

Physicochemical Properties, Amino Acid Composition and Volatile Components of Fermented Fish (*Pla-ra*) Accelerated by Starter Cultures

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Abstract

Fermented striped snake-head fish, known as *Pla-ra*, is a popular traditional food that is eaten throughout Thailand. Lactic acid bacteria (LAB) are found as the dominant microorganisms in many fermented fish products. The primary role of LAB is to ferment the available carbohydrates, and thereby causes a decrease in pH. The combination of low pH and organic acids (mainly lactic acid) are the main preservative factors found in fermented fish products. The purpose of this study was to determine the change in physicochemical properties, amino acid composition, and active volatile compounds of *Pla-ra*, by adding halophilic bacteria and LAB into the fish fermentation. The results revealed that the pH of the 8 *Pla-ra* treatments were found between 5.33 - 5.63, and the salt content of all *Pla-ra* treatments, ranged from 20.60 to 22.28 %. The increase in total acidity and the degree of hydrolysis (DH), is generally accompanied by a decrease in pH. The FMP treatment had the highest total acidity, protein, salt, DH and amino acid contents, but had the lowest pH. The important amino acids in all 8 *Pla-ra* treatments were glutamic acid, lysine, and leucine. There were 43 volatile compounds detected in *Pla-ra*, and the 7 prime ones were aldehyde, alcohol, ester, furan, pyrazine, sulfur, and ketone. *Micrococcus* sp., *Bacillus subtilis* and *Pediococcus halophilus* strains, and roasted rice powder were added to the fish fermentation, which greatly enhanced the flavour and volatile compounds in *Pla-ra*.

Keywords: Fermented fish, *Pla-ra*, Roasted rice powder, Lactic acid bacteria, Volatile compounds

Introduction

Fermented fish, known as *Pla-ra* or *Pla-daek*, is a traditional food which is popular throughout Thailand, especially in the northern and northeastern parts of the country. *Pla-ra* is classified by its main ingredients [1], which are roasted rice powder and rice bran. The *Pla-ra* with roasted rice powder, produces a yellow soft texture and distinctive smell, and is generally used as a paste that is created by using striped snake-head fish or catfish, is usually produced in central Thailand. While the *Pla-ra* with rice bran, is manufactured using smaller fish in the northeastern part of Thailand, and has a pure black colour with a stronger smell and softer texture [2]. *Pla-ra* has a very strong smell, and its salty and sour flavours depend on the amount of salt added, and the amount of lactic acid produced during the fermentation process. The fermented products are usually stored at ambient temperatures and can last for a year or longer in the tropical climates of Southeast Asia [3]. There are differing methods to produce *Pla-ra*, and one is that there are 2 phases of its production. The first phase is to ferment the fish with salt until it softens, and the second is to ferment it with rice bran or roasted rice powder to achieve its scent and flavor [4]. Alternatively, the ingredients are all mixed together at the same time and allowed to naturally ferment for 12 - 18 months, depending on the desirable organoleptic property, at an ambient temperature of 28 - 30 °C [5].

The production of fermented fish is still manufactured on a small scale or as a local cottage industry. Many Asian and African countries still follow spontaneous fermentation, while Europe uses defined starter cultures to maintain the consistency of the products [6]. Moreover, the long period of *Pla-ra* fermentation is also of scientific interest in making products that meet consumer preferences. According to the producers, although *Pla-ra* is deemed to be edible after 2 - 3 months of fermentation, 6 -12 months are required to enhance palatability [7]. However, the inconsistent product quality and extremely long fermentation time are among the factors that limit the growth of fermented fish industries. The introduction of starter cultures

in the fermentation industry has led to greater quality, consistency, and safety of the fermented fish. The mass production of fish products has drastically increased over the last decade [6]. Zang *et al.* [8] reported that when starter cultures are added directly to the raw materials, they allow the fermentation process to be better controlled so that the quality characteristics can be standardized. Many attempts have been made to improve the fermentation process, by using selected starter cultures to accelerate the process and improve the odour and flavour of the fermented fish sauce [9].

Lactic acid bacteria (LAB) are also found as the dominant microorganisms in many fermented fish products. The primary role of LAB is to ferment the available carbohydrates and thereby cause a decrease in the pH. The combination of low pH and organic acids (mainly lactic acid) is the main preservation factor of fermented fish products [10]. *Pediococcus halophilus* is most prevalent when *Pla-ra* is fermented for 3 to 5 months. A study found that 90 % of the samples gathered from markets contain this species of bacterium, so it was concluded that it plays an important role in the fermenting process, especially for *Pla-ra*'s flavour and aroma [11]. Fish proteins are gradually hydrolysed by both bacterial and fish proteinases during fermentation. This process relies on natural fortuitous microorganisms with strong degradation and hydrolysis capabilities, including *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, and halophilic lactic acid bacteria. These bacteria could play a significant role in the bioconversion of fish substrate into fermented fish with colour, flavour, and aroma [9].

The physico-chemical characterization of various types of fermented fish is dependent on a variety of factors, which include the type of fish processing used, the percentage of salt in the product, the number of days of fermentation, and the presence of microorganisms in the product [12]. Rodpai *et al.* [3] investigated the microbial diversity of fermented freshwater fish products in *Pla-ra* from 3 provinces (Khon Kaen, Kalasin, and Roi Et) in the northeast of Thailand. They found that *Halanaerobium fermentans* and/or *Tetragenococcus muriaticus* (*Pla-ra* samples from Khon Kaen Province), *Lactobacillus rennini* (*Pla-ra* from Roi Et Province), and *T. muriaticus* (*Pla-ra* from Kalasin Province) were the dominant bacterial species. The microbial diversity between these samples may be due to the use of different raw materials, salt concentrations, processes, and the fermentation durations.

The purpose of this study was to determine the chemical properties, flavour and volatile compounds of *Pla-ra* by adding halophilic bacteria and LAB, as starter cultures into the fish fermentation. These bacteria produce enzymes that break down peptide bonds in proteins. These enzymes produce various peptides and amino acids, which affects the flavour of the fermented fish, and these are a precursor that reacts with other food constituents such as sugars and fats that form new substances with flavour, colour, and the individuality of *Pla-ra* [13]. In addition, bacteria produce amylase enzymes to decompose starch from roasted rice into lactic acid, causing a lower pH in *Pla-ra*, thereby reducing the fermentation time of it.

Materials and methods

Materials

Fresh striped snake-head fish (*Channa striata*) was purchased from a local fish shop (Kong Krailat, Sukhothai province, Thailand). The fresh fish was descaled, gutted, and sliced into an approximate size of 2x2 inches, and then immediately transferred to the laboratory in ice. The Sin Thao salt used in this study, was purchased from a local supermarket in Phitsanulok, Thailand. The roasted rice powder was prepared by roasting Khao Dawk Mali105 broken rice at 90 °C for 20 min, and then ground into a granular form. *Micrococcus* sp. (TISTR1404), *Bacillus subtilis* (TISTR001) and *Pediococcus halophilus* (TISTR430) were purchased from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand.

Chemicals

Ethanol, sodium hydroxide, nitric acid, hydrochloric acid, acetonitrile and methanol were purchased from RCI Labscan, Bangkok, Thailand. Ammonium thiocyanate and phenolphthalein were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Silver nitrate was purchased from QReC, New Zealand. Sodium chloride was purchased from Ajax Finechem Pty Ltd., Australia. Ferric alum was purchased from RX Chemicals, India. Phosphate buffer was purchased from G-Bioscience, USA. Trinitrobenzenesulfonic acid solution (TNBS) was purchased from Fluka, Germany. Sodium acetate and triethylamine were purchased from Thermo Fisher Scientific, USA. n-Alkanes was purchased from Sigma-Aldrich, USA.

Culture preparation

All 3 strains were cultured in agar media for maximum activity, before being added to the fish fermentation process. The inoculum should be in the log 6-log 7 CFU range, which is the appropriate

amount of microbial activity for fish fermentation. Several studies have made the addition of microorganisms to food at log 6 CFU. *Micrococcus* sp. inoculum was prepared according to Saratale *et al.* [14], *Bacillus subtilis* according to Zhong *et al.* [15] and *Pediococcus halophilus* according to Gurtler *et al.* [16].

Fish fermentation process

The fish fermentation process was divided into 2 phases, namely salt fermentation and roasted rice powder fermentation. In the salting phase, striped snake-head fish fillets were mixed with salt in a ratio of 3:1 w/w. After that, 1 mL *Micrococcus* sp., *Bacillus subtilis*, and *Micrococcus* sp. + *Bacillus subtilis* were added to the fish to obtain an initial culture amount of log 6-log 7 CFU/g, where the sample with no culture added, was regarded as the Control. There were 4 treatments for the salting phase, and each treatment created 2 sets. After the fish, the salt and the starter culture were combined, it was then packed tightly in a 1 kg jar, and kept at room temperature for 3 months during the first phase of the fermentation process. The fermented fish was removed from the jar, to enable the second phase of the fermentation to be carried out with the roasted rice powder. The first 4 treatments were mixed with ground roasted rice at a ratio of 4:1 w/w, and 1 mL (log 6-log 7 CFU) of *Pediococcus halophilus* was added. The other 4 treatments were only mixed with ground roasted rice at a ratio of 4:1 w/w. The 8 treatments were prepared for the roasted rice fermentation phase as shown in **Table 1**. The mixture was then packed tightly in a 1 kg jar, and kept at room temperature for another 3 months of fermentation process, where the characteristic taste of *Pla-ra* is attained. The total fermentation period of the *Pla-ra* was 6 months. The *Pla-ra* from each treatment was collected and stored in a freezer at $-18\text{ }^{\circ}\text{C}$, before further analyses of its physical and chemical properties. The fish fermentation process is illustrated in **Figure 1**.

Table 1 Details of fermented fish treatment.

Sample	Treatment	Non <i>P. halophilus</i>	<i>P. halophilus</i>
Control	fish + salt + roasted rice powder	/	-
FP	fish + salt + roasted rice powder	-	/
FM	fish + salt + roasted rice powder + <i>Micrococcus</i> sp.	/	-
FMP	fish + salt + roasted rice powder + <i>Micrococcus</i> sp.	-	/
FB	fish + salt + roasted rice powder + <i>B. subtilis</i>	/	-
FBP	fish + salt + roasted rice powder + <i>B. subtilis</i>	-	/
FMB	fish + salt + roasted rice powder + <i>Micrococcus</i> sp. + <i>B. subtilis</i>	/	-
FMBP	fish + salt + roasted rice powder + <i>Micrococcus</i> sp. + <i>B. subtilis</i>	-	/

Note: / means adding, - means no adding

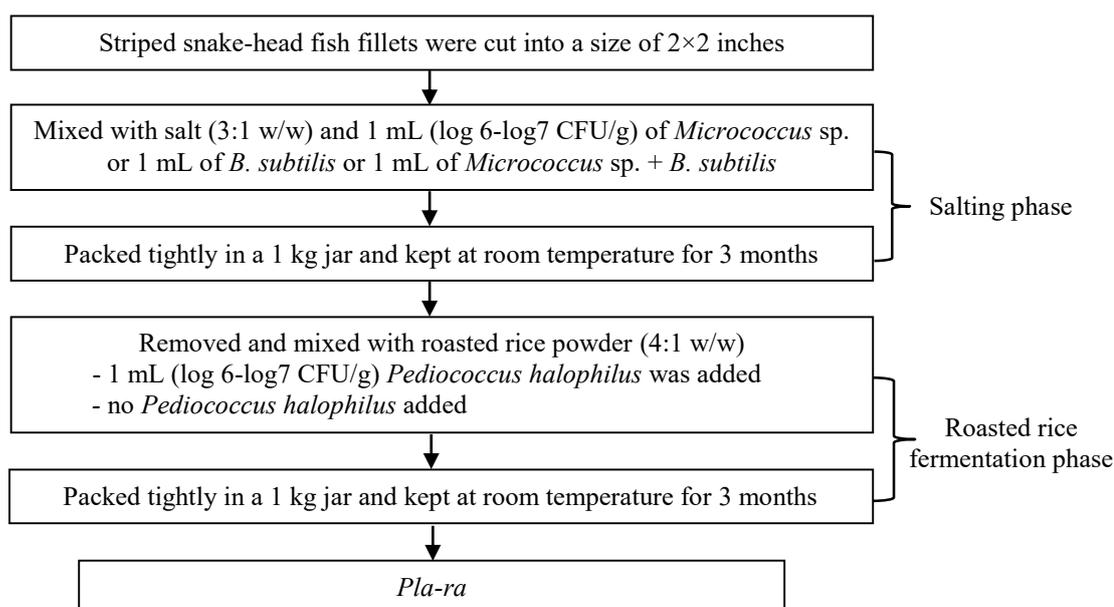


Figure 1 Fish or *Pla-ra* fermentation process.

Physical properties

The colour L^* , a^* and b^* values of each *Pla-ra* sample were measured with a colourimeter (HUNTER LAB, DP 9000, U.S.A.).

Chemical properties

Determination of pH, protein content and total acidity

The pH levels of *Pla-ra* samples were determined using a pH meter (Sartorius, Docu pH meter). The protein content was analyzed using the Kjeldahl method AOAC [17], using a conversion factor of 6.5. Total acidity (TA) was determined according to the official standard method of the AOAC [17].

Determination of salt content

Salt content was determined according to the AOAC [17] procedure. A 1 g sample was treated with 10 mL of 0.1 M silver nitrate and 10 mL of nitric acid, and then the mixture was heated gently on an induction-cooker for 10 min. This was followed by the mixture being cooled with water, then 50 mL of distilled water and 5 mL of ferric alum indicator were added. The mixture was titrated with standard 0.1 M ammonium thiocyanate until the solution became permanent brownish-red. The results were calculated and expressed as % NaCl.

Degree of hydrolysis analysis

The degree of hydrolysis (DH) of the protein hydrolysates, can be determined by measuring the amount of free amino acids using the method described by Wisuthiphaet *et al.* [18]. DH of the fish protein hydrolysate was analyzed according to the trinitro-benzene-sulfonic acid (TNBS) method. The 2 mL of 0.2 M phosphate buffer pH 8.2 and 2 mL of 0.1 % TNBS solution were added to 0.25 mL of fish protein hydrolysate then incubated at 50 °C for 30 min. That was followed by 5 mL of 0.1 M HCl being added to terminate the reaction, then the tubes were allowed to rest at room temperature for 15 min. The mixture was analyzed using the spectrophotometric method, which measured the absorbance at a wavelength of 420 nm. Free α -amino acid was obtained using the standard curve of leucine and the DH was calculated using the equation:

$$DH = [(L_t - L_0) / (L_{\max} - L_0)] \times 100$$

where, L_t is the amount of α -amino acid of fish protein hydrolysates hydrolyzed for t h, L_0 is the amount of α -amino acid of raw material fish and L_{\max} is the amount of α -amino acid of fish protein hydrolysates completely hydrolyzed by 6 M HCl (protein solution: HCl = 1:10) at 100 °C for 12 h.

Amino acids

Amino acids were analysed following AOAC [17] by weighing 0.5 g of each sample into a 25 mL beaker to which 10 mL of 6 N hydrochloric acid was then added. The samples were heated at 100 °C for 24 h and then filtered to obtain a filtrate to which 5 mL of a drying solution (methanol, picolotiocyanate, triethylamine) was added, and then dried. The derivative solutions (methanol, sodium acetate, and triethylamine) were added and the samples were left for 20 min. Approximately 200 mL of 1 M acetate solution was added and an aliquot of each sample was injected into a High Performance Liquid Chromatography (HPLC) instrument. The following HPLC settings were used: at room temperature, using the pico column tag $3.9 \times 150 \text{ mm}^2$, a flow rate of 1.5 mL/min, a pressure limit of 3,000 psi (1 psi = 6.89476 kPa), gradient program, mobile phase 60 % acetonitrile, 1 M sodium acetate buffer, and UV detector with wavelength 254 nm.

Volatile compounds

The volatile compounds were measured using a gas chromatograph coupled to a mass detector spectrometer (GC-MS, 5977B MSD, Agilent, USA) by applying the method of Yang *et al.* [19].

Isolation of volatiles

A 3 g sample of minced *Pla-ra* was placed into a 20 mL vial, then incubated at 70 °C for 40 min using a digital dry bath. Volatile compounds were collected for 30 min using a Twister® /Stir Bar (PDMS fiber, film thickness 1.0 mm, 20 mm length GERSTEL Twister® kit, USA), after incubation.

GC-MS analysis

The fiber was inserted into the thermal desorption unit (TDU) for desorption. The initial temperature of the TDU mode was set at 40 °C, then increased to 250 °C at 240 °C/min for 3 min via the splitless mode and then the transfer temperature was set at 280 °C. The GERSTEL Cooled Injection System (CIS) mode was initially set at 20 °C, then increased to 250 °C at 12 °C/min for 10 min before the volatile compounds could be collected then transferred to a column. A 30 m × 0.25 mm × 0.25 µm film thickness Agilent J&W DB-WAX capillary column, was used for the separation of the volatile compounds. The flow rate of the carrier gas (helium) was 1.5 mL/min. The GC oven gradient program was as follows: The initial temperature was 30 °C and maintained for 5 min, then increased to 50 °C at 3 °C/min and held there for 3 min, then increased to 150 °C at 5 °C/min, and finally raised at 20 °C/min to 240 °C and held for 5 min. The mass spectrometer conditions were set as follows: Electron impact (EI) mode with electron energy of 70 eV, the scan range of full scan was 29 - 400 m/z, the temperature of the ion source was 250 °C, and the temperature of the quadrupole detector was 150 °C. To calculate the retention index (RI), the n-alkanes (C6-C28, Sigma-Aldrich, USA) were measured with the same GC-MS conditions. The volatile compounds were measured by using a comparison with the mass spectral database (NIST 11, National Institute of Standards and Technology Mass Spectrometry Data Center, Gaithersburg, MD, USA).

Statistical analysis

All measurements were performed identically 3 times and the experimental results are expressed as means. The data was analyzed using the analysis of variance (ANOVA), and the means were separated by Duncan's multiple range tests at the 95 % significance level.

Results and discussion

Physical properties

The colour and characteristics of the *Pla-ra* are shown in **Figure 2** and the obtained L*, a* and b* values are presented in **Table 2**. The fermented fish with the highest L* (54.48) ($p \leq 0.05$) was that of the Control, but it was not different from the FMB and FP ($p > 0.05$), which L* values were 52.58 and 52.68, respectively, while the L* of the FBP (50.28) ($p \leq 0.05$) was the lowest. The FBP's L* value was not significantly different from the FMP (50.31) ($p > 0.05$). However, no significant difference was found in the a* value of each fermented fish. The a* values were found to be in the range of 2.52 - 3.63. The b* values of the FB was 20.76 and it was the highest b* values ($p \leq 0.05$) among the 8 *Pla-ra* treatments. The FB's b* value was not significantly different from the FP, FM, FBP, FMB and FMBP ($p > 0.05$). The treatment with the lowest b* (18.42) ($p \leq 0.05$) was the FMP, but it was not different from the Control, FP, FM, FBP, FMB and FMBP. All of the 8 treatments were yellowish-brown, which is mainly attributed to the addition of roasted rice powder. Furthermore, the colour of the product depends on the species of fish used as well as the processing methods. Poorly fermented products tend to be grayish or dark, but if the fish is cut and dried either in an oven or in the sun, they are usually light brown in colour. Moreover, the L*, a* and b* values usually increased during fermentation. The increase in L* value might be a result of protein denaturation induced by the formation of acid. The increase in a* and b* values were possible due to the non-enzymatic browning reaction of the free amino acid group as well as the lipid oxidation products increased, particularly with increasing fermentation time. The Maillard reaction between a carbonyl compound and amino acids is the major non-enzymatic browning in foods [20]. Kim *et al.* [21] investigated the colour of the irradiated salted and fermented anchovy sauce. The colour values L*, a*, and b* were increased by gamma irradiation at 0 week, while the reduction of the colour values were observed in all the samples that showed a trend of darkening during storage. However, the colour values were higher in the gamma-irradiated samples than the control. Whereas, in this study the L* of the Control was higher than the samples, and no significant difference was found in the a* value of each sample, while the b* of the Control showed no significant difference ($p > 0.05$) in some of the samples.

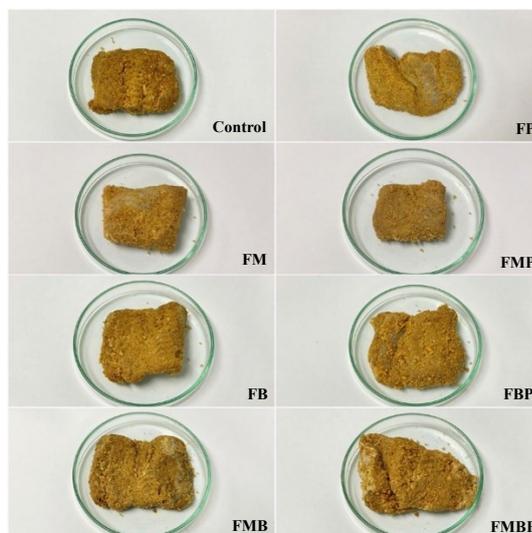


Figure 2 Colour and physical appearance of *Pla-ra*.

Note: Control: Fish + salt + roasted rice powder, FP: Fish + salt + roasted rice powder + *P. halophilus*, FM: Fish + salt + roasted rice powder + *Micrococcus* sp., FMP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *P. halophilus*, FB: Fish + salt + roasted rice powder + *B. subtilis*, FBP: Fish + salt + roasted rice powder + *B. subtilis* + *P. halophilus*, FMB: fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* and FMBP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* + *P. halophilus*

Chemical properties

pH

The pH is an important parameter that affects the fermentation process. At the beginning of fermentation, the digestive enzyme responsible for autolysis (trypsin and chymotrypsin) was stable at neutral condition [22], which would help in increasing protein hydrolysis. The pH has a significant effect on DH, and the pH tended to decrease with the progress of hydrolysis. The pH of all the *Pla-ra* measurements are shown in **Table 2**. The pH of the 8 *Pla-ra* treatments were found to be in the range of 5.33 - 5.63, while the pH of the Control and FM were 5.62 and 5.63, respectively. Although these 2 values were not significantly different from each other ($p > 0.05$), they were the highest pH among the *Pla-ra* treatments, while the pH of the FMP (5.33) ($p \leq 0.05$) was the lowest. It was observed that the pH of all the treatments with *Pediococcus halophilus* were significantly lower than the uninoculated treatments. Thus, *Pediococcus halophilus* is a LAB, which produces lactic acid during the fish fermentation. Moreover, the addition of roasted rice powder decreased the pH and increased the production of lactic acid, which causes the bacteria to digest various nutrients, especially the glycogen formed in the fish by glycolysis. The acid content increases due to the addition of roasted rice powder, which is caused by the bacteria turning the starch from the roasted rice powder into lactic acid. There are also other acids derived from fatty acids caused by the deamination reactions. All 8 samples had the pH values between 5 and 6, which conformed to the standard value of *Pla-ra* [1], causing a lower pH in the *Pla-ra* that indicated that the fermentation process for 6 months was completed.

Protein content

The protein content of the Control was the highest (11.10 %) ($p \leq 0.05$). The FB had the lowest protein content (8.31 %) ($p \leq 0.05$), but it was not significantly different from the FMP, FBP and FMBP ($p > 0.05$) as shown in **Table 2**. The samples with the addition of the starter culture had a lower protein content than the naturally fermented fish without inoculation (Control) during fermentation. This was due to both the *Micrococcus* sp. and *Bacillus subtilis* aided in the protein degradation during the fish fermentation. *Pediococcus halophilus* is LAB bacteria that is also involved in fish protein degradation. Fish protein cells were destroyed, and decomposed outside the cells, causing the fish protein content to decrease. The protein content was varied in the fermented fish because of different types of raw materials used and also the processing method (salt concentration and fermentation condition). Various amounts of different proteins were found in these fermented fish products. Pattarawiwat and Itthipong [2] reported that fermented fish made from snake-head fish, had a high protein content of 17.95 %, which is a higher protein

content than other types of fermented aquatic products such as Somfug, fish sauce and shrimp paste, and is higher than the protein content in this study.

Total acidity

The total acidity of the *Pla-ra* is shown in **Table 2**. The FBP sample had the highest total acidity content (2.87 %) ($p \leq 0.05$) among the 8 *Pla-ra* treatments, but it was not significantly different from the FMP and FMBP (2.74 and 2.85 %, respectively) ($p > 0.05$). The total acidity of the FMB (2.08 %) was the lowest ($p \leq 0.05$), regarding the samples with and without *Pediococcus halophilus* inoculation. The samples with *Pediococcus halophilus* had a higher total acidity content than the samples without this bacterium, because it is classified as LAB. This bacterium can produce lactic acid during the fermentation of the fish, especially in 3 - 5 months of this process, which is coincided to be the ideal fermentation period in this study. Lactic acid is produced by LAB, which converted starch from roasted rice powder into lactic acid, and also caused the bacteria to digest various nutrients, especially glycogen formed in the fish via glycolysis. In addition, there are also acids that are derived from fatty acids by deamination reactions [23]. The highly significant negative correlation between pH and total acidity values, demonstrated that the increase in total acidity is generally accompanied by a decrease in pH [24].

Salt (NaCl) content

The fermented fish with the highest salt content (22.28 %) ($p \leq 0.05$) was that of the FMP. The salt content of the Control, FBP, FMB and FMBP were 21.46, 21.18, 21.24 and 21.51 %, respectively. These 4 values showed no significant difference between each other and the FMP ($p > 0.05$). The FB had the lowest salt content of 20.60 % ($p \leq 0.05$), which is not significantly different from the other samples ($p > 0.05$), except the FMP (**Table 2**). All of the 8 fermented fish treatments had slightly different salt contents. These results showed uniformity of the fermented fish pieces and the rate of salt diffusion into them.

The standard salt content of fermented fish in the Thai Community Product Standard is between 12 - 20 % [1]. The salt content of all the fermented fish treatments ranged from 20.60 to 22.28 %, indicating:

Table 2 Physical characteristics and chemical composition of *Pla-ra*.

Composition/ Characteristics	Non- <i>Pediococcus halophilus</i>				<i>Pediococcus halophilus</i>			
	Control	FM	FB	FMB	FP	FMP	FBP	FMBP
Colour								
L*	54.48 ± 0.88 ^a	51.53 ± 1.06 ^{bc}	51.47 ± 0.62 ^{bc}	52.58 ± 1.51 ^{ab}	52.68 ± 0.88 ^{ab}	50.31 ± 0.94 ^c	50.28 ± 1.41 ^c	52.26 ± 0.94 ^{bc}
a* NS	2.53 ± 1.13	3.5 ± 1.16	2.52 ± 0.90	3.55 ± 1.42	2.84 ± 1.02	3.63 ± 0.91	3.39 ± 0.95	3.59 ± 0.50
b*	18.64 ± 1.11 ^b	19.53 ± 1.18 ^{ab}	20.76 ± 0.57 ^a	19.75 ± 1.23 ^{ab}	20.29 ± 1.08 ^{ab}	18.42 ± 1.19 ^b	18.97 ± 0.70 ^{ab}	19.34 ± 0.81 ^{ab}
pH	5.62 ± 0.02 ^a	5.63 ± 0.01 ^a	5.54 ± 0.02 ^b	5.52 ± 0.02 ^b	5.45 ± 0.02 ^c	5.33 ± 0.01 ^f	5.37 ± 0.02 ^c	5.42 ± 0.00 ^d
Total acidity (%)	2.08 ± 0.11 ^d	2.27 ± 0.05 ^e	2.33 ± 0.03 ^c	2.38 ± 0.08 ^c	2.57 ± 0.10 ^b	2.74 ± 0.06 ^a	2.87 ± 0.01 ^a	2.85 ± 0.05 ^a
Protein content (%)	11.10 ± 0.48 ^a	9.64 ± 0.43 ^c	8.31 ± 0.03 ^c	8.91 ± 0.09 ^d	10.14 ± 0.34 ^b	8.73 ± 0.11 ^{de}	8.38 ± 0.03 ^c	8.47 ± 0.06 ^{de}
Salt (NaCl) (%)	21.46 ± 0.53 ^{ab}	20.91 ± 0.71 ^b	21.01 ± 0.26 ^b	21.24 ± 0.82 ^{ab}	20.60 ± 0.46 ^b	22.18 ± 0.71 ^a	21.18 ± 0.14 ^{ab}	21.51 ± 0.46 ^{ab}
DH (%)	42.45 ± 0.81 ^g	43.04 ± 0.35 ^g	49.46 ± 0.15 ^c	50.25 ± 0.10 ^d	45.48 ± 0.38 ^f	54.07 ± 0.17 ^a	52.51 ± 0.26 ^e	51.46 ± 0.28 ^c

Note: Control: Fish + salt + roasted rice powder, FP: Fish + salt + roasted rice powder + *P. halophilus*, FM: Fish + salt + roasted rice powder + *Micrococcus* sp., FMP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *P. halophilus*, FB: Fish + salt + roasted rice powder + *B. subtilis*, FBP: Fish + salt + roasted rice powder + *B. subtilis* + *P. halophilus*, FMB: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* and FMBP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* + *P. halophilus*

- All values are means of triplicates ± standard deviation. Means with different letters (a - g) within the same row were significantly different by Duncan's multiple range test ($p \leq 0.05$).

- NS = not significant ($p > 0.05$)

that the salt content in all 8 treatments was slightly higher than the specified standard. Salt is an important ingredient in the fish fermentation as it helps to accelerate the process and inhibit the spoilage bacteria at a concentration of 20 % [25]. The different salt concentrations found in different fermented fish samples, have a pronounced influence on the microbial growth and the rate of fermentation, and the sensory quality and safety of the product [26]. Other microorganisms such as LAB, yeasts, molds and halophilic bacteria are also able to grow. The lactic acid is produced from LAB and it inhibits the growth of spoilage pathogens. Salt contributes to the growth of LAB, therefore, the salt content is slightly higher than the standard in this study, which was considered to have a positive effect on the shelf-life of the *Pla-ra*. Salt is well-known for its role as a flavouring agent and as a food preservative, both in industrial food processing and home cooking.

Degree of hydrolysis

The FMP had the highest DH (54.07 %) ($p \leq 0.05$), while the Control had the lowest DH (42.45 %) ($p \leq 0.05$), but it was not significantly different from the FM (43.04 %) ($p > 0.05$) (Table 2). The treatments inoculated with *Pediococcus halophilus* showed higher DH than those not inoculated. This was mainly due to the acid produced by LAB, that was involved in the protein degradation during the fish fermentation. LAB added to the samples included *Micrococcus* sp. and *Bacillus subtilis* were important protein-degrading bacteria, which increased the efficiency of protein degradation, thereby increased the DH.

The DH is a true reflection of the progress of hydrolysis and thus its selection as the primary indicator, was very appropriate. For a given substrate and enzyme at a given pH, the comparison of different hydrolysates properties is based on the DH, which is the primary determinant. The pH is naturally found to exert an independent influence on the properties of the hydrolysates. It is therefore recommended that the pH should be controlled, and in most cases remain constant during the hydrolysis processes. The pH has a significant effect on the DH, which increased as pH decreased and the reaction time increased [27].

Amino acid composition

LAB and endogenous enzyme activities can convert organic substances into simple compounds such as peptides, amino acids, other nitrogenous compounds and fatty acids [28]. The alteration in the number of each amino acid, is caused by the difference in the free amino acid balance produced by autolysis and microbial activity, which significantly affected the taste of the fermented fish products. The glutamic acid creates a meaty aroma, while some of other amino acids such as glycine, alanine, serine, and threonine give a sweet taste, while valine, phenylalanine, and histidine give a bitter taste [29].

The amino acid content of the *Pla-ra* is shown in Table 3. The 9 non-essential amino acids and 9 essential amino acids were measured in *Pla-ra*. The results showed that the important amino acids in all 8 *Pla-ra* treatments are glutamic acid (meaty aroma), lysine (caramel aroma) and leucine (bitterness odour). The glutamic acid of all treatments was the highest, followed by lysine and leucine, respectively. Glutamic acid is a non-essential amino acid, which is classified as an acidic amino acid [30], while lysine and leucine are essential amino acids. Lysine is a basic amino acid and leucine is a neutral nonpolar amino acid, which is essential for maintaining a 3D protein structure. Seven of the eight *Pla-ra* samples with the starter cultures had a higher amino acid content than the Control. The FMP had the highest amino acid content of 4,214.31 mg/100 g, while the Control had the lowest amino acid content of 3,055.12 mg/100 g. The inoculated samples had a high amino acid content, due to the acceleration of bacterial metabolic activity and protein degradation during the fish fermentation. Generally, proteolytic enzymes such as lysosomal cathepsins and proteases which are found in fish muscles, are involved in protein hydrolysis during the fish fermentation. These enzymes are released from the lysosomes of fish muscles, and perform better in an acidic environment. Therefore, the *Micrococcus* sp. and *Bacillus subtilis*, which are protein-degrading bacteria, and *Pediococcus halophilus* which is a halophilic LAB, were added to the fish fermentation process that increased the lactic acid, and decreased the pH, is an important factor that promoted the degradation of the fish protein, causing higher amounts of amino acids in the *Pla-ra* [9].

Table 3 Amino acid contents in *Pla-ra*

Amino acid (mg/100 g)	<i>Non-Pediococcus halophilus</i>				<i>Pediococcus halophilus</i>			
	Control	FM	FB	FMB	FP	FMP	FBP	FMBP
Non-essential								
Glutamic acid	457.11	556.65	545.24	618.18	519.28	656.43	628.47	627.29
Alanine	241.06	291.07	281.64	320.51	269.16	327.36	309.40	317.32
Aspartic acid	201.54	216.27	219.10	251.29	210.61	260.86	239.37	248.34
Serine	165.38	193.46	200.36	212.24	180.58	213.19	207.81	219.11
Tyrosine	151.06	178.02	186.24	169.43	163.40	188.31	175.47	182.00
Proline	101.76	125.31	121.29	128.56	120.34	139.61	130.16	135.72
Glycine	96.69	127.90	120.46	134.64	113.47	146.42	131.58	139.48
Arginine	10.21	13.64	14.36	16.19	12.35	19.38	16.63	17.26
Cysteine	7.49	8.76	10.26	11.32	8.14	11.93	10.78	11.47
Essential								
Lysine	403.24	519.17	501.86	529.82	479.27	589.35	551.16	578.42
Leucine	358.36	445.65	467.24	518.65	404.12	523.28	509.48	513.41
Threonine	172.25	186.86	189.08	199.81	178.38	201.13	193.46	196.41
Isoleucine	167.65	183.46	183.27	189.23	178.19	194.38	180.54	198.09
Phenylalanine	142.08	172.58	180.36	184.28	161.16	195.52	186.61	191.06
Valine	137.21	167.14	171.01	170.87	156.03	187.63	178.21	182.24
Methionine	131.11	156.66	161.00	168.27	150.23	179.04	162.81	174.18
Histidine	87.12	114.38	119.47	123.68	98.04	132.30	112.24	126.65
Tryptophan	23.80	36.26	32.12	43.62	32.72	48.19	41.34	46.11
Total	3055.12	3693.24	3704.36	3990.59	3435.47	4214.31	3965.52	4104.56

Note: Control: Fish + salt + roasted rice powder, FP: Fish + salt + roasted rice powder + *P. halophilus*, FM: fish + salt + roasted rice powder + *Micrococcus* sp., FMP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *P. halophilus*, FB: Fish + salt + roasted rice powder + *B. subtilis*, FBP: Fish + salt + roasted rice powder + *B. subtilis* + *P. halophilus*, FMB: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* and FMBP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* + *P. halophilus*

Volatile compounds

There were 43 volatile compounds detected in the *Pla-ra*, using a GC-MS (Table 4). Among all the samples, the 7 most abundant volatile components detected were aldehyde, alcohol, ester, furan, pyrazine, sulfur, and ketone.

The majority of aldehydes originate from oxidation and degradation of lipids, and generally enhance the flavour of meat products [31]. Due to the low threshold values of aldehydes, they are important aroma compounds found in food products. They can contribute to a desirable aroma and flavour, as well as a rancid odour during the spoilage of fat and fatty foods. The aldehyde compounds such as hexanal, pentanal, nonanal and 3-methylbutanal that are detected in meat or fermented fish products, are produced by the Strecker reaction. The 3-methylbutanal had the highest content of the aldehydes in all of the *Pla-ra* treatments, which was followed by hexanal (Table 4). In the regular catabolic pathway of amino acids, leucine is converted to α -ketoisocaproic acid (4-methyl-2-oxopentanoic acid) by deamination, which is then converted to acetoacetate and acetyl-CoA. The latter compound can enter the TCA cycle. As an alternative to the major catabolic route, α -ketoisocaproic acid can be used as the starting molecule for the generation of the branched aldehyde, 3-methylbutanal [32]. Hexanal was mainly derived from omega-6 unsaturated fatty acids, and had a green grassy fragrance [33].

Alcohols were not as important as aldehydes and ketones in the creation of the aroma of the fermented fish products, but the alcohols play a key role in producing its flavour [33]. In particular, 1-octen-3-ol, which is mainly derived from the oxidation of unsaturated fatty acids and provides a grassy, fishy mushroom-like odour [34], is an important flavour compound in fermented mandarin fish and other fermented products [35]. The 1-octen-3-ol was also detected in this study.

In general, esters derived from the microbial esterification of acids with alcohols provide a fruity aroma and mask the rancid taste, and are important components of the fermented food's unique flavour [35]. Isoamyl acetate, isoamyl valeriate and isopropyl myristate were detected in some *Pla-ra* treatments in this study.

Heterocyclic pyrazine compounds such as methylpyrazine were detected in low quantities in 7 of the 8 *Pla-ra* treatments, except the FMBP one. Four other types of pyrazine were able to be detected, but some could not be found in any of the treatments due to its very low quantities. Pyrazines have a low-threshold, and a pleasant roasted flavour which are considered to be important for roasted and cooked-meat/plant-like odorants. Pyrazines are a result of the Maillard reaction, which involves free amino acids, peptides, proteins, and reducing sugars, were formed by a reaction between heat-induced carbohydrate degradation products and Strecker products of amino acids or ammonia [36]. The pyrazine detected in this study, was the result of the addition of roasted rice powder as an ingredient for the fish fermentation. In addition, the formation of pyrazines has been reported to be associated with the Maillard reaction, through the Strecker degradation as various nitrogen sources that include amino acids as a precursor, and metabolic activities of the microorganisms during fermentation [37].

Furan is formed from the dehydrated or fermented condensates of carbohydrates in the Amadori rearrangement pathways [38]. This volatile substance is produced by glucose pyrolysis and the Maillard reactions during fish fermentation. The furans resulted from thermal degradation and rearrangement of cellulose and hemicellulose, via a classical carbohydrate degradation of roasted rice powder during roasting. In this study, 2-pentylfuran was detected in all treatments, except the FB, FMB and FBP, and contributed a beany, grassy and licorice (as soybean oil) flavours [38].

The 2 sulphur-containing compounds detected in *Pla-ra* included dimethyl disulfide and dimethyl trisulfide, which are often derived from sulphur amino acids, have a garlic and cooked cabbage aroma, respectively, and also constituted significant fractions of volatile compounds. Some heterocyclic sulphur compounds, usually having a barbecue flavour, are also reported to be derived from amino acids (cysteine, cystine and methionine) or thiamine [38].

Ketones are produced by the microbial enzymatic action on lipids or amino acid degradation, or by the Maillard reactions [37]. Although ketone compounds are often detected, their aromatic contribution may be minor due to their high threshold [35]. In this study, 6-methyl-5-hepten-2-one, 3-hydroxy-2-butanone and 2-heptanone were detected in some *Pla-ra* treatments.

The volatile compounds formed by various reactions that occur during the fermentation process, are the Maillard reaction and the Strecker degradation reactions of amino acids. These were also caused by the breakdown of fatty acids and the generation of amino acids during fermentation from fish protein degradation via bacteria and enzymes produced during fish fermentation. The addition of the 3 starter cultures, *Micrococcus* sp., *Bacillus subtilis* and *Pediococcus halophilus* affected the flavour and volatile compounds in the *Pla-ra*. In addition, roasted rice powder as an ingredient in the *Pla-ra* also contributed flavour and volatile compounds.

Zhou *et al.* [39] assessed the influence of lactic acid bacteria (LAB) on the physicochemical characteristics of low-salt fermented Mackerel (*Pneumatophorus japonicus*) seasonings. Key volatile compounds and their formation pathways were analyzed. They found that a total of 27 odor-active compounds were detected in the mackerel seasonings, most of which were aldehydes and alcohols. The key odor-active compounds, which were determined through the calculation of OAV, included (E)-2-nonenal, (E,E)-2,4-decadienal, (E)-2-decenal, 1-octen-3-ol, and (E,E)-2,4-nonadienal, which were mainly derived from lipid oxidation.

Giri *et al.* [32] investigated the suitability of 4 species of trash fishes for the production of fish miso, a Japanese fermented fish meat paste compared with soy and rice miso from the point of view of product aroma. A total of 107 volatile compounds have been identified, where 94 were common for all the miso samples. Considering the lower threshold perception and higher odour active values 2-methylbutanal, 3-methylbutanal, methional, isoamyl acetate, dimethyl disulfide, dimethyl trisulfide, 2,3-butanedione, 3-methylethyl butanoate, 3-methyl-1-butanol, ethyl hexanoate, 1-octen-3-ol, heptanol, heptanal and 2-undecanone were identified as key compounds for the miso products.

Table 4 Volatile compounds in *Pla-ra*.

No.	Volatile compounds	RI ^a Wax	Odour description	%Area							
				Non- <i>Pediococcus halophilus</i>				<i>Pediococcus halophilus</i>			
				Control	FM	FB	FMB	FP	FMP	FBP	FMBP
1	Acetone	820	Solvent	ND	6.64	8.47	6.55	3.94	6.59	5.82	4.32
2	3-Methylbutanal	928	Almond, nutty	2.55	1.57	ND	1.84	3.02	2.09	2.13	2.01
3	2-Propanol	921	Solvent, musty	2.87	ND	ND	6.23	2.33	ND	7.32	4.89
4	Ethanol	938	Alcoholic	ND	8.08	6.48	ND	ND	3.03	ND	ND
5	Pentanal	988	Pungent, almond, rubber	0.43	1.02	ND	1.02	0.9	0.44	0.98	1.91
6	Toluene	1048	Sweet, pungent, benzene	ND	0.38	0.57	0.41	0.53	ND	ND	0.41
7	1-Propanol	1049	Plastic, musty	0.45	1.65	1.46	0.98	0.4	0.68	1.63	0.62
8	Dimethyl disulfide	1078	Cooked cabbage, onion	2.31	1.17	0.86	0.61	3.5	1.72	0.61	0.36
9	Hexanal	1091	Fishy, grassy	1.72	1.39	0.49	1.43	5.48	0.97	2.73	6.94
10	2-Methyl-1-propanol	1103	Solvent-like	0.21	0.52	0.59	0.56	0.27	0.19	0.66	0.57
11	1-Butanol	1131	Solvent	ND	0.53	ND	ND	ND	0.24	ND	ND
12	Isoamyl acetate	1130	Banana, fresh, pear odour	ND	ND	ND	0.39	ND	ND	0.35	0.38
13	1,4-Dimethylbenzene (p-Xylene)	1150	Cold meat, oily	0.10	0.18	0.13	0.12	ND	0.05	0.10	0.16
14	3-Methylundecane			ND	ND	0.20	0.24	ND	ND	0.21	0.20
15	2-Heptanone	1164	Soapy, fruity	0.22	ND	ND	ND	0.36	ND	ND	ND
16	Heptanal	1195	Dry fish	ND	0.23	ND	0.10	ND	ND	0.12	0.31
17	Limonene	1211	Fresh, sweet	0.30	0.49	0.19	0.28	0.25	0.20	0.23	0.23
18	3-Methyl-1-butanol,	1219	Rancid, pungent, balsamic	0.30	2.72	2.70	4.63	2.49	2.85	3.72	2.96
19	2-Pentylfuran	1234	Green bean-like	0.09	0.12	ND	ND	0.20	0.07	ND	0.19
20	1-Pentanol	1249	Green, wax	1.25	2.35	0.74	ND	1.69	0.90	0.65	1.14
21	Methylpyrazine	1278	Fishy, nutty, ammoniacal	0.07	0.14	0.12	0.11	0.09	0.06	0.07	ND
22	Isoamyl valerierate		Fruity, sweet	ND	ND	ND	0.55	ND	ND	0.43	ND
23	3-hydroxy-2-butanone (Acetoin)	1280	Buttery, creamy, dairy	ND	ND	0.37	ND	ND	ND	ND	0.70
24	2,3-Dimethylpyrazine	1340	Pungent, almond, rubber	ND	ND	0.05	0.04	ND	ND	ND	ND
25	1-Haxanol	1340	Green, grassy	2.57	5.07	0.85	0.55	1.71	1.48	0.55	0.38
26	6-Methyl-5-hepten-2-one	1341	Sweet, fruity	0.07	0.16	0.09	0.09	ND	0.07	0.10	ND
27	Dimethyl trisulfide	1390	Fishy, sulfury	0.15	0.06	ND	ND	0.21	0.08	ND	ND
28	Trimethyl pyrazine	1399	Bread, burnt, musty, peanut	ND	ND	0.13	0.14	ND	ND	0.12	0.14
29	Nonanal	1407	Green, fatty	ND	0.38	0.14	0.25	0.16	0.19	0.26	0.31
30	1-Octen-3-ol	1444	Mushroom, fishy, grass, fatty	0.20	0.39	0.19	0.29	0.41	0.19	0.30	0.47
31	1-Heptanol	1450	Fresh, nutty	0.18	0.31	0.06	0.06	0.41	0.18	0.07	0.1
32	Tetramethyl pyrazine	1487	Fermented soy	0.11	0.04	0.12	0.05	ND	ND	0.07	0.07
33	3,5-diethyl-2-methylpyrazine		Nutty, meaty, vegetable	ND	ND	0.06	ND	ND	ND	0.04	ND
34	Benzaldehyde	1539	Bitter almond	0.88	0.6	0.82	1.05	0.85	0.56	0.70	0.51
35	1-Octanol	1542	Fatty, green	0.20	0.20	0.03	0.05	0.28	0.11	0.03	0.04
36	Isopulegol	1580	Minty, cooling, medicinal	ND	0.08	0.05	ND	ND	ND	ND	ND
37	2-Furanmethanol	1665	Burnt sugar	0.06	0.10	ND	0.19	ND	0.06	0.10	ND
38	Phenylethyl alcohol	1934	Honey, rosy	0.29	0.31	0.36	1.19	0.20	0.68	0.62	0.73
39	Phenol	1997	Herbal, anisic	2.31	4.66	6.27	2.21	5.42	3.58	6.33	0.9
40	Isopropyl myristate		Fatty, faint, oily	1.08	1.04	ND	0.17	0.27	0.19	ND	0.13
41	1-Hexadecanol	2390	Waxy, clean, greasy	1.00	0.36	ND	ND	0.52	0.62	ND	ND
42	Indole	2421	Pungent, floral, naphtha	0.21	0.14	0.16	0.09	0.18	0.12	ND	ND
43	2-Hexadecanol			0.08	0.07	0.09	0.06	0.08	0.10	ND	ND

Note: Control: Fish + salt + roasted rice powder, FP: Fish + salt + roasted rice powder + *P. halophilus*, FM: fish + salt + roasted rice powder + *Micrococcus* sp., FMP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *P. halophilus*, FB: Fish + salt + roasted rice powder + *B. subtilis*, FBP: Fish + salt + roasted rice powder + *B. subtilis* + *P. halophilus*, FMB: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* and FMBP: Fish + salt + roasted rice powder + *Micrococcus* sp. + *B. subtilis* + *P. halophilus*

^a All compounds were measured by using a comparison with the mass spectral database (NIST 11)

Conclusions

All of the 8 *Pla-ra* treatments were yellowish-brown in colour, due to the addition of roasted rice powder. The pH of all the *Pla-ra* treatments were found to be in the range of 5.33 - 5.63, which conformed to the standard levels. This indicated that the fish fermentation process of 6 months was completed, thereby reducing the fermentation time of it. The samples with the addition of starter cultures had a lower protein content than the Control during fermentation. The samples with *Pediococcus halophilus* had a higher total acidity content and DH than the samples without this bacterium, because it is classified as LAB. This was mainly due to the acid produced by the LAB that was involved in the protein degradation during the fish fermentation. An increase in the total acidity content and DH, is generally accompanied by a decrease in pH. The salt content of all the *Pla-ra* treatments ranged from 20.60 to 22.28 %. A slightly higher salt content than the standard in this study, was considered to have a positive effect on the shelf-life of the *Pla-ra*. The FMP treatment had the highest total acidity, protein, salt, DH and amino acid contents, while having the lowest pH. The 9 non-essential amino acids and 9 essential amino acids were detected in *Pla-ra*. The important amino acids in all 8 *Pla-ra* treatments were glutamic acid, lysine, and leucine. Forty-three volatile compounds were detected in the *Pla-ra*, with the 7 most abundant ones being aldehyde, alcohol, ester, furan, pyrazine, sulfur, and ketone. The volatile compounds were formed by various reactions that occurred during the fermentation, which included the Maillard reaction and the Strecker degradation reaction of amino acids. The addition of the 3 starter cultures, *Micrococcus* sp., *Bacillus subtilis* and *Pediococcus halophilus* into the fish fermentation, as well as roasted rice powder provided a great effect on the flavour and volatile compounds in the *Pla-ra*. However, the ratio of fish to salt for the fish fermentation may be studied in the future, due to this study having a slightly higher salt content in the *Pla-ra* than the standard.

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