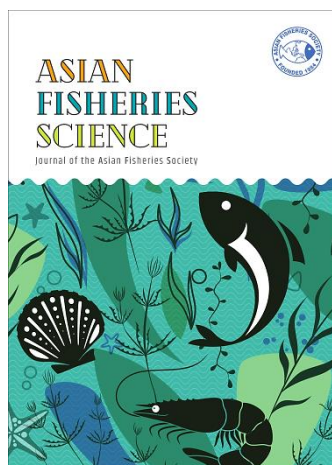




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Antioxidative Activities and Lactic Acid Bacteria Composition of Fermented Frigate Tuna *Auxis thazard* (Lacepède, 1800) at Different Salt-fish Ratios

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Abstract

The antioxidative activities and lactic acid bacteria (LAB) composition of fermented frigate tuna *Auxis thazard* (Lacepède, 1800), locally known as *tinabal* at different salt-fish ratios were investigated. Fresh frigate tuna were gutted and cleaned before the addition of salt at different salt-fish ratios (w/w), (1:3), (1:4), (1:5), (1:6) and fermented at ambient temperature. The LAB viable count increased, and the pH levels decreased with increasing per cent titratable acidity. Isolated LABs were presumptively identified as *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Pediococcus pentosaceus*, *Leuconostoc* spp., *Lactobacillus fermentum*, and *Streptococcus* spp. Ethanolic extracts from F3 (1:6) showed favourable antioxidative activities against 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) radical and hydrogen peroxide (H₂O₂) radical. Sample F1 (1:4) showed a high reduction of ferric ions, while F2 (1:5) showed favourable activities against singlet oxygen (¹O₂) radical. In general, F2 (1:5) has favourable LAB composition with a wide range of LABs and high LAB counts. Also, F2 (1:5) has favourable scavenging activities coupled with an increasing degree of hydrolysis (DH) during fermentation. Results showed increasing activities in *tinabal* during the fermentation process, making it a potential source of antioxidants for industrial uses. Additionally, the LABs in F2 (1:5) indicates its usefulness as a viable source of LAB for applications in other fermented products as starters for improved product quality. These results make *tinabal* a possible functional food product that could benefit consumers.

Keywords: salting, antioxidant assay, LAB, degree of hydrolysis, *Tinabal*, natural fermentation

Introduction

Fermentation is a traditional food processing technique common in Asia, producing products that have a distinct and unique flavour, texture, and aroma (Raksakulthai and Haard, 1992). These characteristics are mainly caused by protein degradation by autolytic and bacterial enzymes occurring during fermentation (Rajapakse et al., 2005). Fermented products are sources of proteins, carbohydrates, vitamins, and minerals. These products have been reported to exhibit strong antioxidative activities which contribute to the extended shelf life of the product (Faithong and Benjakul, 2012). *Tinabal* fermentation is highly dependent on lactic acid bacteria (LAB) microflora. These microorganisms produce organic acids, bacteriocin, diacetyl, hydrogen peroxide, and free fatty acids that could inhibit the proliferation of spoilage and

pathogenic organisms in fermented foods (Leroy and De Vuyst, 2004).

Tinabal is commonly consumed as viand or condiment and traditionally prepared from frigate tuna *Auxis thazard* (Lacepède, 1800) added with salt at the ratio of 1 part salt to 3 parts fish (1:3), and contained in sealed plastic containers for 7 days of ripening or fermentation of the product. *Tinabal* is classified into two distinct types: *Tinabal manko Auxis thazard* (Lacepède, 1800) and *Tinabal molmol* (*Scarus* sp.), which are obtained from the coastal waters in Visayan Region, Philippines (Calanoga, 1995). The existing technology for fermented fish products in the region is a simple and cost-effective process which provides a livelihood to small scale processors along the coastal areas. More so, fermented fish products supplement the nutritional needs as a protein source of the locals.

Naturally occurring LAB in fermented fish products have been previously reported. Tamang (2014) reported the dominance of *Lactobacillus* and *Pediococcus* species in fermented foods. The same author further indicated that these species are acid-tolerant, fastidious, and strictly fermentative type of LAB. Calanoga (1995) reported the naturally occurring LAB in *tinabal* such as *Pediococcus pentosaceus*, *Streptococcus equinus*, *Leuconostoc* species and *Lactobacillus* species and the said species were found throughout the fermentation process. In addition, Banaay et al. (2013) indicated that the growth of spoilage and pathogenic bacteria in *tinabal* were inhibited at the initial stage of fermentation due to the antagonistic effect of lactic acid produced and high salt concentrations.

Biological activities of fermented products such as whole fish, fish paste, and fish sauce have been previously reported. Kleekayai et al. (2015) reported strong antioxidative activities of Thai fermented shrimp paste, *Kapi* against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate (ABTS⁺) radicals, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radicals as well as ferric reducing activity power (FRAP). In addition, a study conducted by Pongsetkul et al. (2015) in *Kapi* showed strong antioxidative activity against singlet oxygen radicals. Giri et al. (2012) reported bioactive properties of fermented Japanese horse mackerel (*Trachurus japonicus*, Temminck & Schlegel 1844) paste to have increasing antioxidative activities against different types of free radicals including hydroxyl radicals during prolonged fermentation. Hartmann and Meisel (2007) reported that fermented products such as *tinabal* are good sources of amino acids and peptides as products of fermentation. These investigations revealed antioxidative activities in fermented fish products related to fermented frigate tuna. Other biological activities such as hydrogen peroxide scavenging activity and singlet oxygen scavenging activity specific to *tinabal* have not been previously reported. Therefore, this work was designed to investigate the antioxidative activities and determine the LAB composition of *tinabal*. Specifically, this study aimed to determine the dominant LAB isolates during fermentation, assess the antioxidative activities against free radicals and reactive oxygen species (ROS), and determine the degree of hydrolysis of proteins. Measurement of pH, and per cent titratable acidity of the different salt-fish ratios during fermentation was also monitored.

Materials and Methods

Materials

Raw material frigate tuna *Auxis thazard* (Lacepède, 1800) for making *tinabal molmol* were obtained from the community fish landing centre (CFLC) of Maybato, San Jose, Antique, Philippines. The frigate tuna

normally spawns along its migratory route from August to January. The researchers further consulted experts for the identification of the raw material used. Prior to processing, the freshness test was conducted by following the quality index method (QIM) by assessing the general appearance, eyes, and gills of the fish samples. The marketable-sized fresh frigate tuna (28–32 cm FL) with different salt-fish ratios (w/w) (1:3, 1:4, 1:5, 1:6) were layered and packed in airtight plastic containers and were allowed to ferment for 7 days at ambient temperature. Fermentation of *tinabal* for 7 days is the common practice of the local *tinabal* processors in the Visayan Region, Philippines. Based on the preliminary study conducted by the researchers for *tinabal*, it was observed that histamine content of the fermented frigate tuna samples is considered safe for consumption at day 7 of fermentation. In addition, a similar study has shown that histamine content of the fermented frigate tuna is considered safe for consumption at day 7 of fermentation (Calanoga, 1995). After termination of the fermentation process, all samples were stored at -17 °C until further analysis was conducted in triplicate. Process flow of *tinabal* processing is reflected in Figure 1.

Culture media and chemicals

De Man, Rogosa and Sharpe (MRS) agar and broth were purchased from Hi-Media (Mumbai, India). Chemicals and reagents that were used in the antioxidative assays were 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), L-ascorbic acid, ferric chloride hexahydrate, horseradish peroxidase (HRPO), L-leucine, picrylsulfonic acid solution (TNBS), sodium dodecyl sulphate (SDS), 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (TROLOX), potassium persulfate, 2,4,6 - Tri(2-pyridyl)-s-triazine (TPTZ), Rose Bengal, L-histidine were analytical grade purchased from Sigma-Aldrich (Singapore). Absolute ethanol (99.99 %) and sodium hydroxide were purchased from Scharlau (Spain). Other chemicals and culture media used in the assays were of analytical grade or higher.

Analysis of pH and titratable acidity

Measurement of per cent titratable acidity (TA) was determined by titration to pH 8.2 with 0.1 M NaOH according to the method of Benjakul et al. (1997). It was calculated by multiplying the molarity of titrant used, volume of the titrant used, and molecular weight of lactate and dividing it by the weight of the sample to get the per cent TA. The results were expressed as per cent Lactic Acid. The pH levels of *tinabal* were determined following the method of AOAC (2001) using a digital pH meter (HM Digital, California, U.S.A.). Measurement of pH and per cent TA was monitored at every sampling point during fermentation.

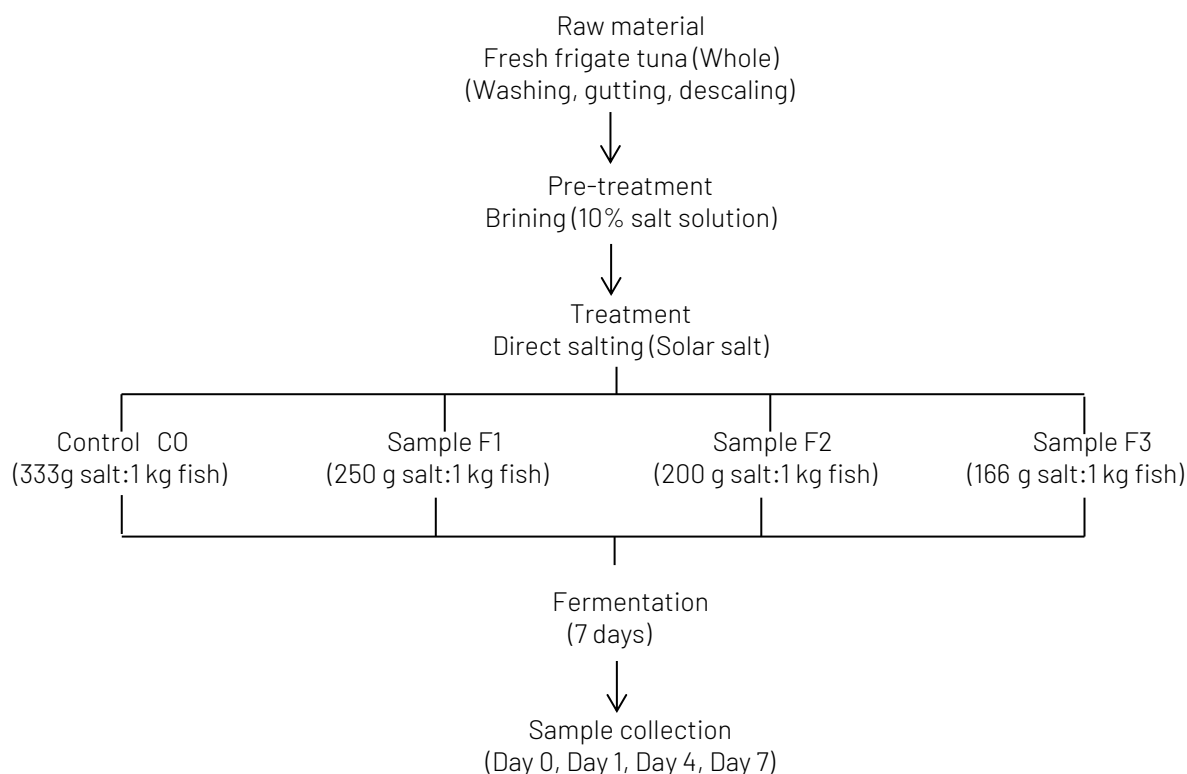


Fig. 1. Schematic diagram of *tinabal* preparation.

Enumeration, isolation and identification of LAB

One gram of each sample was aseptically mixed with 9.00 mL sterile natural saline solution (0.85 % w/v, NaCl). Lactic acid bacteria were isolated from *tinabal* by appropriate dilutions (ten-fold) in saline solution, pour plated on MRS agar, and were incubated at 37 °C for 2–3 days (AOAC, 2001; US-FDA, 2001). Enumeration of LAB was done in every sampling point. Colonies were picked according to their colony characteristics and were propagated twice to check the purity of the isolates, and stored in soft agar with 80 % glycerol (Tagg and McGiven, 1978) until needed. The morphological, physiological, and biochemical examination of isolates were determined by the standard procedure of Gram staining, spore staining, catalase test, gas production test and motility test (US-FDA, 2001; Downes and Ito, 2001). Lactic acid bacteria identification was carried out as per the schemes outlined in the Bergey's manual of systematic bacteriology (Sneath et al., 1986).

Measurement of the degree of hydrolysis

Per cent degree of hydrolysis (DH) was determined according to the sample extraction method by Benjakul et al. (1997) and the TNBS method by Adler-Nissen (1979). Extraction of peptides from the sample was carried out by weighing 1.00 g of *tinabal*, added with 9.00 mL of 70 mM SDS. The mixture was then

homogenised at 6,000 rpm for 15 min and centrifuged at 10,000 ×g for 15 min. The samples (0.250 mL) were then subjected to the TNBS method by adding 2.00 mL of 0.2125 M sodium phosphate buffer pH 8.2, and 2.00 mL of 0.10 % TNBS solution, and incubated at 50 °C in the dark. The reaction was terminated with 0.1 N HCl and read at 340 nm using BMG Labtech microplate reader (Baden-Wurttemberg, Germany). A standard solution was prepared using L-leucine with 0–10 mM concentrations. The per cent DH was calculated by dividing the hydrolysis equivalent (meq.g⁻¹) of the sample with the hydrolysis equivalent at complete hydrolysis (8.0 meq.g⁻¹) as estimated by Adler-Nissen (1979).

Antioxidant assays

Preparation of ethanolic extracts

Ethanolic extracts from *tinabal* were prepared as per the method of Peralta et al. (2005). Frigate tuna (1.0 g) was weighed using Shimadzu Electronic Balance ATX224 (Kyoto, Japan), added with 50.00 mL of 85 % ethanol. The mixture was homogenised (6000 rpm for 15 min) and centrifuged (8,500 ×g for 15 min) using Centurion C2006 (West Sussex, United Kingdom) centrifuge. The supernatant was collected and subjected to the rotary evaporator (Hei-Vap Precision, Germany) to eliminate the solvent and determine the weight of the crude extract, and was dissolved again in 85 % ethanol to attain 5 mg.mL⁻¹ concentration of the extracts.

ABTS⁺ radical scavenging activities of the ethanolic extracts were measured by the method of Re et al. (1999). ABTS⁺ solution was prepared by mixing equal amounts of 7 mM ABTS and 2.45 mM potassium persulfate and incubated for 16 h at room temperature. The radical solution was then diluted to attain an absorbance of 0.70 ± 0.02 at 734 nm by phosphate buffer saline pH 7.4. The samples were tested with the radical solution in a 96 well plate. The changes in the reaction mixture were monitored after 10 min of incubation in the dark at room temperature read at 734 nm using BMG Labtech microplate reader (Ortenberg, Germany). Ascorbic acid (1 mM) was used as a positive control. A standard curve using Trolox was prepared with 0–15 μM concentration. The percentage inhibition was calculated, and the antioxidative activities were expressed as μmol Trolox equivalent per mL of fish ($\mu\text{mol TE}\cdot\text{mL}^{-1}$).

Ferric reducing power assay

The reducing power of the ethanolic extracts was determined by the method of Benzie and Strain (1996). The assay was based on the reduction of TPTZ-ferric ion (Fe^{3+}) to TPTZ-ferric ion (Fe^{2+}) in the presence of antioxidants in the tested ethanolic extracts of *tinabal*. The working FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 10 mM ferric chloride hexahydrate in 10:1:1 ratio respectively. To monitor the TPTZ- Fe^{2+} generated, ethanolic extracts were mixed with FRAP reagent and allowed to react at 37°C for 5 min in a 96-well plate and read at 593 nm using BMG Labtech microplate reader (Ortenberg, Germany). Trolox (10 mM) was used as a positive control. The FRAP value was calculated according to the FRAP value of the standard used. Ascorbic acid with FRAP value of 2 (Benzie and Strain, 1996) was prepared as standard in 50–1000 μM concentrations.

Hydrogen peroxide scavenging assay

H_2O_2 scavenging activities of the ethanolic extracts were determined according to the method of Baharun et al. (1996). The ethanolic extracts were added with the reaction mixture containing 89 mM NaCl in 0.1 M phosphate buffer pH 7.0, 23 mM hydrogen peroxide, 0.05 mg horseradish peroxidase (HRPO) and 0.1 mg phenol red in 0.1 M phosphate buffer and kept at room temperature for 15 min. The reaction was then terminated after addition of 1.33 M NaOH and was read at 610 nm using BMG Labtech microplate reader (Ortenberg, Germany). Standard was prepared using Trolox with 0–10 mM concentrations. Ascorbic acid (10 mM) was used as a positive control. The radical scavenging activities were expressed as $\text{mmol TE}\cdot\text{mL}^{-1}$.

$^1\text{O}_2$ scavenging activities of the ethanolic extracts were determined by the method of Lugmann et al. (2016). The ethanolic extracts (106.5 μL) were added with the reaction mixture consisted of 25.0 μL of 12.5 mM histidine and 20.0 μL of 100 mM of RNO and kept in the dark for 10 min. It was then added with 20.0 μL of 100 mM H_2O_2 and immediate addition of 28.5 μL of 100 mM sodium hypochlorite. After 10 min of incubation in the dark, absorbance was read at 440 using BMG Labtech microplate reader (Ortenberg, Germany). The blank was prepared by replacing the sample with 0.1 M sodium phosphate buffer, and 1 mM Rose Bengal was used as positive control prepared in the same manner. A standard curve was prepared using Trolox with 0–10 mM concentration. The scavenging activities were expressed as $\text{mmol TE}\cdot\text{mL}^{-1}$.

Statistical analysis

All data were analysed for multivariate analysis of variance (MANOVA), and One-way analysis of variance (ANOVA) using SPSS software version 20.0. Significance of means was determined using Duncan's multiple range test (DMRT) at a 95 % significant difference.

Results

pH and titratable acidity

The pH levels and titratable acidity of *tinabal* were observed to determine per cent lactic acid generated in the product, as shown in Table 1 and 2. The final pH of control, F1, F2, and F3 were 5.4, 5.36, 5.5, and 5.49, respectively. F1 has the highest pH decrease (0.55) relative to initial pH, while the control had the least pH decrease (0.45). All samples including the control decreased in pH level as the fermentation progressed, with a significant difference in the pH levels between samples in every sampling period during fermentation ($P < 0.05$) as presented in Table 1. The interaction effect between the fermentation time and salt-fish ratio on pH levels was significant ($P < 0.05$). The pH levels in the samples significantly decreased during the fermentation process, while the pH level increased as the salt-fish ratio decreases ($P < 0.05$).

The final per cent TA of control, F1, F2, and F3 were 0.4664, 0.5401, 0.5217, and 0.4971 respectively. Sample F1 had the highest per cent TA increase (0.178), and control had the lowest TA increase (0.0491). Regardless of the salt-fish ratios, the main effect of fermentation time on the per cent TA of sample F1 (1:5) was significant ($P < 0.05$).

Table 1. pH levels of fermented frigate tuna *Auxis thazard* at 7 days of fermentation.

Salt-fish ratio	Fermentation time (Mean $\pm$ SD)			
	Day 0	Day 1	Day 4	Day 7
Control	5.86 $\pm$ 0.03 ^{Ca}	5.85 $\pm$ 0.05 ^{Ca}	5.51 $\pm$ 0.06 ^{Ba}	5.4 $\pm$ 0.04 ^{Aa}
F1	5.91 $\pm$ 0.03 ^{Cb}	5.87 $\pm$ 0.03 ^{Ca}	5.53 $\pm$ 0.01 ^{Ba}	5.36 $\pm$ 0.4 ^{Aa}
F2	5.97 $\pm$ 0.02 ^{Cc}	5.88 $\pm$ 0.01 ^{Ba}	5.52 $\pm$ 0.04 ^{Aa}	5.5 $\pm$ 0.02 ^{Ab}
F3	5.96 $\pm$ 0.02 ^{Bbc}	5.95 $\pm$ 0.03 ^{Bb}	5.51 $\pm$ 0.03 ^{Aa}	5.49 $\pm$ 0.01 ^{Bb}

*UPPERCASE superscripts in the same row denote significant differences in samples during fermentation (Duncan multiple range test, $P < 0.05$).

*lowercase superscripts in the same column denote significant differences in between samples in sampling period (Duncan multiple range test, $P < 0.05$).

Table 2. Per cent titratable acidity of fermented frigate tuna *Auxis thazard* at 7 days of fermentation.

Salt-fish ratio	Fermentation time (% Mean $\pm$ SD)			
	Day 0	Day 1	Day 4	Day 7
Control	0.42 $\pm$ 0.04 ^{Aa}	0.42 $\pm$ 0.12 ^{Aa}	0.44 $\pm$ 0.04 ^{Aa}	0.47 $\pm$ 0.06 ^{Aa}
F1	0.36 $\pm$ 0.1 ^{Aa}	0.37 $\pm$ 0.08 ^{Aa}	0.44 $\pm$ 0.09 ^{Ab}	0.54 $\pm$ 0.05 ^{Ba}
F2	0.41 $\pm$ 0.09 ^{Aa}	0.45 $\pm$ 0.02 ^{Aa}	0.46 $\pm$ 0.08 ^{Aa}	0.52 $\pm$ 0.07 ^{Aa}
F3	0.37 $\pm$ 0.12 ^{Aa}	0.39 $\pm$ 0.02 ^{Aa}	0.47 $\pm$ 0.11 ^{Aa}	0.50 $\pm$ 0.1 ^{Aa}

*UPPERCASE superscripts in the same row denote significant differences in samples during fermentation (Duncan multiple range test, $P < 0.05$).

*lowercase superscripts in the same column denote significant differences in between samples in sampling period (Duncan multiple range test, $P < 0.05$).

Microbial composition of fermented frigate tuna

In this study, results reveal that at day 4 of fermentation, sample F2 had the highest LAB count (6.87 log CFU.g⁻¹) and control sample had the lowest count (6.09 log CFU.g⁻¹). Most of the colonies present in all samples at day 7 of fermentation showed very high LAB viable counts (too numerous to count TNTC, > 2,500,000 EAPC). Samples from F1, F2 and F3 showed low LAB counts (< 2,500 EAPC) in the initial days of fermentation. The initial screening of randomly selected LABs resulted in 127 colonies that were subjected to the catalase test, motility Gram staining. After this screening, 32 isolates (25 %) were found to be catalase negative, Gram positive, and these isolates were categorised as those belonging to LAB group. The 32 isolates were biochemically identified as *L. casei*, *L. delbrueckii* subsp. *delbrueckii*, *P. pentosaceus*, *Leuconostoc* spp., *L. fermentum*, and *Streptococcus* spp. following the schemes in Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986). Presence of presumptively identified LAB is presented in Table 3.

Degree of hydrolysis

Per cent DH is the measure of the cleavage of peptides. Results showed increasing per cent DH as

fermentation progressed, with sample F3 the highest per cent DH (68.64 %) at day 7 of fermentation (Fig. 2). In addition, the results reveal that the increasing fermentation time affected the DH of *tinabal* ($P < 0.05$). Furthermore, the decreasing salt-fish ratio also affected the DH of the fermented product ($P < 0.05$).

Antioxidative activities of fermented frigate tuna

The antioxidative activities of the ethanolic extracts of *tinabal* at different salt-fish ratios are shown in Figure 2. ABTS⁺ can be solubilised in hydrophilic and hydrophobic system, which these compounds could be successfully assessed (Re et al., 1999). Results showed that the activities in all samples of *tinabal* were similar and increased as fermentation progress (Fig. 3). F2 had the highest increase from 3.48 to 3.53 $\mu\text{mol TE.mL}^{-1}$ sample, while the control sample had the lowest increase from 3.50 to 3.53 $\mu\text{mol TE.mL}^{-1}$ sample. Results revealed that sample F3 at day 7 had the highest concentration (3.56 $\mu\text{mol TE.mL}^{-1}$), and sample F2 at the initial day had the lowest concentration (3.48 $\mu\text{mol TE.mL}^{-1}$). The results in Figure 2 showed that scavenging activities of all the ethanolic extracts increased as the fermentation progressed ($P < 0.05$). Regardless of the salt-fish ratios, the main effect of fermentation time on ABTS⁺

Table 3. Presence of different LAB species isolated from different salt concentrations of fermented frigate tuna *Auxis thazard* at 7 days of fermentation.

Presumptively identified LAB	Control				F1				F2				F3			
	Day 0	Day 1	Day 4	Day 7	Day 0	Day 1	Day 4	Day 7	Day 0	Day 1	Day 4	Day 7	Day 0	Day 1	Day 4	Day 7
<i>L. casei</i>	+	+	+							+		+				+
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i>									+							
<i>P. pentosaceus</i>						+	+									
<i>Leuconostoc</i> spp.										+						
<i>L. fermenti</i>				+										+		
<i>Streptococcus</i> spp.	+	+	+	+	+			+	+		+	+	+	+	+	+

+, indicate presence of LAB during fermentation

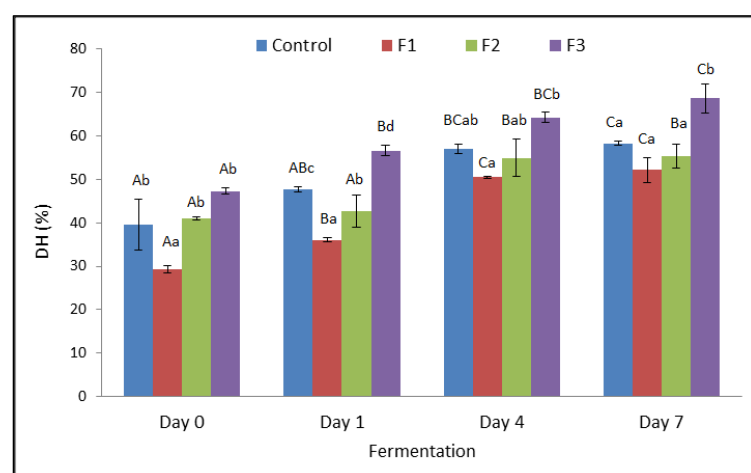


Fig. 2. Degree of hydrolysis of proteins from extracts of the different salt concentrations of fermented frigate tuna *Auxis thazard* at 7 days of fermentation. *UPPERCASE superscripts denote significant differences during fermentation of every sample (Duncan multiple range test, $P < 0.05$), *lowercase superscripts denote significant differences of samples at different sampling period (Duncan multiple range test, $P < 0.05$).

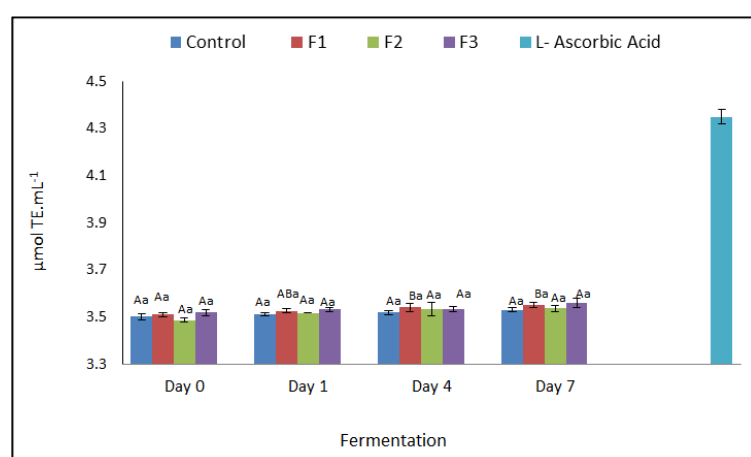


Fig. 3. Trolox equivalent antioxidant capacity on ABTS+ of the ethanolic extracts from the different salt-fish ratios of fermented frigate tuna *Auxis thazard* during fermentation. *UPPERCASE superscripts denote significant differences during fermentation of every sample (Duncan multiple range test, $P < 0.05$), *lowercase superscripts denote significant differences of samples at different sampling period (Duncan multiple range test, $P < 0.05$).

radical scavenging activities of F1 with 1:4 salt-fish ratio is increasing significantly ($P < 0.05$).

Reduction of ferric ions in fermented frigate tuna

In terms of reducing the power of the ethanolic extracts, the activities of all *tinabal* samples increased as fermentation progressed (Fig. 4). Results revealed that the control sample at day 0 had

the lowest FRAP value (1.78), and extracts of sample F1 had the highest FRAP value (3.6) at day 7 of fermentation. Results further revealed that sample F2 had the lowest increase (2.46 to 3.08), and control had the highest increase (1.78 to 3.58) in terms of FRAP values during 7 days of fermentation. Regardless of the salt-fish ratios, the main effect of the fermentation time on the ferric reducing power of control was significant ($P < 0.05$).

Scavenging of reactive oxygen species (ROS) such as hydrogen peroxide and singlet oxygen was observed in *tinabal* ethanolic extracts. In terms of scavenging H_2O_2 capacity of the ethanolic extracts, activities were observed in all samples, having sample F3 with the highest activity (Fig. 5). Values that vary from 5.6–7.55 mmol TE.mL⁻¹, with F1 sample at day 7 having the lowest (5.6 mmol TE.mL⁻¹) and sample F3 at the 7th day of fermentation having the highest ROS scavenging activity (6.66 mmol TE.mL⁻¹).

High singlet oxygen scavenging activities were observed in all samples, having F2 with the highest

activity (Fig. 6). The 1O_2 scavenging activity increases as fermentation progresses ($P < 0.05$) for all samples, and the values were within the range of 4.19–7.79 mmol TE.mL⁻¹ sample. Sample F2 showed the lowest concentration (4.19 mmol TE.mL⁻¹) at the start of the fermentation process but had the highest concentration (7.79 mmol TE.mL⁻¹) on the 7th day of the fermentation process. It was also observed that the interaction effect between fermentation time and salt-fish ratios on singlet oxygen scavenging activities of *tinabal* was significant ($P < 0.05$).

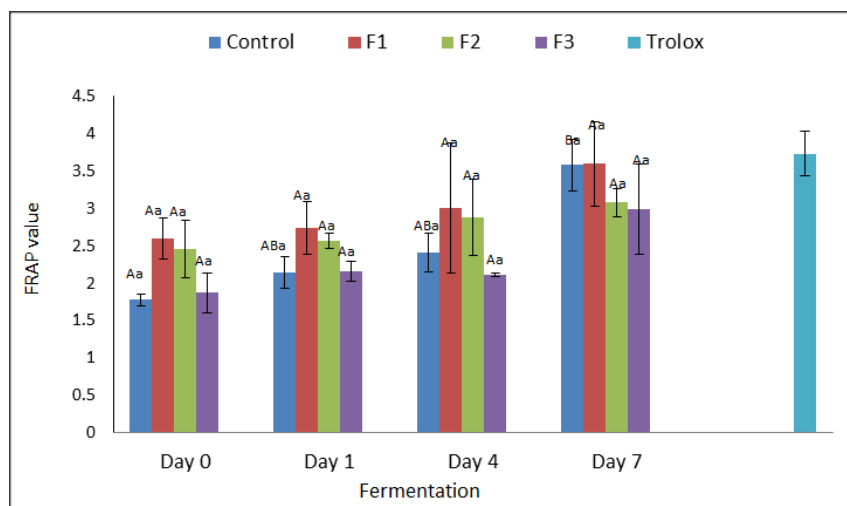


Fig. 4. FRAP values of ethanolic extracts from the different salt-fish ratios of fermented frigate tuna *Auxis thazard* during fermentation. *UPPERCASE superscripts denote significant differences during fermentation of every sample (Duncan multiple range test, $P < 0.05$), *lowercase superscripts denote significant differences of samples at different sampling period (Duncan multiple range test, $P < 0.05$).

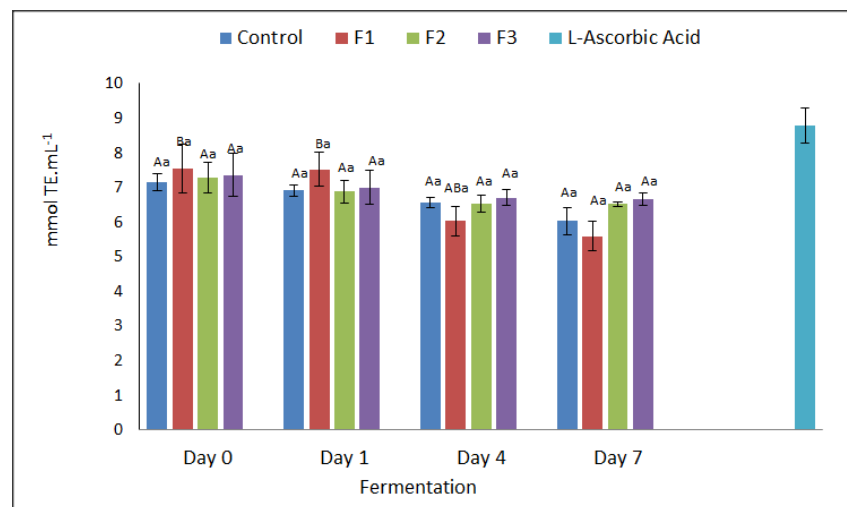


Fig. 5. Trolox equivalent antioxidant capacity on H_2O_2 of ethanolic extracts from the different salt-fish ratios of fermented frigate tuna *Auxis thazard* during fermentation. *UPPERCASE superscripts denote significant differences during fermentation of every sample (Duncan multiple range test, $P < 0.05$), *lowercase superscripts denote significant differences of samples at different sampling period (Duncan multiple range test, $P < 0.05$).

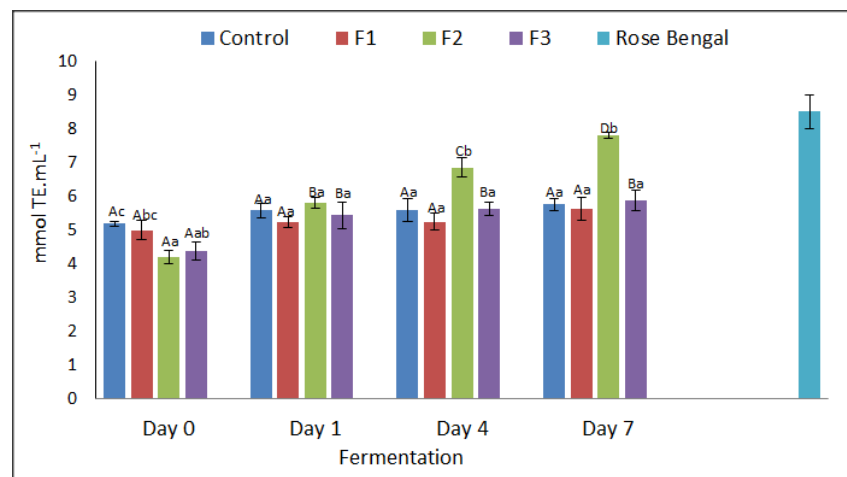


Fig. 6. Trolox equivalent antioxidant capacity on 1O_2 of ethanolic extracts from different salt-fish ratios of fermented frigate tuna *Auxis thazard* during fermentation. *UPPERCASE superscripts denote significant differences during fermentation of every sample (Duncan multiple range test, $P < 0.05$), *lowercase superscripts denote significant differences of samples at different sampling period (Duncan multiple range test, $P < 0.05$).

Discussion

The samples in the present study were observed to drop in pH at day 4 of fermentation that is within the optimum pH level. The drop in pH is in accordance with the study of Yang et al. (2018) that the optimum pH level for LAB growth is 5.5 to 5.8. The decrease in pH (6.5 to 4.3) of naturally fermented products is usual as indicated by Achinewhu and Oboh (2002). The decrease in pH with fermentation period in *tinabal* agrees with the results reported by Nakano et al. (2017) on the chemical properties of common fermented fish sauces in Asia. The same author further indicated the decrease in pH depends on the salt concentration and fermentation process of commercial fermented fish and fish sauce products. The decreasing pH of *tinabal* is in accordance with the study of Feti et al. (2017) indicated that pH of Indonesian fermented fish sauce, *bakasang* decreases with increasing salinity. The same study further suggested that fermented fish produced using 20 % - 30 % salt had optimum microbial and chemical properties. In the present study, the interaction of the fermentation and salt-fish ratios on pH was significant ($P < 0.05$). The increase in fermentation time affected the pH levels of *tinabal*. Increasing the fermentation time results in a decrease in pH due to the accumulation of H^+ ions as by-product of proteolytic activities and lactic acid from cellular respirations (Aluko, 2018). Whereas, the decrease in salt-fish ratio affected the increase in pH of the samples in every sampling period of the present study. The increase in pH when salt is reduced could be due to dissociation of amino acids and small peptides in the presence of salt through osmosis (Sanchez, 2008). Salt favoured the growth of LABs, and organic acids (pyruvic acids and lactic acids) as its by-product lowered the pH levels as similarly shown in the study of Tabatabaei-Yazdi et al. (2013); indicating different salt concentrations added to a product may affect the growth, activity, and role of microorganisms involved in fermentation.

The increase in per cent titratable acidity was due to the increase in the production of organic acids as a by-product of enzymes and microorganisms respiration (Tomovska et al., 2016). Correspondence of the decrease in pH and increase in per cent TA could be due to the organic acids produced during fermentation as also indicated by Bertoldi et al. (2004) that TTA increased with decrease in pH of red meat tuna fermentation. Similar studies on the reduction of salt on fermented foods showed minimal changes in the physicochemical properties such as pH and titratable acidity of the product during fermentation. Bautista-Gallego et al. (2013) revealed that reduction of NaCl in fermented meat drops the pH level and increases titratable acidity with improved firmness of the product. Tassuo et al. (2007) reported that reformulation with reduced salt content shows a reduction of pH while increasing the lactic acid and

other organic acid production of fermented products. The decrease in pH is due to the by-products of proteolytic activities such as H^+ ions and lactic acid that accumulated with time of fermentation (Aluko, 2018). It is interesting to note that although lower salt was added, pH level of the samples was within the usual pH range.

High LAB counts observed at initial days of fermentation in the control could be due to LABs that are high salt tolerant. High composition of LAB-like species may have proliferated at the initial stage of fermentation. Stamer et al. (1971) indicated that growth and fermentation patterns of LAB depend on temperature, salinity, and quality of the raw material. Possibly, high microflora in raw material and salt could be attributed to the high LAB counts in control. This observation could also be due to the possible microorganisms present in solar salt used in fermentation (Gancel et al., 1997). The high LAB counts observed on the sample in the initial days of fermentation could be due to the wide range of bacterial microflora including LAB that are present in the gills and skin surface of the raw material (Austin, 2002). Interestingly, Tamang et al. (2016) indicated that concentrations in at least 10^6 log CFU per gram of probiotic bacteria such as *Lactobacillus* spp. could provide health benefits to consumers since this amount of viable organisms are sufficient in providing probiotic effects. In addition, this viable count requirement is recommended by regulatory authorities before health claims are made in these products.

Presence of the different LAB genus *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, and *Pediococcus* were identified in the present study. These findings agree with the findings of Ringo et al. (2018) where all the LAB are found to be indigenous species in the gastrointestinal tract and surface of finfishes. Presumptively identified species are as follows; *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus fermentum*, *Pediococcus pentosaceus*, *Leuconostoc* spp., and *Streptococcus* spp. All biochemical tests conducted for the dominant LAB confirmed the description for the bacterial genus in Bergey's manual (Sneath et al., 1986). Presence of these LABs (Table 3) in *tinabal* agrees with Banaay et al. (2013) who revealed that some of these mentioned LABs are naturally occurring in traditional fermented frigate tuna which include *P. pentosaceus*, *S. equinus*, *Leuconostoc* spp. and *Lactobacillus* spp. Also, a study by Desnair et al. (2013) indicated the dominance of Gram positive cocci in fermented milk fish, *bekasam*, which are all non-motile and non-spore forming. Accordingly, the presence of LAB during fermentation contributes to the breakdown of proteins and development of flavour and aroma (Salampessy et al., 2010). In the present study, *Streptococcus* species were dominating the microbial composition of *tinabal*. Interestingly, this genus is considered as initiators of

fermentation, and it persists during fermentation of products (Marco et al., 2017). Some *Streptococcus* spp. are found to be beneficial in fermented products such as *S. equinus*, and *S. thermophilus* and are commonly used in fermented milk and cheese as starters (Ashraf and Shah, 2011). The study conducted by Cooper-Bribiesca et al. (2018) on *S. infantarius* as the starter of *Nejayote* fermentation also revealed significant results on *S. infantarius* as a potential fermentation starter. *Lactobacillus* species that are present from the initial throughout the end of fermentation is due to the characteristics of this genus, being acid and salt-tolerant (Steinkraus, 1983). *Pediococcus* spp. comprise only a small amount of microflora in the sample and is similar in another finding by Olympia et al. (1992) on LAB composition of fermented milkfish *Chanos chanos* (Forsskål, 1775) "burong bangus". Other LAB species such as *L. delbrueckii* and *L. fermenti* were also observed in the control, F2, and F3 samples but were few in number. These observations can be supported by Palludan-Muller et al. (1999), who reported that these LAB species are observed in low salt fermented fish product from Thailand such as *som-fak*, *plaa-som*, *plaa-ra*, and *plaa-chom*. In this study, sample F2 had the most diverse LAB composition with high LAB counts based on the results mentioned above (Table 3). These LABs present in sample F2 indicate high quality and composition of LABs that are known to exist in a wide range of fermented products. Interestingly, these results show that a wide variety of LABs is present in the lower salt-fish ratio (F2, 1 salt:5 fish).

The high DH in F3 with a low salt-fish ratio of *tinabal* could be due to the low salt concentration that generated high peptides and amino acids from endogenous and exogenous enzymes present during fermentation. Possibly, hydrolysis of protein in the higher salt-fish ratio is not as efficient as the hydrolysis of proteins at 1:6 salt - fish ratio. Ormanci and Colakoglu (2015) further indicated that high salt concentration contributes to protein denaturation, which decreases moisture through cell osmosis and makes the product firm. The high salt concentrations lead to loss of nutrients during salting in which the amino acids transfer from the tissue to salt (Ferraro et al., 2006). Chamcheun (2015) reported that, in most fermented products, the degree of hydrolysis increases as the fermentation time increases. A study conducted by Ardiansyah et al. (2015) also indicated high per cent DH in low salt fermented fish sauce (60 to 70 %), with the products showing high levels of essential amino acids. The degree of hydrolysis in fish protein is slower due to the decrease in proteolytic enzyme activity during the storage period (Sikorski et al., 1995). The decrease in pH, as observed in the present study (see decisions above) could have caused the inactivation of the proteolytic enzymes that are responsible for the degradation of proteins (Aluko, 2018). Gildberg (2001) stated that the degree of hydrolysis is initiated by the proteolytic enzymes during the fermentation process, which is agreed by

(Majumdar et al., 2015) who stated that proteolytic enzymes are available in the raw material prior to fermentation. These endogenous enzymes are believed to play the critical roles in protein digestion which these enzymes were primarily acting on muscle degradation during the fermentation process (Orejana and Liston, 1982). Since the fermentation process in this study was stopped after 7 days of storage, it could, therefore, be possible that the DH of the samples after this period could be higher. In addition, Wang et al. (2017) noted that denatured proteins are easily hydrolysed in the prolonged fermentation process. They also noted that the gradual change in pH during fermentation affect the peptide composition of traditional fermented products. Based on the results, sample F3 have favourable DH throughout fermentation, showing that lower salt-fish ratio (F3) favour a high degree of hydrolysis in *tinabal*. However, the DH for the sample F1 (1:4) and F2 (1:5) had lower DH as compared to control. This could be due to the degradation of the peptides and amino acids that are affected by high salt concentrations through dimerisation reactions of protein and salt (Yamaguchi et al., 2009).

The scavenging activities of ethanolic extracts observed were lower than the activities observed from the positive control (L-ascorbic acid) with known wide range antioxidative activities. This indicates that the ethanolic extracts were effective in scavenging ABTS⁺ radicals, but not as efficient as the positive control. ABTS⁺ scavenging activities of F3 had the highest activity on day 7 of fermentation. These results could be due to the compounds with higher H⁺ donating ability to ABTS⁺, generated from the degradation of proteins to peptides (Kleekayai et al., 2015). In addition, lower salt content in F3 could have salting-in effects in proteins, in which these low-salt concentrations interact with metal-binding proteins that lead to its higher solubility and stability (Arakawa and Timasheff, 1991). Furthermore, the activities observed are due to the reaction of salt on proteins on the discolouration of meat as this agrees with the study of Yamaguchi et al. (2009) who revealed that the mechanism of salt in protein browning affected the complex reaction rates of peptides. In the present study, the minimal increase in the antioxidant activity of the fermented frigate tuna samples during fermentation could be due to the shorter period of fermentation than most of the fish product fermentation processes. This could mean that ABTS⁺ scavenging activities could have higher values if fermentation time is prolonged. However, histamine levels could increase with prolonged fermentation time and would greatly affect the quality of the product as observed in the initial study conducted for *tinabal*.

The high reducing power of sample F1 was higher than other salt-fish ratios, and this suggests the higher capability of the ethanolic extract of providing electron. This could be due to the higher salt

concentration that breaks water structures that generates more hydrogen, which reduces ferric ions that prevents oxidation from occurring (Nose et al., 2004). The observed ferric reducing activities of *tinabal* agree with Liu et al. (2010) that indicated high availability of H^+ ions are generated from proteolytic activities occurring during fermentation. In addition, these proteases are generally present in the raw material such as frigate tuna (Wiriaphan et al., 2012). These findings agree with the results of Je et al. (2009), indicating that compounds with higher reducing power have the capabilities of donating electron or hydrogen and therefore can serve as an indicator for its potential as an antioxidant. The peptides and amino acids with lower pH are effective at iron reduction, which is similar to the reduction potential of ascorbic acid used as a standard in the present study (Guo et al., 2019). In addition, reducing power exhibited by the ethanolic extracts could be due to the high availability of hydrogen ions from the enzymatic reaction of proteases that may have released some peptides (e.g. lysine, arginine, and glutamic acid) (Liu et al., 2010), and could also be influenced by the protease present in the raw material i.e. frigate tuna (Wiriaphan et al., 2012). The high FRAP values for F1 (1:4) could be due to the action of salt that breaks water structures and generates more hydrogen, which plays a role in reducing ferric ions (from Fe^{3+} to Fe^{2+}) that prevents oxidation from occurring (Nose et al., 2004). On the other hand, extracts with lower salt fish-ratio have lower FRAP values at the end of fermentation. Results show that lower salt-fish ratio ethanolic extracts had weak antioxidative activities since lower salt is available for generating hydrogen ions responsible for the reduction of ferric ions present in the sample. Ethanolic extracts from sample F1 had the highest and favourable FRAP value with high reducing capacities. This indicates that the F1 with 1 salt:4 fish ratio ethanolic extracts generate higher H^+ ions responsible for reducing ferric ions in the sample.

The H_2O_2 scavenging capacities of ethanolic extracts at different salt-fish ratios indicate potential to scavenge hydrogen peroxide radicals (Maleki et al., 2015). The decreasing results observed could be due to the behaviour of hydrogen peroxide as a reactive non radical that can permeate biological membranes that could be converted to more reactive species such as hydroxyl and singlet oxygen radicals (Choe and Min, 2005). Hydrogen peroxide is a precursor of hydroxyl radicals, thus, removal of H_2O_2 is necessary for antioxidant defence in food systems (Pongsetkul et al., 2015). The results in Figure 5, indicates that the fermented frigate tuna ethanolic extracts have minimal capacity to scavenge hydrogen peroxide. In addition, the decrease in the H_2O_2 radical scavenging activity observed during fermentation could be due to the behaviour of hydrogen peroxide as a reactive non radical, that can permeate biological membranes, and converted to more reactive species such as hydroxyl radicals and singlet oxygen (Choe and Min, 2005). The

results show that the H_2O_2 scavenging activities could be positively correlated with the increasing degree of hydrolysis. This observation is due to the enzymatic hydrolysed peptides and amino acids that are present in the product (Fig. 2).

Singlet oxygen is a reactive molecule that reacts with electron-rich double bonds and unsaturated fatty acids; thus removal of this type of ROS is needed (Choe and Min, 2005). The observed activities in *tinabal* could be due to the generated peptides and amino acids that have bioactive properties generated from fermentation, as shown in Figure 2. The results from F2 (1:5) singlet oxygen scavenging activities show the potential of the ethanolic extracts to scavenge this ROS, which is a by-product of cellular respiration, that initiates lipid oxidation. The scavenging activities are lower than the activities observed from the positive control (Rose Bengal) with known wide range antioxidative activities against scavenging ROS. This indicates that the ethanolic extracts are effective in scavenging singlet oxygen, but not as efficient as the positive control. The high ROS scavenging activities in F2 with lower salt-fish ratio could be due to the action of peptides and amino acids and not directly influenced by salt. This agrees with Cui et al. (2016) who revealed that high salt content could promote lipid oxidation, but this could be controlled by metal chelators that could be present in the sample. The same author further indicated that salt in higher doses act as pro-oxidants that promotes lipid oxidation in fermented foods. Thus, the reduction of high salt to low salt concentrations in food is necessary. The ROS scavenging activities exhibited by the ethanolic extracts from *tinabal* suggests its potential to scavenge hydrogen peroxide (Maleki et al., 2015). In this study, sample F2 with 1 salt:5 fish ratio has favourable scavenging activities against single oxygen radicals. This type of ROS is responsible for the degradation of bioactive molecules and causes cell damage that leads to spoilage of this product. This indicates that *tinabal* with 1:5 salt-fish ratio ethanolic extracts have potential in scavenging these ROS.

In general, the different antioxidative capacities of the ethanolic extracts in the present study were due to different peptide and amino acid composition of the hydrolysates produced as products of the fermentation process as shown in Figure 2. These observations agree with the findings of Lertittikul et al. (2007) which showed that the different compositions of the bioactive compound greatly impact the antioxidative activity of the low molecular weight peptides and amino acids present in the fermented fish sample. The high DH observed in sample F3 correlated well with the high ABTS⁺ and H_2O_2 scavenging activities. This indicates that the generation of peptides could be attributed to the high scavenging activities observed. The exact mechanisms of antioxidant activity by peptides generated from fermentation is not fully understood

(Guo et al., 2019), yet studies prove that these peptides act can serve as lipid peroxidation inhibitors, scavengers of free radicals, and chelators of transition metal ions (Sarmadi and Ismail, 2010).

In general, ethanolic extracts from sample F2 (1:5 salt-fish ratio) exhibited favourable antioxidant activities in terms of scavenging singlet oxygen radicals that are responsible for the cell damage and oxidative stress in fermented products. The increasing DH observed in sample F2 indicates the presence of cleaved peptides responsible for the radical scavenging activities. In this study, salt-fish ratio (1:5) contains potential natural antioxidants as peptides that are responsible for the antioxidative activities observed.

Conclusion

In conclusion, *tinabal* fermented with 1:5 salt-fish ratio (F2) showed high LAB counts with wide range of LAB species presumptively identified as *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus fermentum*, *Leuconostoc* spp., *Pediococcus* spp., and *Streptococcus* spp. *Tinabal* fermented with 1:5 salt-fish ratio (F2) ethanolic extracts showed strong antioxidant activities against singlet oxygen that is the major cause of oxidative stress and damage to bioactive compounds in fish and fish products. In addition, the increasing degree of hydrolysis in F2 indicated the generation of peptides and amino acids responsible for the antioxidative activities observed. It is suggested that prolonging fermentation time could improve the antioxidative activities in terms of scavenging ABTS⁺, ferric ions, hydrogen peroxide, and singlet oxygen, and also increase the per cent DH of the product. Analyses on amino acid and molecular weight of peptides should be further investigated. However, consumer acceptability on the lower salt concentration should be addressed. This fermented fish product has the potential to be produced in large scale since inland communities could have access to these products with appropriate product packaging for safety. Therefore, *tinabal* fermented at 1:5 salt-fish ratio (F2) could serve as a source of antioxidants and LABs for industrial uses. It will not only provide nutritional benefits but also would promote consumer health as a functional food.

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