cfu/g. Based on this and previous studies, it is suggested that *Clostridium and Staphylococcus* are mainly related to the production of acetic acid in Cambodian fermented fish products. These

microorganisms might play an important role in the preservation and maturation of the fermented fish products. It is also noteworthy that gram-negative rods were minor in all type of foods, indicating the presence of suppressive effects toward these putrefactive bacteria.

To investigate microbiota more in detail, we conducted the pyrosequencing analysis of 16S rDNA V1-V2 regions amplified from Cambodian fish fermented products *prahok* and *toeuk trey*. Twenty-five to 68 genera and 35 to 107 genera were identified by using RDP classifier from *prahok* and *toeuk trey*, respectively. The results revealed that the most dominant species in the bacterial flora of *prahok* was *Clostridium* and *Halanaerobium*. *Tetragenococcus* was detected in *toeuk trey* made from fresh water fish, it seemed to be increased during fermentation process. *Closridium* and *Escherichia/Shigella* were inversely decreased in the final product. *Halanaerobium* was a major species in *toeuk trey* made from sea fish. The data were also analyzed by combining with the qPCR results, revealing that bacterial population of *prahok* was increased during the fermentation process of three months, and then decreased. For *toeuk trey*, the 6 months fermented sample and final products were lower in bacterial population than 1st stage fermentation.

Taken together, the data acquired from this research is very important and useful for Cambodian peoples' dietary life. Moreover, this research provided information on the fundamental signatures of not only Cambodian but also the entirety of Asian fermented fish products, benefitting the manufacturing processes, including microbial control and quality stabilization.

ACKNOWLEDGMENT

I wish to express my sincere gratitude and appreciation to my supervisor, Prof. Toshiki ENOMOTO for the valuable suggestions, ceaseless guidance, efficacious suggestion and constant motivation throughout my entire course studies and especially during the preparation of the thesis, without which this work would never be possible.

I express my sincere acknowledgement to Prof. Toshihiro YANO and Assoc. Prof. Takashi KOYANAGI for their valuable suggestions and comments regarding my thesis work and technical guidance and constructive criticism throughout the Ph.D study period.

My sincere thanks to Assoc. Prof. Kenji MATSUMOTO, Assoc. Prof. Yuji HONDA and Assoc. Prof. Yumiko YOSHIKI, Food Functional Group for their supports and guidance to me in all parts of the Ph.D study work.

I would like to extent my sincere gratitude to the Japanese International Cooperation Agency (JICA) for providing me a scholarship to study Ph.D degree in Food Science and Technology at Ishikawa Prefectural University (IPU) in Japan. Especially, sincere thanks to Jica Hokuriku staffs who has help me and warmly cooperated during study period.

Sincere heartfelt thanks to all Professors in Department of Food Science, Ishikawa Prefectural University of the mind for their precious help in all over graduate studies.

I wish to thanks to Dr. Sarun CHAN and Dr. Sopheareth MAO, Ministry of Agriculture

Forestry and Fisheries and Department of Agro-Industry, for their supports and guidance in all parts of the study tours in Cambodia.

Sincere thanks to all of the friends of the mind for their help in all over during studies. Special thanks to Ms. Mimoe SHIOMOTO who has cooperating with me to work, for her encouragements and guidance to me to work in laboratory.

Sincere thanks to supervisor and technicians of Ishikawa Industrial Research Institute laboratory that help me to use an apparatus for my work.

Lastly, I wish to express deepest gratitude to my husband, daughters, mother in-law, brother and sisters for their moral support and encouragement during my study here.

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