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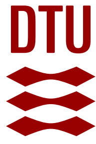

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Ka-pi-plaa **fermented using beardless barb fish**

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Published in:
International Journal of Food Science and Technology

Link to article, DOI:
10.1111/ijfs.15484

Publication date:
2022

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Sripokar, P., Hansen, E. B., Zhang, Y., Maneerat, S., & Klomklao, S. (2022). Ka-pi-plaa fermented using beardless barb fish: Physicochemical, microbiological and antioxidant properties as influenced by production processes. *International Journal of Food Science and Technology*, *57*(2), 1161-1172. https://doi.org/10.1111/ijfs.15484

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Article type : Original Article

Ka-pi-plaa fermented using beardless barb fish: Physicochemical, microbiological and antioxidant properties as influenced by production processes

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/IJFS.15484

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Abstract

Production of Thai fish paste *Ka-pi-plaa* fermented using beardless barb, *Cyclocheilichthys apogon* was monitored. Physicochemical, microbiological and antioxidant properties were compared after each process, i.e., autolysis, salting, sun-drying and fermentation. Color parameters L^* decreased while a^* and b^* increased during production ($P<0.05$). The *Ka-pi-plaa* finished product presented an intense brown color as shown with the increase in browning intensity (A_{420}). Contents of formal nitrogen, ammonia nitrogen and amino nitrogen showed continuous increase ($P<0.05$) indicating the formation of peptides and free amino acids, which were verified by protein patterns. Populations of total, halophilic, proteolytic, lipolytic and lactic acid bacteria generally increased. Halophilic bacteria grew rapidly after salting. Lactic acid bacteria counts were correlated with the pH change. It suggested that a few biochemical reactions occurred during production, including protein hydrolysis by microbial and fish proteases, lipid oxidation as presented by the increasing thiobarbituric acid reactive substances value, and Maillard reaction based on the determined precursors and products. Antioxidant activities generally increased during production particularly fermentation, suggesting *Ka-pi-plaa* possessed 2,2-diphenyl-l-picrylhydrazyl and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) scavenging and metal chelating activities, and ferric reducing power. This study provides important information on the relationship among production steps, various properties and chemical reactions for fish fermentation.

Keywords: *Ka-pi-plaa*, Fermentation, beardless barb, Protein degradation, Antioxidant

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Introduction

Fermentation is a key technology to preserve perishable food products and it has been used for centuries (Ciou *et al*., 2020). Consumption of fermented foods has beneficial impact on human health. One of the reasons lies in the action of some active microorganisms in fermented food that exhibit probiotic effect; Moreover, the bacterial metabolism also contributes to the synthesis of bioactive compounds during fermentation (Wilburn and Ryan, 2017; Kumaunang et al., 2019). Fermented fish, a traditional food product, is produced from the hydrolysis of fish by fish and microbial enzymes with the presence of highly concentrated salt (NaCl). In some Asian countries especially Thailand, traditional fermented fish paste products such as *Ka-pi-plaa* has been consumed as food condiment with wide use in cuisine and food processing because of its excellent taste, odor and flavor. To be specific, the color of commercial *Ka-pi-plaa* varies from pleasant gray to brownish, and it has a strong and consistent umami paste-like flavor. This product is generally made from small fish from both freshwater and seawater, such as anchovies, sardine (*Sardinella gibbosa*), bronze featherback (*Notopterus notopterus*) or beardless barb (*Anematichthys apogon*).

The process of production of *Ka-pi-plaa* includes a step of mixing fish after autolyis for 24 h with salt (fish : salt = 5-7 : 1), a step of sun drying, a step of thorough blending to produce semi-solid fish paste, and a final step of compacting in container for fermentation, where the paste is kept at room temperatures (28-30°C) for ≥10 days to generate a desirable aroma and flavor. Proteolysis is a key biochemical reaction in the process, which is catalyzed by the endogenous proteases in both fish and bacteria (halophilic and/or anaerobic bacteria) that survive during the process conditions especially the high salt content (Peralta *et al*., 2008; Dallagnol *et al*., 2021). Lipolysis is a process in which free fatty acids are first released and then oxidized to develop aromatic compounds (Kanjan and Sakpetch, 2020). The formed products from proteolysis and lipolysis are compounds with a low molecular weight such as aldehydes, amines, amino acids, organic acids and peptides (Sripokar *et al*., 2015; Klomklao *et al*., 2018). These produced compounds not only affect the texture, odor and flavor of fish paste (Pongsetkul *et al*., 2017a), but also its bioactivity because the hydrolysis helps to unfold and expose amino acid residues in fish proteins to allow them to react with oxidants (Faithong and Benjkaul, 2014; Prihanto *et al*., 2021). For example, the peptides and amino acids produced from the enzymatic hydrolysis during fish fermentation have been found to improve the antioxidant properties of fish products (Yang et al., 2020). Thus, the production/fermentation process of fish paste *Ka-pi-plaa* is accompanied by the

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changes in physicochemical, microbiological and antioxidant properties, which is of vital importance to investigate.

Beardless barb (*Anematichthys apogon*) is a type of small freshwater fish found in Thale Noi, the largest waterfowl reserve in Thailand. Beardless barb is one of the traditional fish species for *Ka-pi-plaa* production, however, no information has been reported regarding the changes and relationships of physicochemical, microbiological and antioxidant properties during the production process of *Ka-pi-plaa* used beardless barb. Thus, this paper aimed to monitor the physicochemical characteristics, microbial populations and antioxidant activities during *Ka-pi-plaa* production period including autolysis, salting, sun-drying and fermentation steps.

Materials and methods

Fish collection

Fresh beardless barb fish (average body weight of 250-300 g) were purchased from Thale Noi, Phatthalung province, Thailand. The fish were placed in ice (fish/ice ratio of 1:2, w/w) and transported to the lab in Department of Food Science and Technology, Thaksin University.

Preparation of fish paste *Ka-pi-plaa*

When arrival, the fish were washed, cut and degutted (sample from this step was named as "R"). They were stored at room temperature for 24 h for autolysis (sample from this step was named as "A"). After autolysis, the texture become soft and watery. Fishy, ammonia-like and rancid smells are found in autolysis fish. Then, the autolysis fish were mixed with salt at a ratio of 7:1 (w/w) and smashed and pounded thoroughly (sample from this step was named as "S"). The mashed and salted fish were spread out on fiberglass mats to dry under sunlight, and at night the fish sample was gathered and re-stored in earthen jar overnight and laid out again under the sun during daytime. This sun-drying step took 2-3 days until the sample disintegrate with a moisture content of 40-50%) (sample from this step was named as "D"). Afterwards, the sun-dried fish sample were transferred into sealed earthen jars for fermentation of 15 days at room temperatures (28-30ºC). During the fermentation, fish sample were taken at day 2 (sample was named as "F2"), day 5 (sample was named as "F5"), day 10 (sample was named as "F10") and day 15 (sample was named as "F15") for measurements. Thus, during the production, there were 8 different fish samples collected, i.e., raw materials (S), after autolysis (A), after salting (S), after sun-drying (D), and at different fermentation days (F2, F4, F10, F15). All these samples were analyzed and

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compared.

Determination of physicochemical properties during production

Moisture content assay. Moisture content was determined using AOAC method (2016).

Water activity (A_w) assay. A_w was analyzed on a water activity analyzer (Thermoconstanter, Novasina, Switzerland).

pH value assay. The pH value was measured on a pH meter (Sartorius, Gottingen, Germany) using the method developed by Thongruck et al. (2017).

Titratable acidity. The titratable acidity was measured by titrating the sample with 0.1 N NaOH using phenolphthalein as an indicator. The titratable acidity was calculated as lactic acid and expressed as percentage (w/w).

Color assay. Color was measured using a colorimeter (ColourFlex, Hunter Lab Reston, VA, USA). The results were shown in L^* indicating lightness, a^* indicating redness/ greenness, and b^* indicating yellowness/blueness.

Preparation of water extract of fish paste. Water extract of sample was prepared using procedures described by Pongsetkul *et al.* (2017b) with slight modifications. Fish paste (2 g) was mixed with distilled water (50 ml), and homogenized at a speed of 10,000 rpm for 2 min. After 15-min centrifugation at 8,000 rpm at room temperature (RC-5B plus centrifuge, Sorvall, Norwalk, CT, USA), the supernatant was collected, and the pellet was re-extracted in the same manner to collect more supernatant. Finally, all the collected supernatants were combined and adjusted to 50 ml and analyzed to determine Maillard reaction products (MRPs), browning intensity, the nitrogen contents, antioxidant activities.

Determination of intermediate MRPs. The absorbance of water extract of fish paste samples was measured at 280 and 295 nm (UV-1601 spectrophotometer, Shimadzu, Kyoto, Japan) using method developed by Pongsetkul *et al.* (2014), to measure the formation of intermediate products from Maillard reaction.

Determination of browning intensity. The browning intensity of water extract was measured at 420 nm (UV-1601 spectrophotometer) using method developed by Pongsetkul *et al.* (2017b).

Determination of formal nitrogen, ammonia nitrogen and amino nitrogen contents. These parameters were measured as described by the Thai Industrial Standard (1983) and Klomklao *et al.* (2006) and Pongsetkul *et al*., (2017c).

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Determination of TCA-soluble peptide content. Fish paste sample was homogenized with cold 5% TCA (at a ratio of 3 g : 27 ml) at 11,000 rpm for 1 min. After 30-min storage in ice, it was subjected to 20-min centrifugation at 5000 g at 4°C (RC-5B plus centrifuge). The peptide content was measured using procedures developed by Pongsetkul *et al.* (2017a) and was expressed as mmol tyrosine equivalent/g dry sample.

Determination of protein patterns. *Ka-pi-plaa* samples were measured for protein patterns using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and 4% stacking gel and 10% running gel were used (Laemmli, 1970; Pongsetkul *et al*., 2017a). Samples (3 g) were solubilized in 27 ml of 5 % (w/v) SDS. The mixture was homogenized for 1 min at a speed of 13,000 rpm and incubated at 85ºC for 1 h to dissolve total proteins. Samples (15 µg protein) determined by the Biuret method (Robinson and Hogden, 1940) were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gels were stained with 0.05 % (w/v) Coomassie Blue R-250 in 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min. Finally, gels were destained with a mixture of 50 % (v/v) methanol and 7.5 % (v/v) acetic acid for 30 min and destained again with a mixture of 5 % (v/v) methanol and 7.5 % (v/v) acetic acid for 1 h.

Determination of peroxide value (PV). PV was determined according to the method of Low and Ng (1978). The PV is expressed as milliequivalents (meq) of free iodine per kg of lipid.

Determination of thiobabituric acid reactive substances (TBARS). TBARS value was determined using procedures described by Ghani *et al*. (2019) and was expressed as mg malondialdehyde (MDA)/kg dry sample. This method measures the oxidation products of unsaturated fatty acids in the fish paste production. Standard curve was prepared using malondialdehyde bis (dimethyl acetal) at concentration of 0-2 ppm.

Determination of free fatty acid (FFA). The FFA content (as oleic) was determined using the titration method described in AOAC method 940.28 (AOAC, 2003).

Determination of antioxidant activities during production

Water extracts of *Ka-pi-plaa* samples were subjected to free radicals (DPPH and ABTS) scavenging activities, FRAP and metal chelating activity following the method of Poonsin *et al*. (2018). Free radicals (DPPH and ABTS) scavenging activities and FRAP were expressed as nmol Trolox equivalents (TE)/g dry sample. Metal chelating activity was expressed as nmol EDTA

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equivalent (EE)/g dry sample.

Determination of microbiological properties during production

Determination of total viable count. Total viable count was determined using PCA without 10% NaCl in step R (raw material) and A (autolysis) and PCA with 10% NaCl in step M (mixing with salt), D (drying) and F (fermentation for 2, 5, 10 and 15 days). The recipe contained 25 g of fish paste sample and 225 mL of peptone water containing 10% (w/v) NaCl. Mixture was mixed at high speed for 3 min (Stomacher 400 Lab Blender, Seward Ltd., Worthing, UK), and was diluted in serial tenfold steps for analysis by the spread plate technique. Incubation was carried out at 30°C for 3-5 days (BAM 2001). Microbial load was expressed as colony forming units/g dry sample (CFU/g dry sample).

Determination of halophilic bacteria count. Diluted sample (0.1 ml) was applied on the surface of JCM media (pH 7.2, composed of (l^{-1}): 100 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g glutamic acid, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$ and 20 g agar), spread and incubated for 5-7 days at 30°C (Pongsetkul *et al.*, 2017a).

Determination of proteolytic bacteria count. A standard PCA containing 10% (w/v) NaCl and 1% (w/v) sodium caseinate (pH 7.5) was used. Incubation was performed at 30°C for 3-5 days (Pongsetkul *et al.*, 2017a). Clear zone around colonies on plate were counted as proteolytic bacteria.

Determination of lipolytic bacteria count. A standard PCA containing 10% (w/v) NaCl and 1% (w/v) tributyrin (pH 7.5) was used. Incubation was performed at 30°C for 3-5 days (Pongsetkul *et al.*, 2017a). Clear zone around colonies on plate were counted as lipolytic bacteria.

Determination of lactic acid bacteria (LAB) count. De Man, Rogosa, and Sharpe (MRS) agar containing 1% (w/v) $CaCO_3$ and 10% (w/v) NaCl (pH 7.5) was used. Aliquot of 0.1 ml diluted sample was applied and incubated at 30°C for 3-5 days (Pongsetkul *et al.,* 2017a).

Statistical analysis

All experiments were performed in triplicate. A completely randomized design was used. Statistical analysis was by one-way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS 11.0, SPSS Inc., Chicago, IL, USA). Mean value comparison was performed using Duncan's multiple range test (Steel and Torrie, 1980).

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Results and discussion

Changes in physicochemical properties of *Ka-pi-plaa* during production

Moisture content and Water activity (A_w)

Changes in moisture content of *Ka-pi-plaa* is displayed in Fig. 1A. The highest moisture content was found for fresh fish (78.28%), and it decreased significantly in autolysis and salting steps ($P<0.05$). The possible reason is that during autolysis, fish proteins are hydrolyzed by both proteases existed endogenously and exogenously, which could lead to the decrease in protein functionality especially the water holding capacity (Dallagnol *et al*., 2021). Moisture content after the salting step was low to be 62.74%. It was because salt penetrated into fish meat while water in fish mussel was removed due to osmotic pressure. Sample after drying with sunlight had a moisture content of 47.02%. Moreover, there was no changes were found during 15 days of fermentation ($P>0.05$). Moisture content of *Ka-pi-plaa* was consistent, which was also associated with the texture of fish paste products that can vary from soft and pasty to dry and hard. After 15 days of fermentation, *Ka-pi-plaa* had the moisture content of 47.50%, and it correlated with the product texture observed to be slightly dry and hard. Results suggested that the autolysis, salting and drying steps were the major ones in terms of the decrease of moisture content.

Changes in water activity (A_w) is displayed in Fig. 1B. The change showed similar trend to the moisture content for the production steps. During autolysis, salting and drying period, A_w of samples decreased gradually. No difference was found among samples obtained from fermentation period. The finished *Ka-pi-plaa* product had an A_w value of 0.69. It has been reported that intermediate moisture foods generally have the A_w ranging of 0.6-0.7 (Fellows, 2017). Thus, the A_w of *Ka-pi-plaa* samples suggested them to be intermediate moisture food products; And therefore, the growth of undesirable microbials such as food pathogens were expected to be retarded.

pH and titratable acidity

Changes in pH and titratable acidity (TA) of *Ka-pi-plaa* is displayed in Fig. 1C and 1D. The pH of fresh fish was 6.98, but autolysis step had significantly changed the pH to 7.45 ($P<0.05$). The increases in pH could be explained by the accumulated ammonia, TMA and other alkali substances formed from the fish spoilage (Khairina *et al.,* 2017). Besides, drying step slightly decreased the pH of *Ka-pi-plaa* sample to 7.17, and a continuous decrease in pH was

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observed during fermentation for 15 days (P<0.05), among which the finished product had a pH of 6.62. This occurred because the titratable acidity increased from 0.01% (raw material) to 0.15% on day 15. The increase in titratable acidity and the decrease in pH value are closely related to enzymatic activity in the raw materials and microorganisms that grow during fermentation, particularly lactic acid bacteria. Importantly, some bacteria produce free fatty acids with the help of lipase enzymes, giving an increase in TA. Kanjan and Sakpetch (2020) found that *Staphylococcus simulans* PMRS35 isolated from Budu, which efficiently produces lipase in a high salted environment.

Color, MRPs and browning intensity

The changes of color, MRPs and browning intensity of *Ka-pi-plaa* during the production are depicted in Table 1.

The lightness (L^*-value) of fresh beardless barb fish was 51.83, which was the highest. The L^*-value decreased in the next steps and decreased gradually during 15-day fermentation (P<0.05). On the contrary, both a^*- and b^*-values increased throughout the manufacturing steps (P<0.05). It is worth noting that there were no significant changes of L^*-, a^*-, and b^*-values observed for *Ka-pi-plaa* samples obtained among 5-, 10- and 15-days' fermentation time (P>0.05). The decreased L^*-values and increased a^*- and b^*-valued indicated a darker color formed, which was probably due to the browning pigments produced from Maillard reaction (Prihanto *et al.,* 2021) among the free amino acids and reducing sugars released from fermentation process (Yang *et al.,* 2020).

Values of MRPs (A_{280} and A_{295}) of *Ka-pi-plaa* were also compared (Table 1). A significant continuous increase in absorbance at 280 nm was observed during autolysis, salting, drying and the 5 days' fermentation (P<0.05), and A_{280} was relatively stable during 5 to 15 days' fermentation (P>0.05). Similarly, an increase in A_{295} was observed from initial step to fermentation within 15 days (P<0.05), but there was no difference between fermentation at 10 and 15 days (P>0.05). The increase in A_{280} and A_{295} suggested the possible formation of precursor compounds for the Maillard reaction (Pongsetkul *et al.,* 2017b). The result was in agreement with the color observation (L^*-, a^*-, and b^*-values) described above.

Changes in browning intensity (A_{420}) showed a similar increasing trend. Briefly, A_{420} increased with processing of the production steps, and the value reached 0.126 at day 15 of fermentation. Absorbance at 420 nm has been used to indicate the browning developed at the final

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stage (Pongsetkul *et al.,* 2017b). Maillard reaction was found to be responsible for the brown color in fermented products (Dissaraphong *et al*., 2006). Most of nitrogenous compounds generated, particularly during fermentation, are free amino acids and small peptides, which contributed to brown color development via Maillard reaction (Dissaraphong *et al*., 2006). Carbohydrate derivatives and other substances present in the metabolic pathways could also act as reactants in the Maillard reaction (Kawashima and Yamanaka, 1990). Moreover, oxidation products, such as aldehyde were able to react with free amino acids, liberated during fermentation. Thus, the results of color parameters (L^*, a^*, and b^*), MRPs and browning intensity all suggested that the autolysis, salting, sun-drying and fermentation time had significant influence on the color development of *Ka-pi-plaa*, resulting in a intense brown color from the production.

Peroxide value (PV) and TBARS

PV values of beardless barb during *Ka-pi-plaa* production are shown in Fig. 1E. PV of the fresh beardless barb was 9.19 meq/kg lipid, suggesting that lipid oxidation occurred after harvest or during transportation in ice. PV slightly increased after autolysis, mixing with salt, drying with sunlight and continuously increased during fermentation for 5 days ($P<0.05$). Subsequently, a decrease in PV was noticeable at day 10 and 15 of fermentation ($P<0.05$). Thiansilakul *et al.* (2010) reported that an increase in PV was noticed in red tilapia within the first 3 days of iced storage, and gradually decreased thereafter. Hydroperoxide is the primary product of lipid oxidation, and the determination of the peroxide value can be used as an oxidative index for the early stage of lipid oxidation (Ramadan and Mörsel, 2004). The decrease in PV after 10 and 15 days of fermentation was probably due to the decomposition of hydroperoxide to form low molecular weight compounds e.g., aldehydes and ketones.

The TBARS values were compared among the samples collected during *Ka-pi-plaa* production (Fig. 1F). Fresh fish had a TBARS value of 1.51 mg MDA/kg dry sample. After autolysis, TBARS increased significantly ($P<0.05$), suggesting lipid oxidation in autolysis step, but no significant difference was observed in samples collected after salting ($P>0.05$). Sun-drying step increased TBARS value, and the fermentation process continuously increased the TBARS value of fish paste *Ka-pi-plaa*. The highest TBARS value (3.51 mg MDA/kg dry sample) was observed for *Ka-pi-plaa* collected after 15 days' fermentation ($P<0.05$). The result indicated that lipid oxidation mainly occurred at the autolysis step. Fish generally have 22 to 48% of highly unsaturated fatty acids and thus fish is prone to oxidation (Secci and Parisi, 2016). During the

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autolysis, fish muscle matrix gets loosened to release and expose the lipids that were bound with the muscle matrix to oxidation, as observed in TBARS values.

Free fatty acid (FFA) content

Changes in FFA content in *Ka-pi-plaa* during production are depicted in Fig. 1G. FFA content of fresh fish was 0.30%. Continuous increases in FFA content of *Ka-pi-plaa* during autolysis, drying and fermentation process were observed ($P<0.05$). After 15 days of fermentation, the product had FFA content of 5.75%. The result indicated that hydrolysis of lipids occurred to a great extent at the end of the fermentation. Visessanguan *et al*. (2006) found that FFA content of *Nham,* a Thai fermented pork sausage*,* at the beginning of the process represented 0.3% and increased to 3% at the end of fermentation. Lipolytic activity during *Ka-pi-plaa* production was attributed to both lipases of the muscular tissue and microbial origin (Pacheco-Aguilar *et al*., 2000). Visessaguan et al. (2006) found that the accumulation of FFA in Nham could be attributable to the lipase activity in muscle and microorganisms, which were enhanced with extended fermentation. Nayak *et al.* (2003) also reported that extracellular lipase, produced by certain microorganisms, such as *Pseudomonas fragi* also contributed to the lipolytic breakdown of fish lipids. The increase in FFA content was correlated well with the increase in lipolytic bacteria (Fig. 4D) during fermentation. Moreover, free fatty acids formed were likely prone to oxidation. This resulted in the release of free fatty acids, which were susceptible to oxidation as indicated by the increase in PV and TBARS values (Fig. 1E and 1F).

Contents of formal, ammonia and amino nitrogen

Contents of formal, ammonia and amino nitrogen throughout the production steps are presented in Fig. 2A, B and C, respectively, and in general, they showed similar trend. There was a sharp increase found after autolysis process for the formal, ammonia and amino nitrogen contents, compared to the initial fish sample ($P<0.05$). All the three nitrogen content parameters remained relatively stable in the following salting and sun-drying steps. During the fermentation from 2 to 15 days, these nitrogen values also showed a general increase trend as fermentation time increased.

Formal nitrogen content is an index to indicate the degree of protein hydrolysis (Ciou *et al*., 2020). Previous research studies have found the formal nitrogen content could increase with the increase of fermentation time for fish fermentation (Klomklao *et al*., 2006; Faithong and

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Benjakul, 2014). Thus, the result in present study indicated that fish protein degraded continuously during the whole production process. Ammonia nitrogen content is associated with the breakdown of protein into free amino acids and volatile nitrogen (Ciou *et al*., 2020). An increase in ammonia nitrogen content could be because of the action of fish endogenous and microbial enzymes (Pongsetkul *et al*., 2017c). This study also suggested that processing steps and fermentation time had great impact on hydrolysis of fish protein. An increase in amino nitrogen content is associated with polypeptide degradation (Pongsetkul *et al*., 2017c). In the *Ka-pi-plaa* production, proteins were hydrolyzed to peptides and amino acids, and similar results were observed in the fermentation of small shrimp (*Acetes vulgaris*) and krill (*Mesopodopsis oreintalis*) (Faithong and Benjakul, 2014; Pongsetkul *et al*., 2017b).

The produced short peptides and free amino acids contribute to the flavor and taste of *Ka-pi-plaa*, partially because of Maillard reaction where free amino groups and carbonyl compounds undergo glycation (Moldoveanu, 2021). Moreover, the breakdown of protein was in accordance with the development of browning. Thus, Maillard reaction occurred during *Ka-pi-plaa* production as the high temperature in sun-drying and the fermentation performed in sunlight facilitated Maillard reaction.

TCA-soluble peptide content & protein pattern

Change in TCA-soluble peptide content is displayed in Fig. 3A. Fresh beardless barb fish contained TCA-soluble peptides (41.83 μmol/g dry sample) that was from the endogenous oligopeptides in fish tissue during post-harvest handling (Dallagnol *et al*., 2021). A dramatic increase in TCA-soluble peptides was observed after autolysis ($P<0.05$). There were no differences among the autolysis, salting and sun-drying steps ($P>0.05$). However, protein hydrolysis continued gradually during the fermentation step, for which the TCA-soluble peptide content of sample collected at 15 days' fermentation was 345.25 μmol/g dry sample. Thus, as the fermentation proceeded, small peptides were produced with time as a function of the activity of fish endogenous and microbial proteinases; And the peptides had influence on the properties, bioactivities and quality of *Ka-pi-plaa*.

Fig. 3B shows the protein patterns of samples collected in the production process. The main proteins are myosin heavy chain (MHC) and actin in initial raw fish sample. After autolysis and drying, MHC protein obviously degraded, and it was hydrolyzed completely after 2-day fermentation. Actin protein showed band intensity in all samples, but it was found in low quantity

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during the fermentation period because of hydrolysis. It is worth noting that peptides ≤ 32 kDa increased as fermentation proceeded. It was reported that MHC is susceptible to enzymatic degradation compared to other muscle proteins like actin, troponin and tropomyosin (Singh and Singh, 2020). The results suggested that proteins of beardless barb fish continuously degraded into smaller oligopeptides throughout the production process, which further affected the *Ka-pi-plaa* properties.

Changes in microbiological properties of *Ka-pi-plaa* during production

Changes in the population of various microorganisms in the samples collected during *Ka-pi-plaa* production are shown in Fig. 4. The change patterns for different microbes exhibited different trends. In general, the microbiological activities increased with the production procedures, which might also contribute to the development of flavor and taste of *Ka-pi-plaa*.

Fig. 4A shows the total viable counts (TVC). Initial TVC of fresh beardless barb fish was 3.45 log CFU/g dry sample, which was the lowest. After autolysis, the sample showed an increased TVC, which was the highest among all samples. After salting and sun-drying steps, TVC slightly decreased ($P<0.05$), and during the fermentation for 2 to 10 days, there was no significant change in TVC ($P>0.05$). The TVC was calculated to be 5.92 log CFU/g dry sample in the fish paste collected after 15 days of fermentation. The increase in the TVC at autolysis step of *Ka-pi-plaa* production was probably because of the bacterial and autolytic spoilage when fish samples were placed at room temperatures where mesophiles grow rapidly (Pongsetkul *et al.*, 2017b). In the salting, drying and fermentation processes, the reduction of TVC was due to the inhibition of natural flora by the highly concentrated salt which was also important for food preservation via inhibition of pathogen growth (Pongsetkul *et al.*, 2017b). Moreover, salting and drying caused lower moisture content and A_w (Fig 1A and 1B), which could reduce the activities of microorganisms.

Fig. 4B shows the population of halophilic bacteria that are key microorganisms in fermentation to degrade proteins (Sim *et al.*, 2015). The number of halophilic bacteria increased in each production step. Especially after salting step, there was a significant increase of halophilic bacteria population ($P<0.05$). The results suggested that halophilic bacteria were dominant microorganisms in *Ka-pi-plaa*.

The change of proteolytic bacteria or lipolytic bacteria counts exhibited similar trend throughout *Ka-pi-plaa* production steps (Fig. 4C and 4D). The initial counts of the proteolytic and

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lipolytic bacteria were the lowest at 3.14 and 2.71 log CFU/g dry sample, respectively. After the autolysis step, both bacteria count sharply increased (P<0.05) but slightly decreased after salting and sun-drying steps. The reasons could be the presence of high salt and the low A_w in samples that retarded the growth of both proteolytic and lipolytic microorganisms. However, both of them slightly increased during fermentation of *Ka-pi-plaa*, which contributed to the decomposition and degradation of proteins and lipids in fish to develop flavor and aroma in *Ka-pi-plaa* product. The active bacteria can be both halophilic and proteolytic/lipolytic. For example, halophilic bacteria, *Virgibacillus halodenitrificans MSK-10P* was isolated from traditional fermented fish, *Kapi* in Southern Thailand that presented high proteinase activity (Kumaunang *et al*., 2019). Kanjan and Sakpetch (2020) also reported that *Staphylococcus simulans* PMRS35 had good lipolytic activity and moderate proteolytic activity.

Fig. 4E shows the changes of lactic acid bacteria (LAB) in *Ka-pi-plaa* during production. The initial LAB count of raw material was 2.02 log CFU/g dry sample that was the lowest. The number of LAB increased gradually in the production steps of autolysis, salting, drying and the first 5 days of fermentation (P < 0.05). In addition, the increase of LAB count coincided well with the decrease of pH during production, as LAB produced acids like lactic acid and acetic acid in *Ka-pi-plaa* (Thongruck *et al.*, 2017). LAB is important and desirable in fish fermentation, which help to inhibit microbial spoilage, enhance flavor and reduce pathogenic microorganisms (Thongruck *et al.*, 2017). However, no further change in LAB count occurred during fermentation up to 15 days (P > 0.05). *Ka-pi-plaa* contained low amount of carbohydrate. This could result in the reduction of LAB during fermentation because of insufficient substrates for their growth.

Overall, halophilic bacteria were the dominant microorganisms, which could grow and produce some proteolytic or lipolytic enzymes. These microbial enzymes and endogenous enzymes play a profound role in degradation of proteins and lipids in fish throughout the process. Those degradation products might contribute to the typical properties of final *Ka-pi-plaa*, especially flavor or taste.

Changes in antioxidant properties of *Ka-pi-plaa* during production

The changes in antioxidant activities of the *Ka-pi-plaa* water extracts were determined by free radicals (DPPH, ABTS) scavenging activities, FRAP and metal chelating activity, as shown in Fig. 5. In general, with the proceeding of production, the antioxidant activities gradually increased. The results suggested that *Ka-pi-plaa* was a good source to provide natural antioxidants.

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The initial DPPH radical scavenging activity was only 12.00 nmol TE/g dry sample, and a significant increase was detected after autolysis step ($P<0.05$) (Fig. 5A), but no significant change was found after salting step ($P>0.05$). There was a remarkable increase in DPPH radical scavenging activity found after sun-drying step ($P<0.05$), and the activity slightly increased throughout fermentation. It was suggested that the peptides and free amino acids generated in *Ka-pi-plaa* could donate hydrogen atoms to retard the propagation of free radicals. For fermented products, hydrolysis was generally progressed throughout the prolonged fermentation, leading to the increases in hydrolyzed peptides and amino acids. Low molecular weight peptides and amino acids have been found to possess antioxidant activity (Klomklao et al., 2018). Pongsetkul *et al.* (2017b) also reported an increase of DPPH radical scavenging activity for the water extract of salted shrimp paste when fermentation proceeded.

Fig. 5B shows the ABTS radical scavenging activity of the water extract samples. Initial ABTS radical scavenging activity was the lowest with 1518.46 nmol TE/g dry sample, and it increased in the following steps ($P<0.05$). At day 15 of fermentation, the highest ABTS radical scavenging activity was measured with 3377.12 nmol/g dry sample. It can be inferred that antioxidant compounds, including peptides, fatty acids and MRPs contributed to the increases of this activity, since ABTS assay determines both hydrophilic and lipophilic antioxidants (Pongsetkul *et al*., 2017b; Klomklao and Benjakul, 2018). Increases in antioxidative activities were correlated well with the increase in MRPs during *Ka-pi-plaa* production, especially during fermentation.

The increase of ferric reducing antioxidant power (FRAP) of *Ka-pi-plaa* water extract showed a gradual increase trend in Fig. 5C. FRAP of final product was the maximum with a value of 6289.06 nmol TE/g dry sample. The reducing ability of *Ka-pi-plaa* was possibly due to the presence of peptides, which donated electrons to free radicals, leading to the prevention or retardation of propagation. The change of metal chelating activity (Fig. 5D) showed slight increase after autolysis (1627.53 nmol EE/g dry sample) and salting (1648.62 nmol EE/g dry sample), and significant increase was measured after drying step (3426.18 nmol EE/g dry sample) ($P<0.05$). Fermented fish products, especially *Kapi* have been reported to possess FRAP and metal chelating activity (Faithong et al., 2014; Pongsetkul et al., 2017b). Based on the results, it may be inferred that *Ka-pi-plaa* produced from beardless barb was shown as the potential source of natural antioxidants with different modes of action in prevention of lipid oxidation.

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Conclusions

A salted and fermented fish paste product, *Ka-pi-plaa*, was produced and investigated for various characteristics through all production steps. The color of products developed to be dark brown and Maillard reaction products were also determined. Each production step played important role in the change of various physiochemical, microbiological and antioxidant properties of *Ka-pi-plaa*. Autolysis is key step to increase the nitrogen content, peptide production and the growth of bacteria. Salting is important to change the halophilic bacteria count. Sun-drying step is vital to moisture content, pH and growth of bacteria. Fermentation is accompanied by the hydrolysis of proteins and improvements in antioxidant activities. These changes directly affected the quality of finished *Ka-pi-plaa* product.

Conflict of interest

The authors report no conflict of interest.

Author contributions

Pakteera Sripokar: Conceptualization, Investigation, Writing-original draft, Visualization. Egon Bech Hansen: Methodology. Suppasil Maneerat: Visualization. Yi Zhang: Revision, Supervision. Sappasith Klomklao: Conceptualization, Supervision.

Ethical approval

Not applicable.

Funding information

This study was funded by Thailand Science Research and Innovation with the Royal Golden Jubilee Ph.D. Programme awarded to Pakteera Sripokar (PHD/0074/2559). The National Research Council of Thailand and Thaksin University were also acknowledged.

Data Availability Statement

The data sets of the present study are available from the corresponding authors on reasonable request.

Compliance with ethical standards

Not applicable.

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Figure legend

Fig. 1. Changes in moisture content (A), water activity (A_w) (B), pH (C), titratable acidity (D), peroxide value (PV) (E) and thiobarbituric acid reactive substance (TBARS) (F) during *Ka-pi-plaa* production. Bars represent the standard deviation (n=3). R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days. Different letters on the bars indicate the significant differences (P<0.05).

Fig. 2. Changes in formal nitrogen (A), ammonia nitrogen (B) and amino nitrogen (C) contents during *Ka-pi-plaa* production. Bars represent the standard deviation (n=3). R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days. Different letters on the bars indicate the significant differences (P<0.05).

Fig. 3. Changes in TCA-soluble peptide content (A) and protein patterns (B) during *Ka-pi-plaa* production. R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days. Different letters on the bars indicate the significant differences (P<0.05).

Fig. 4. Changes in total viable count (A), halophilic bacteria (B), proteolytic bacteria (C), lipolytic bacteria (D) and lactic acid bacteria (E) during *Ka-pi-plaa* production. Bars represent the standard deviation (n=3). R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days. Different letters on the bars indicate the significant differences (P<0.05).

Fig. 5. Changes in DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), Ferric reducing antioxidant power (C) and metal chelating activity (D) during *Ka-pi-plaa* production. Bars represent the standard deviation (n=3). R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days. Different letters on the bars indicate the significant differences (P<0.05).

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Table 1 Changes in colors, A_{280}, A_{295} and browning intensity (A_{420}) during *Ka-pi-plaa* production.

Samples	***L****	***A****	***B****	**OD$_{280}$**	**OD$_{295}$**	**OD$_{420}$**
R	51.83±1.27a	-2.43±1.02c	- 0.93±1.59d	0.160±0.01d	0.539±0.02f	0.067±0.74e
A	46.83±1.61b	-2.45±1.22c	2.30±1.56c	0.200±0.11c	0.771±0.07e	0.088±1.13d
S	44.20±0.20c	-2.23±1.07c	2.13±1.8c	0.204±0.01bc	0.774±0.74e	0.088±0.23d
D	47.20±1.34b	-0.43±1.53b	4.23±1.05b	0.216±0.53b	0.805±0.00d	0.094±0.01cd
F2	42.20±0.82d	0.23±0.15b	5.67±0.86ab	0.217±0.23b	0.846±0.38c	0.100±0.00bc
F5	40.33±1.19e	1.23±0.75ab	5.90±0.95ab	0.237±0.00a	0.874±0.99b	0.107±0.00b
F10	39.30±0.79e	1.33±1.45ab	5.83±0.80ab	0.243±1.10a	0.917±1.08a	0.119±0.01a
F15	38.90±0.36e	2.40±1.28a	6.57±0.51a	0.252±0.77a	0.921±1.10a	0.126±0.17a

*Values are given as mean ± SD (n=3).

R: raw material, A: after autolysis, S: after salting, D: after sun-drying, F2, F5, F10 and F15: after fermentation for 2, 5, 10 and 15 days.

Different letters in the same column indicate the significant difference ($P<0.05$).

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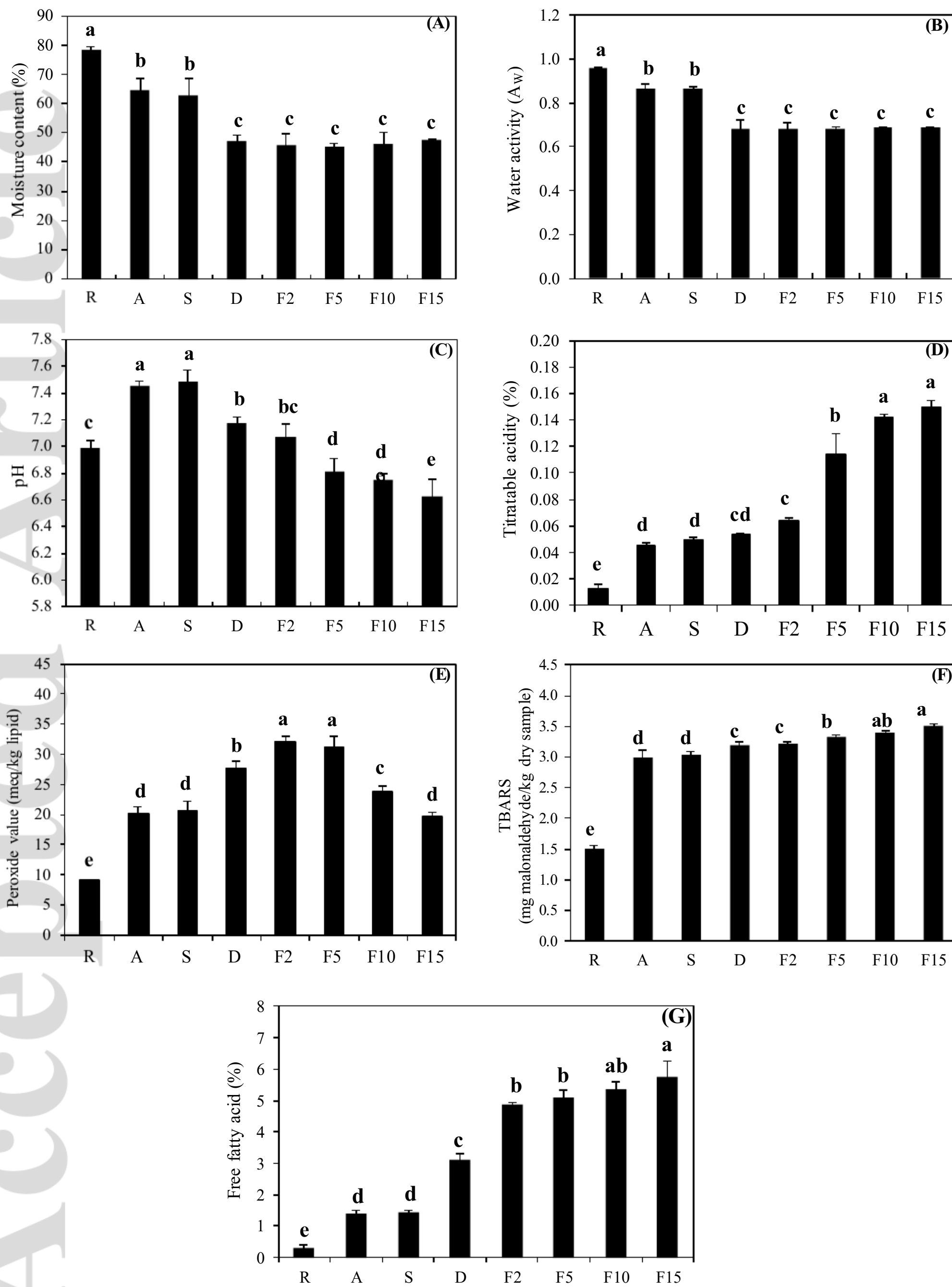

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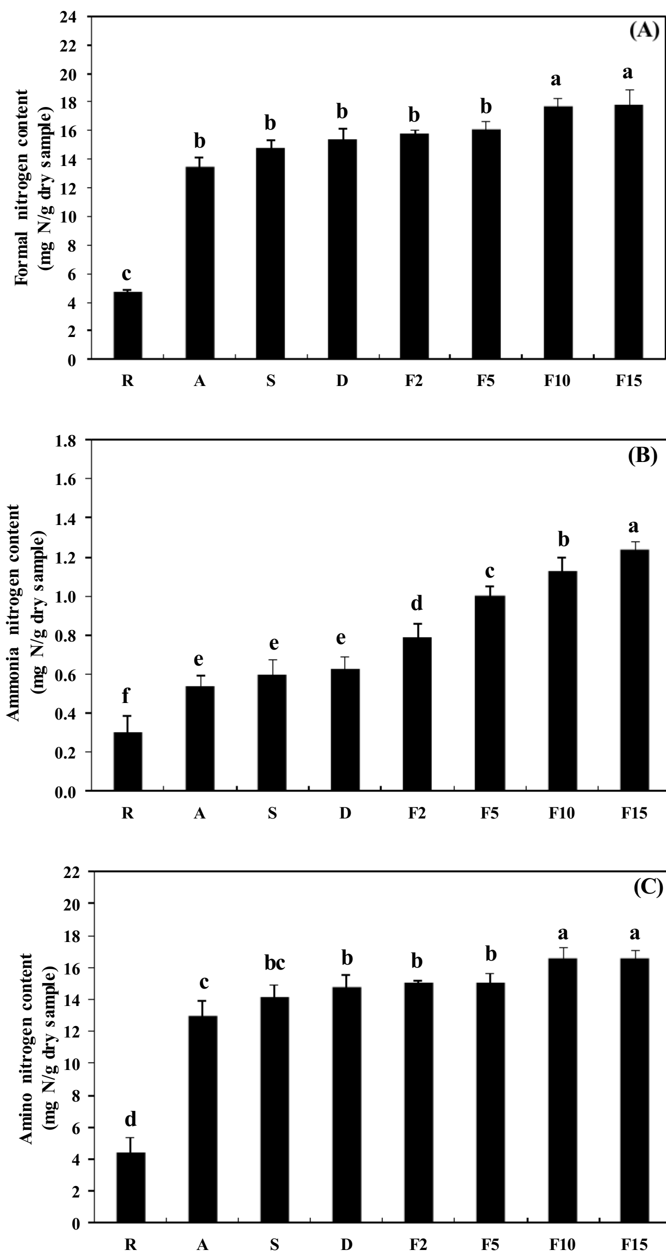

Fig. 2.

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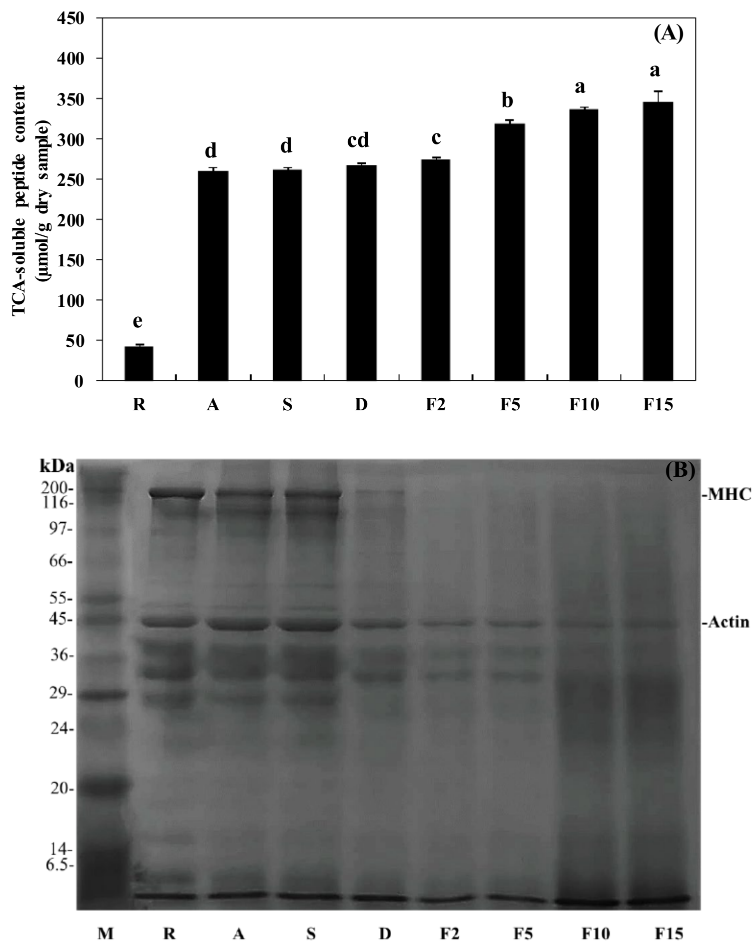

Fig. 3.

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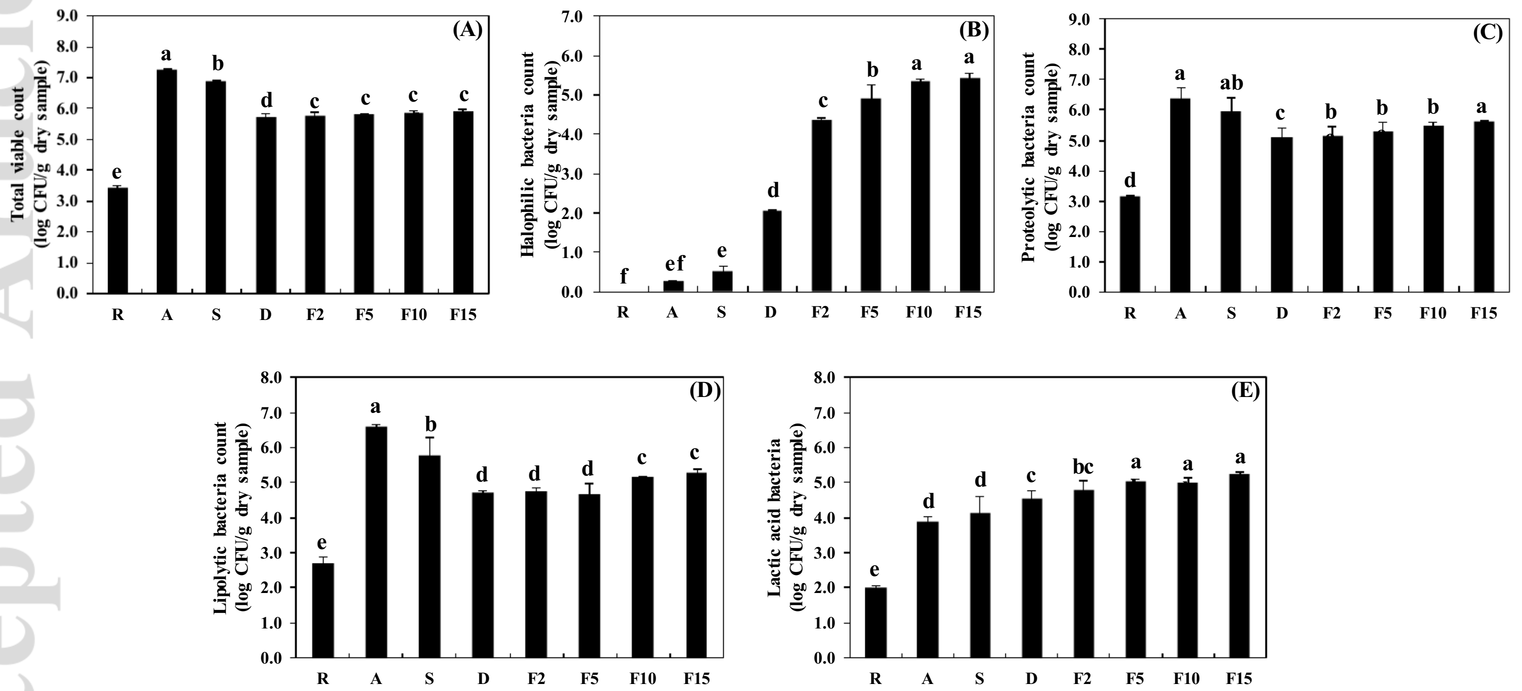

Fig. 4.

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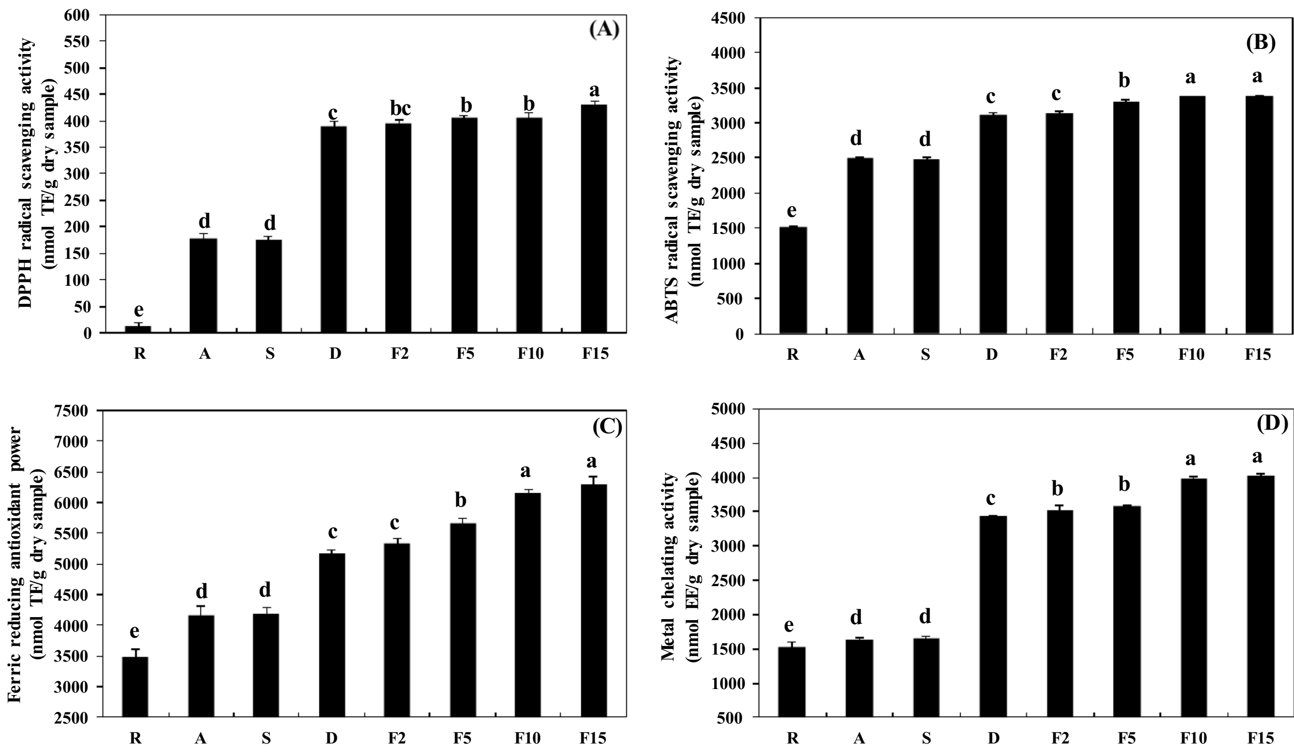

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Fig. 5.

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