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PHYSICOCHEMICAL AND MICROBIAL CHANGES IN SKIPJACK TUNA (*Katsuwonus pelamis*) VISCERA DURING SALT FERMENTATION

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ABSTRACT

Tuna viscera is often regarded as waste by many canning industries around the globe. However, it also represents a potential source of various proteinases that can enhance fish fermentation. Salt-fermented fish viscera locally known as *dayok*, is a traditional fermented food popular in Mindanao, Philippines. This study aimed to investigate the changes in physicochemical properties and microbial populations of salt-fermented skipjack tuna (*Katsuwonus pelamis*) viscera over a 150-day fermentation period. The skipjack tuna viscera primarily consisted of 76.42% moisture, 17.27% protein, 3.64% lipid, and 1.37% ash. Results showed that during the salt fermentation of tuna viscera, pH decreased to 5.71, while titratable acidity (TA) and degree of hydrolysis (DH) increased to 1.64% and 69.60%, respectively. Salt concentration remained stable ranging from 28.10% - 30.28% (w/w) throughout the fermentation period, eliciting a preservative effect. Changes in microbial population revealed a decline in the total viable count to 1.36 log CFU.g^{-1} at the later stages of fermentation, and an increase in lactic acid bacteria (LAB) count to 2.32 log CFU.g^{-1} during the early stages of fermentation. The dominant bacteria identified belonged to the genera *Bacillus*, *Staphylococcus*, and *Enterococcus*, potentially affecting the physicochemical and microbial properties of the salt-fermented tuna viscera. The most abundant bacterial genera may have contributed to the enhancement of flavor and aroma of the final product. This study will lay a foundation for standard tuna viscera fermentation systems and quality improvement of fish paste and fish sauce made from tuna viscera.

Keywords: Lactic acid bacteria, *Katsuwonus pelamis*, fish viscera, *dayok*, salt fermentation, skipjack tuna, waste utilization

INTRODUCTION

Fermentation is a traditional method for making fish paste and fish sauce, typically using anchovies, mackerel, or herring, and involves mixing of one part salt with two or three parts fish (**Dissaraphong *et al*., 2006**). *Dayok* is a popular salt-fermented fish product in Mindanao, Philippines, which utilizes fish viscera and yields two products at the end of the fermentation period: fish paste and fish sauce **(Besas and Dizon, 2012)**. This traditional method not only helps prevent seafood spoilage but also imparts a distinctive flavor to the fish sauce product, while serving as an excellent source of essential nutrients (**Wang *et al*., 2018**). Carbohydrates, lipids, and proteins in raw fish are broken down into flavor compounds and volatiles during fermentation through the action of indigenous and microbial enzymes **(Pombo *et al*., 2009)**.

Microbial population typically increases in number during the early stages of fermentation but gradually decreases as fermentation progresses due to the dominance of halophilic and halotolerant bacteria, such as lactic acid bacteria and yeast **(Lopetcharat & Park, 2002; Jiang *et al*., 2007)**. Lactic acid bacteria (LAB) play a crucial role in preservation, flavor development, texture improvement, nutrient enrichment, and health benefits of fermented foods **(Cai *et al*., 2024)**. The presence of *Bacillus*, *Lactobacillus*, *Micrococcus*, and *Staphylococcus* in fermented foods has enhanced their commercial value by providing beneficial physicochemical properties responsible for the final quality of the product **(Narzary *et al*., 2021)**. These bacteria produce various hydrolytic enzymes and organic acids which boost acidification in fermented fish, thereby inhibiting spoilage and pathogenic bacteria (e.g., *E. coli*), while enhancing texture, aroma, and nutritional value **(Sakpetch *et al*., 2022)**.

Skipjack tuna (*Katsuwonus pelamis*) is the smallest of the major commercial tuna species and primarily found in the Pacific Ocean, particularly in the Philippines waters **(ISSF, 2024)**. In 2024, skipjack tuna output reached 277,530 metric tons, marking a 31.2% increase, making it the second-highest level of marine fisheries production following yellowfin tuna **(PSA, 2025)**. It is a rich source of protein, low in fat, and contains high levels of omega-3 fatty acids, iron, copper, and zinc, all of which offer significant health benefits for the human body **(Rukshana *et al*., 2021)**. This species is commonly used as a raw material for the tuna canning industry, alongside albacore (*Thunnus alalunga*) and yellowfin tuna (*Thunnus albacares*) **(Zhang *et al*., 2002)**. However, skipjack tuna processing can also be an important source of environmental contamination due to the large amount of waste generated **(Klomklao and Benjakul, 2016)**. The amount of solid waste generated in the tuna canning industry can reach up to 50% by weight, which consists of viscera, skin, heads, and bones **(Montiel-Montoya *et al*., 2022)**. Waste viscera, composed of spleen, stomach, intestines, bile sac, liver, and pancreas **(Dissaraphong *et al*., 2006)**, is a notable by-product rich in valuable proteins, lipids, vitamins, and minerals **(Klomklao & Benjakul, 2016)**. It is also a potential source of various proteinases which can accelerate fish paste and fish sauce production **(Klomklao *et al*., 2006)**. Reducing dietary protein waste has advantages beyond environmental effects, since proteins are necessary for human nutrition **(Peydayesh *et al*., 2023)**.

This study aimed to assess the changes in the physicochemical properties and bacterial populations during salt fermentation of skipjack tuna viscera. The physicochemical changes were correlated with the bacterial profile to determine the optimum conditions for the fermentation process. In addition to enhancing the quality of fish paste and fish sauce made from fish viscera, this study will help establish the groundwork for the standard fermentation of tuna viscera.

MATERIALS AND METHODS

Sample preparation

Skipjack tuna (*K. pelamis*) viscera were collected at Ocean Deli Packing Corporation in General Santos City, Philippines in June 2024. The viscera were fermented for 150 days using a 3:1 ratio of fish viscera to salt. The tuna viscera comprised of intestines, stomach, liver, spleen, and pancreas. Tuna viscera were cleaned and washed with chilled water to remove any remaining blood in the tissues. The cleaned viscera were homogenized using a heavy-duty blender. A total of 1500 g of homogenized viscera was fermented with 500 g of solar salt. This mixture was stored in 1 L sterilized glass jars, with 500 g of fermented tuna viscera in each jar, and securely covered to avoid contamination. The fermented tuna viscera were stored at ambient temperature (27–30 °C). Samples for the physicochemical and microbial analyses were collected on 0, 5, 10, 20, 30, 40, 50, 60, 90, 120 and 150 days of fermentation.

Proximate composition

The protein, moisture and ash contents of the skipjack tuna viscera were determined according to the methods of **AOAC (2005)**, and the lipid content was

determined using the method of **Bligh and Dyer (1959)**. All analyses were done in triplicates and results were expressed on wet weight basis.

Salt content analysis

Salt content in the sample was measured following the **AOAC (2000)** and **Volhard (1874)** method. A 0.5 g of homogenized fermented tuna viscera was mixed with 40 ml of 0.1 N silver nitrate ($AgNO_3$) and 15 ml of concentrated nitric acid (HNO_3). The mixture was gently boiled on a hot plate until all components were dissolved, which took approximately 10 min. After cooling, the mixture was filtered and the remaining components were washed with distilled water until the volume reached 100 ml. Then, 5 ml of ferric alum indicator was added. The mixture was titrated with 0.1 N potassium thiocyanate (KSCN) until a permanent light brown color was achieved. The salt content (% w/w) was calculated using the following formula:

$$Salt\ content\ (\%) = \frac{V_1 - (V_2\ x\ f)\ x\ 0.1\ x\ 58.45}{W_s\ x\ 1000}\ x\ 100$$

Where V_2 is the volume of KSCN consumed in titration (ml), *f* is the correction factor for $AgNO_3$ and KSCN, V_1 is the volume of 0.1 N $AgNO_3$ added to the sample (ml), 58.45 is the molecular weight of NaCl, and W_s is the weight of the sample (g).

Determination of pH and titratable acidity

The determination of pH and titratable acidity (TA) was performed at the sampling points of the fermentation period. For pH determination, the sample was diluted (1:10 dilution) in deionized water and measured using a digital pH meter (Adwa AD11, Hungary) **(AOAC, 2001)**. The percentage TA, expressed as percent lactic acid, was determined by titrating with 0.1 N NaOH standard solution until pH 8.2 **(Sadler and Murphy, 2017)**. TA (%) was computed using the following formula:

$$Titratable\ acidity\ (\%) = \frac{V_1\ x\ 0.1\ x\ 90.078}{W_s\ x\ 1000}\ x\ 100$$

Where V_1 is the volume of NaOH consumed in titration (ml), W_s is the weight of the sample (g), 0.1 is the normality of NaOH used, 90.078 is the molecular weight of lactate ($C_3H_5O_3^-$), and correction factor of 1000 to convert to $mg.g^{-1}$.

Determination of degree of hydrolysis

The Formol titration method and Kjeldahl method were used to measure the degree of hydrolysis (DH) (%) **(Alahmad *et al.*, 2022)**. Fermented tuna viscera samples (1.5g) were added to 45 ml distilled water. The mixture was then neutralized to pH 7.0 with 0.1 N NaOH, and 10 ml of 37% (v/v) formaldehyde was added for 8 min at room temperature. Titration was performed to the endpoint until a faint pink color appeared, using 1% phenolphthalein as an indicator and a 0.1 N NaOH standard solution. The DH (%) was calculated using the following formula:

$$Free\ amino\ acid\ group\ (\%) = \frac{V_1\ x\ C_1\ x\ 14.007}{W_s\ x\ 1000}\ x\ 100$$

$$Total\ nitrogen\ (\%) = \frac{V_2\ x\ C_2\ x\ 14.007}{W_s\ x\ 1000}\ x\ 100$$

$$Degree\ of\ hydrolysis\ (\%) = \frac{\%\ free\ amino\ acid\ group}{\%\ total\ nitrogen}\ x\ 100$$

Where V_1 is the volume of NaOH consumed in titration (ml), C_1 is the normality of NaOH, V_2 is the volume of HCl consumed in titration (ml), C_2 is the normality of HCl, 14.007 is the molecular weight of nitrogen, and W_s is the weight of the sample (g).

Color analysis

The fermented tuna viscera were subjected to color measurement using a colorimeter (Chroma Meter CR-400/410, Konica Minolta®, Japan) to assess the change in color characteristics, including lightness (L*), redness (a*), and yellowness (b*). Samples (10 g) were transferred into a glass Petri dish for color determination **(Klomklao *et al.*, 2006)**. Samples were illuminated with D65-artificial daylight (10° standard angle) in accordance with the manufacturer's instructions.

Microbial analysis

Fermented tuna viscera (25 g) were transferred to 225 ml of 0.85% (w/v) sterile saline solution in an Erlenmeyer flask and vigorously mixed. A 1 ml sample solution was transferred to glass tubes for six-fold dilutions in a 9 ml sterile saline solution. Enumeration of LAB was performed using de Man, Rogosa, and Sharpe (MRS) agar. Sample solution (1 ml) was transferred to Petri dishes, pour-plated, and incubated at 35°C for 3 – 5 days. Total viable counts (TVC) were determined using standard plate count agar containing 10% (w/v) NaCl and incubated for 24 - 48 h at 35°C. All counts were performed in triplicate and data were transformed into logarithms of the number of colony-forming units per gram of a sample (log $CFU.g^{-1}$) **(USFDA, 2001)**.

DNA extraction and sequencing

The metagenomic analysis of the fermented tuna viscera at days 0, 20 and 120 was processed and analyzed at the Philippine Genome Center Visayas, University of the Philippines Visayas, Iloilo, Philippines. Samples were performed following the methods of **Caporaso *et al*. (2012), and Cole *et al*. (2014),** in accordance with the operational instructions of the BIOER BioFastSpin Soil Genomic DNA/RNA Extraction Kit for DNA extraction. The DNA template and specific barcoded primers were selected based on the sequencing region, and a high-efficiency Hi-Fi polymerase was utilized for PCR to ensure amplification accuracy. For lactic acid bacterial analysis, the 16S rRNA V4 region was amplified using the 16SV4 forward primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA and the 16SV4 reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT. Library pooling was assessed using Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA) and the quantification of the pooled sample and control library size (346 bp amplicon size) was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The quantity and purity of genomic DNA were measured using a multiskan sky microvolume spectrophotometer (Thermo Fisher Scientific, USA), to determine the concentration of polymerase chain reaction products, which were then sequenced using the Illumina NextSeq 1000.

Statistical analysis

The data were subjected to one-way analysis of variance followed by Tukey's test and expressed as mean ± standard deviation (n = 3). All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 20.0 for windows (SPSS Inc., USA). *P* values lower than 0.05 were statistically different. For the metagenomic raw sequence data, QIIME2 (version 2024.10.1) was utilized to improve sequencing quality. Taxonomic classification and relative abundance of lactic acid bacteria were determined by comparing all sequences against the Silva 16S rRNA SSU NR database (Silva_138.2).

RESULTS AND DISCUSSION

Proximate composition of the skipjack tuna viscera

The proximate components of skipjack tuna viscera are presented in Table 1. The major composition of the skipjack tuna viscera was moisture (76.42%), followed by protein (17.27%), lipid (3.64%), and ash (1.37%). The moisture content obtained in this study corresponded to the values reported by **Klomklao and Benjakul (2016)** on skipjack tuna viscera at 76.94%. Similar to this study, previous studies found that the protein content of skipjack tuna viscera ranged from 17.51% to 18.11% analyzed on a wet basis (**Detkamhaeng *et al*., 2016**; **Dali, 2024)**. The lipid content of 3.65% was comparable to the reported lipid content of tuna viscera in the range of 1.9 – 5.4% (**FAO, 1988**). The low lipid content of the raw material suggests that it is less likely to oxidize, improving its overall stability during storage (**Ovissipour *et al*., 2012**). Ash contents in fish and shellfish ranged from 0.4% to 2% (**FAO, 1988**), hence, significant level of ash present in skipjack tuna viscera indicates that it is rich in minerals. Overall, the proximate composition of skipjack tuna viscera provides valuable nutritional information, demonstrating its potential as a raw material for food production. It is a suitable raw material for fish sauce production due to its high protein and low lipid content.

Table 1 Proximate composition of skipjack tuna viscera

Parameters	Percentage (%) *
Moisture	76.42 ± 0.08
Protein	17.27 ± 0.55
Lipid	3.65 ± 0.42
Ash	1.37 ± 0.02

* Values are expressed as Mean ± SD (n = 3).

Changes in the physicochemical properties of skipjack tuna viscera during fermentation

Table 2 shows the physicochemical properties of the fermented tuna viscera over a 150-day fermentation period. The pH of the fermented product significantly decreased over time ($p < 0.05$), with a final pH at 5.71 at the end of the fermentation period. TA increased throughout the fermentation period, starting at 1.34% on Day 0 and increased to 1.64% at Day 150. The salt content remained relatively stable during the fermentation period, with salt content ranging from 28.10% to 30.28%. Moreover, the degree of hydrolysis significantly increased over time ($p < 0.05$),

initially at 43.09% and then increased to 69.60% by the end of the fermentation period.

The primary preservation factor in fermented fish products relies on the combination of low pH and organic acids, mainly lactic acid which contributes to the development of flavor and texture in fermented food **(Saithong *et al*., 2010)**. Generally, in order to inhibit the growth of spoilage and pathogenic bacteria, the pH of the fermented fish product should be below 5.0 – 4.5 (**Kilinc *et al*., 2006**). As observed in this study, the fermented tuna viscera remained acceptable, with no indications of spoilage until the end of the fermentation period. The fermentation process, characterized by a decrease in pH and an increase in acidity, kept the product stable and acceptable. **Dissaraphong *et al*. (2006)** obtained a final pH of 5.82 for tuna viscera fermented for 12 months, while this study achieved a slightly lower pH at 5.71 at the end of the fermentation period. The significant decrease in pH in a short period of time may be due to the rapid accumulation of hydrogen (H^+) ions, which are by-products of proteolytic activities, as well as lactic acid production during cellular respiration (**Ortizo *et al*., (2020)**. Furthermore, it may also be related to the type of raw material, i.e. viscera contain endogenous bacteria and enzymes which can hasten the fermentation process. A pH range of 5.0 to 6.5, which is typical for traditional fish sauce, is used to support fermentation and ensure that the product meets commercial standards (**Besas and Dizon, 2012**). A consistent decrease in pH along with a corresponding increase in TA was reported in several studies on fermented fish **(Kilinc *et al*., 2006; Kim *et al*., 2022)**. This study obtained a TA of 1.64% at the end of the fermentation period which is in agreement with those reported in previous studies on fermented fish in the range of 1.6% to 2.0% (**Ezzat *et al*., 2021**; **Kim *et al*., 2022**).

The salt concentration of the fermented tuna viscera remained constant throughout the entire fermentation process. Similarly, salt content remained stable in various studies on fish sauce in the range of 25% to 30% (**Dissaraphong *et al*. 2006; Lopetcharat and Park 2002; Sakpetch *et al*. 2022).** Salt primarily acts through osmotic pressure to inhibit spoilage bacteria, facilitate beneficial fermentation, and enhance flavor **(Besas and Dizon, 2012)**.

A high degree of hydrolysis was observed throughout the fermentation period of skipjack tuna viscera. Solid viscera portions were completely disintegrated, resulting in a soft, creamy purée that is characteristic of a fish paste at the end of the 150-day fermentation period. The high DH observed in this study was comparable to the study of **Rdiansyah *et al*. (2015)**, which reported DH values ranging from 60.36% to 67.86% for *kecap ikan*, a commercial fish sauce product from Indonesia. In addition, **Sakpetch *et al*. (2022)** found that the DH in fish sauce (*budu*) reached 58.39% after 360 days of fermentation, which was lower than the values obtained in this study. Differences in DH values may be due to the type of raw materials used, microbial activity, fermentation conditions, or the presence of proteolytic enzymes (**Alahmad *et al*. (2022**). The high DH obtained in this study can be attributed by the synergistic effect of several proteases produced by bacteria during fermentation (**Wasswa *et al*., 2007**). A clear liquid will develop, and its soluble protein content will increase if fermentation is prolonged for six to twelve months. The breakdown of fish protein by the endogenous enzymes in the fish viscera is a major source of the soluble nitrogen **(Cai *et al*., 2024).** A positive correlation between the increased degree of hydrolysis and high protein percentages was observed during fermentation (**Alahmad *et al*. 2022)**. This is primarily driven by the metabolic activity of microorganisms, which break down complex, insoluble macromolecules into smaller, soluble peptides and amino acids, while simultaneously reducing the overall dry matter (specifically carbohydrates and lipids).

Table 2 Changes in the physicochemical properties of skipjack tuna viscera during salt fermentation

Fermentation time (day)	pH	Titratable acidity (%)	Salt content (%)	Degree of hydrolysis (%)
0	6.21 ± 0.02^{a}	1.34 ± 0.02^{b}	29.64 ± 1.17^{a}	43.09 ± 0.19^{h}
5	6.18 ± 0.01^{a}	1.52 ± 0.03^{a}	28.76 ± 1.08^{a}	51.32 ± 0.49^{g}
10	6.13 ± 0.01^{a}	1.55 ± 0.01^{a}	30.28 ± 2.86^{a}	53.51 ± 0.42^{f}
20	6.11 ± 0.01^{b}	1.60 ± 0.03^{a}	30.12 ± 1.30^{a}	54.90 ± 1.14^{f}
30	6.02 ± 0.02^{c}	1.58 ± 0.07^{a}	29.54 ± 3.35^{a}	55.51 ± 0.91^{f}
40	5.98 ± 0.03^{cd}	1.56 ± 0.03^{a}	29.42 ± 2.80^{a}	57.85 ± 0.68^{e}
50	5.92 ± 0.07^{de}	1.58 ± 0.03^{a}	28.10 ± 0.72^{a}	58.41 ± 0.69^{e}
60	5.90 ± 0.05^{de}	1.61 ± 0.04^{a}	28.47 ± 3.14^{a}	60.55 ± 0.25^{d}
90	5.90 ± 0.01^{de}	1.58 ± 0.04^{a}	28.62 ± 0.84^{a}	63.08 ± 0.76^{c}
120	5.85 ± 0.00^{e}	1.59 ± 0.07^{a}	29.15 ± 3.66^{a}	65.70 ± 0.88^{b}
150	5.71 ± 0.05^{f}	1.64 ± 0.01^{a}	30.27 ± 1.34^{a}	69.60 ± 1.01^{a}

Values are expressed as mean ± SD (n = 3). One-way ANOVA and Tukey's test are used to determine the significant differences among the sampling points. Different superscripts in the same column indicate significant difference ($p < 0.05$).

Changes in the color values of skipjack tuna viscera during fermentation

The color properties of the fermented tuna viscera are shown in Table 3. The lightness (L*) value decreased from 32.25 on Day 0 to 28.99 by Day 150, indicating a gradual darkening over time. The redness (a*) values fluctuated slightly, initially decreasing from 3.93 (Day 0) to 3.14 (Day 50), followed by a gradual increase to 3.81 by Day 150. In contrast, the yellowness (b*) values showed minor variations, initially at 9.0 value to 8.52 value at the end of the fermentation period. Significant differences ($p < 0.05$) were found in the L* and a* values, while the b* value was not significantly different ($p > 0.05$) as the fermentation progressed.

Table 3 Color values for lightness (L*), redness (a*) and yellowness (b*) of skipjack tuna viscera during salt fermentation

Fermentation time (day)	L*	a*	b*
0	32.25 ± 0.35^{a}	3.93 ± 0.34^{a}	9.00 ± 0.55^{a}
5	30.68 ± 1.01^{ab}	3.73 ± 0.08^{ab}	8.43 ± 0.18^{a}
10	31.05 ± 0.33^{ab}	3.41 ± 0.03^{b}	8.28 ± 0.14^{a}
20	30.91 ± 0.52^{ab}	3.53 ± 0.09^{ab}	8.35 ± 0.20^{a}
30	30.96 ± 0.49^{ab}	3.38 ± 0.09^{b}	8.60 ± 0.56^{a}
40	30.81 ± 0.21^{ab}	3.20 ± 0.25^{a}	8.73 ± 0.31^{a}
50	30.42 ± 0.11^{ab}	3.14 ± 0.06^{a}	8.16 ± 0.20^{a}
60	30.14 ± 1.01^{b}	3.49 ± 0.14^{ab}	8.31 ± 0.56^{a}
90	29.84 ± 0.45^{b}	3.28 ± 0.14a	8.36 ± 0.08^{a}
120	29.40 ± 1.53^{b}	3.56 ± 0.28^{ab}	8.49 ± 0.25^{a}
150	28.99 ± 0.13^{b}	3.81 ± 0.11^{b}	8.52 ± 0.28^{a}

Values are expressed as mean ± SD (n = 3) for the color properties. One-way ANOVA and Tukey's test are used to determine the significant differences among the sampling points. Different superscripts in the same column indicate significant difference ($p < 0.05$).

The color parameters of the fermented tuna viscera obtained in this study showed a visible decrease in L* and a* values during fermentation, which is consistent with the brown color development observed in fermented tuna viscera **(Dissaraphong *et al*., 2006)**. This suggests that significant color changes occurred, resulting in a darker color as fermentation progressed, which may be due to the melanoidins produced by nonenzymatic browning reaction, such as the Maillard reaction during storage **(Jiang *et al*., 2007)**. The darkening of color is consistent with typical fish sauce and other fish fermentation processes, where protein degradation and chemical reactions contribute to color development **(Lopetcharat and Park 2002)**. Furthermore, salt concentration also affects color development in fish sauce and fermented fish products. The presence of salt promotes denaturation of proteins and other compounds within the fish during fermentation and basically, the higher salt level accelerates the browning reactions occurring during the fermentation process. Fermented fish products with a high salt content (25%–30% salt) typically have a darker color **(Klomklao *et al*., 2006)**.

Changes in the bacterial population of skipjack tuna viscera during fermentation

The changes in the bacterial population of skipjack tuna viscera over a 150-day fermentation period are shown in Figure 1. Initial microbial load was low, i.e. 2.50 log CFU/g for TVC (Figure 2A) and 2.0 log CFU/g for LAB count (Figure 2B). The low initial microbial load may be due to the use of frozen viscera samples which resulted in freezing injury and the effect of salt **(Lopetcharat and Park, 2002)**. The median TVC was 2.09 log CFU/g (range: 1.36 to 2.49 log CFU/g), while the LAB count had a median of 2.07 log CFU/g (range: 1.97 to 2.32 log CFU/g). Based on the interquartile range (IQR) analysis, the TVC exhibited a larger IQR (0.70 log CFU/g), whereas the LAB count had a narrower IQR of 0.25 log CFU/g. This result indicates that there are fluctuations in the total microbial population (TVC), while LAB represents a more stable microbial population during the fermentation of tuna viscera. Microbial counts decreased over the course of fermentation, probably because of the high salt concentration. High salt concentrations (above 17.5%) can suspend microbial activity, by creating a hypertonic environment that removes water from microbial cells through osmosis **(Besas and Dizon, 2012)**. This slows down or inhibits the growth of microorganisms.

The LAB population increased until Day 20, decreased rapidly on Day 30, and then steadily decreased until the end of the fermentation period (Figure 2B). This observation is consistent with the findings of **Kim *et al*. (2022)** who reported that in *gajami-sikhae* (a Korean fermented fish), LAB counts reached the maximum in 10 days, and then slightly decreased as the fermentation progressed. Similarly, **Wattimena *et al*. (2020)** observed that the total LAB count in tuna viscera fish sauce reached 2.3 log CFU/g during a 6-day fermentation period at 50°C. During the initial stages of fermentation, LAB enter an exponential growth phase, multiplying rapidly under optimum nutritional conditions. Depending on specific fermentation conditions and bacterial strain, LAB population may peak at the optimal fermentation stage and then gradually decrease as the fermentation progresses (**Du *et al*., 2022**). The primary effect of LAB proliferation in fermented foods is to lower the pH of a substrate by producing lactic acid, which readily dissociates into hydrogen ions, lowering the pH level (**Saithong *et al*., 2010**). This phenomenon was observed in this study, wherein the pH level decreased when LAB count peaked at Day 20 of fermentation. LAB plays a vital role in fish fermentation by converting carbohydrates into lactic acid and other key metabolites, which directly contribute to the distinct savory and tangy flavors of fermented fish, such as sauce or pastes **(Cai *et al*., 2024)**.

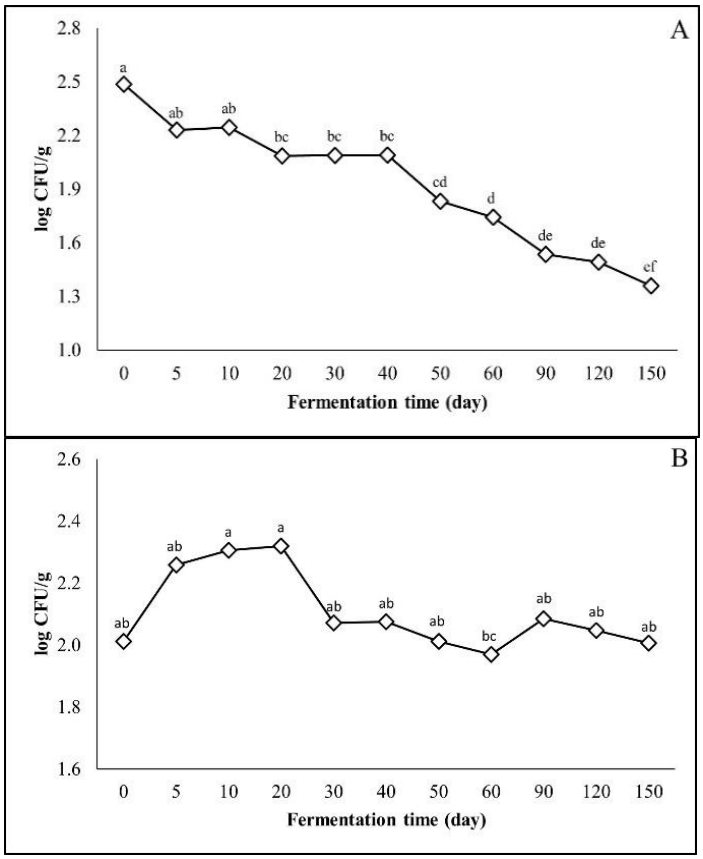

Figure 1 Total viable counts (A) and lactic acid bacterial counts (B) of skipjack tuna viscera during salt fermentation. Different letters indicate significant differences ($p < 0.05$).

Relative bacterial abundance of skipjack tuna viscera during fermentation

The relative abundance of bacteria was surveyed at the initial (Day 0), early (Day 20), and late (Day 120) stages of fermentation (Figure 2). Sequential shifts in the bacterial community were observed. At the beginning, the community was dominated by *Bacillus* (75%), followed by *Staphylococcus* (15%), while *Enterococcus, Mycoplasma*, and other minor genera were present at very low levels (<5%). By the early stage, *Bacillus* decreased sharply to 35%, while *Staphylococcus* increased to 25%, *Enterococcus* rose to 15%, and *Mycoplasma* reached 10%. This shift reflects a transition from a *Bacillus*-dominated microbiota to a LAB-enriched community, which coincides with our observations of increased LAB counts, higher TA, and decreased pH at Day 20. At the late stage, *Bacillus* had moderate abundance (45%), *Enterococcus* remained stable, and *Staphylococcus* (20%) and *Mycoplasma* (2%) declined sharply, suggesting that pathogenic bacteria were suppressed by acid- and bacteriocin-producing bacteria, including LAB and some *Bacillus* species (**Du *et al*., 2022**).

The bacterial community and its succession in fermented skipjack tuna viscera observed in this study differ from those reported in other salt-fermented fish products, but both show enrichment of LAB over time. In anchovy-based fish sauce, which was fermented for an extended period of up to 15 months (**Du *et al*., 2019**), the initial stage was dominated by *Shewanella* (90%), gradually replaced by *Halanaerobium* (up to 86%) during the early to late stages. By the end of fermentation, the halophilic lactic acid bacterium *Tetragenococcus* became predominant, with other genera, including *Pseudomonas*, *Psychrobacter*, *Tissierella*, *Carnobacterium*, and *Gallicola*, also present at notable levels. Similarly, in the fermentation of little yellow croaker and silvery pomfret-based fish sauces, *Psychrobacter* (78%) and *Photobacterium* (47.5%) dominated the early stage, shifting during later stages to the *Tetragenococcus* as well, along with *Staphylococcus* and *Virgibacillus* (**Han *et al*., 2024**). These differences reflect the influence of species-specific raw materials and environmental factors, including salt concentration, nutrient composition, and fermentation duration. The prevalence of LAB in the later stage is primarily due to their ability to create an inhospitable, highly acidic environment that inhibits the growth of competing spoilage and pathogenic bacteria. They can also metabolize proteins and peptides, even in the absence of carbohydrates, producing flavor compounds that enhance the quality of salt-fermented fish products (**Belleggia and Osimani, 2023**).

Although not natural inhabitants of aquatic environments, *Bacillus* species are frequently detected in salt-fermented fish products, including those prepared with rock salt (**Det-Udom *et al*., 2023**). Their presence reflects both contamination of raw materials and their ability to thrive across diverse environmental conditions and withstand the stresses of fermentation (**Raman *et al*., 2025**). In the early stages of fermentation, the activity of endogenous enzymes in the viscera may have promoted *Bacillus* proliferation by providing amino acids and organic acids as energy sources (**Belleggia and Osimani, 2023**). Additionally, under anaerobic conditions, gradual salt penetration and a decrease in pH likely created a favorable environment for *Bacillus* to dominate (**Cai *et al*., 2024**). The genus *Bacillus* is capable of fermenting glucose and amino acids, leading to the production of lactic acid (**Du *et al*., 2019**), which may have promoted the growth of acid-tolerant bacteria like LAB, i.e. *Enterococcus* and *Streptococcus*, in the subsequent stages of fermentation. They can tolerate low to high temperatures, acidic to alkaline pH, and low to high salt concentrations. *Bacillus* species can also produce strong proteolytic enzymes that break down fish proteins, supporting their growth and survival. Their ability to form endospores further allows them to endure fermentation-induced stresses high salt, low nutrient availability, acidic conditions, and the antimicrobial compounds produced by other microorganisms (**Piewngam *et al*., 2018**).

Staphylococcus species were also detected in salt-fermented fish products. Their populations typically increase from the initial to early stages of fermentation and then decline slightly at later stages, likely due to competition with *Bacillus* species and lactic acid bacteria (**Piewngam *et al*., 2018; Zang *et al*., 2018**). A previous study reported that probiotic *Bacillus* can abolish *Staphylococcus aureus* gut colonization by interfering with *S. aureus* quorum-sensing activities (**Piewngam *et al*., 2018**). *Staphylococcus* species are key, early-acting microorganisms in fish fermentation which can utilize proteolytic and lipolytic enzymes to adapt to the environment and produce flavor. The proteolytic activity specifically aids in the degradation of proteins into flavor-presenting amino acids like glutamic acid, which has been linked to enhanced umami taste in fish sauce (**Udomsil *et al*., 2015**). In addition, some *Staphylococcus* strains produce antimicrobial compounds that inhibit spoilage and pathogenic bacteria (**Belleggia and Osimani, 2023**), which may have contributed to the observed low relative abundance of other genera during fermentation.

Two LAB genera, *Enterococcus* and *Streptococcus*, were also detected in this study. *Enterococcus* is commonly found as an indigenous species in the gastrointestinal tract and on the surface of fish (**Ortizo *et al*., 2020**). Mixed cultures of *Enterococcus* have been reported to exhibit strong fermentation performance in salt-fermented fish, including high enzyme activity, enhanced protein breakdown, and production of flavor compounds (**Yuan *et al*., 2023**). In addition, certain *Enterococcus* strains produce bacteriocins and show antagonistic activity against a range of pathogenic bacteria (**Belleggia and Osimani, 2023**). *Streptococcus* species, though commonly found in fermented fish products, their role in fermentation is generally minor. In fact, they were observed at much lower relative abundance compared to *Enterococcus*. These bacteria naturally inhabit the skin and mucous membranes of humans and other warm-blooded animals, so their presence in fermented fish may reflect handling or hygiene practices during processing. Some *Streptococcus* strains, however, have been reported to contribute to texture and flavor development in traditional fermented fish, including *plaa-som, jeotgal, funazushi*, and *rakfisk* (**Li *et al*., 2024**).

Since salt fermentation of fish relies on traditional practices, pathogenic bacteria are frequently detected, possibly originating from raw materials, handlers, or the tools used (**Belleggia and Osimani, 2023**). Among the low-abundance genera observed in this study, *Mycoplasma* and *Clostridium* are noteworthy pathogens. *Mycoplasma* has been reported during the initial fermentation of *myeolchi-aekjeot*, a traditional Korean fermented anchovy sauce, but was no longer detectable after 30 days, likely because the lack of a cell wall makes *Mycoplasma* species highly susceptible to osmotic stress and heat shock (**Jung *et al*., 2016; Browne *et al*., 2017**). On the other hand, *Clostridium* species, which form endospores and resist stresses during fermentation, have been detected in various Indian fermented fish products (**Keisam *et al*., 2019**). Some species, notably *Clostridium botulinum*, are highly pathogenic, which underscores the critical need to minimize their presence in these foods.

In addition, pathogenic species of *Bacillus* and *Staphylococcus*, such as *B. cereus* and *S. aureus* have occasionally been reported in fermented fish products. Their

ability to tolerate and proliferate in high-salt environments (above 7%) likely contributes to their persistence in these products (**Belleggia and Osimani, 2023**).

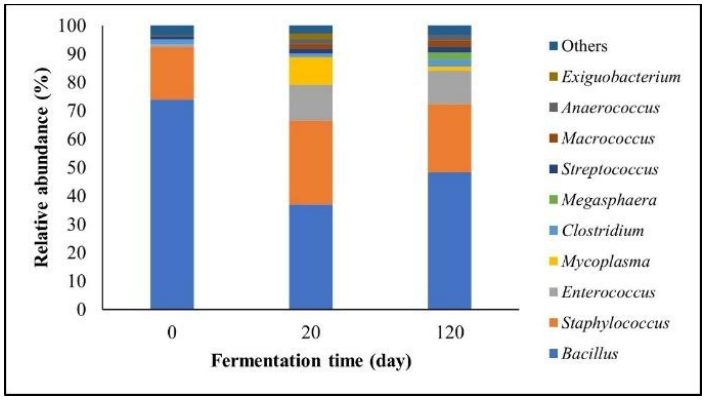

Figure 2 Bacterial community in skipjack tuna viscera during fermentation

Physicochemical and microbial interactions during fermentation of skipjack tuna viscera

This study utilized tuna viscera which is an ideal, nutrient-rich substrate for fermentation due to its high concentration of endogenous proteolytic enzymes, which can facilitate rapid, low-cost hydrolysis. Fish fermentation is fueled by a feedback loop where salt, pH and water activity shape microbial succession, while microbial enzymes constantly alter the chemical environment in the substrate to produce flavor, texture, and preservation. This interaction is presented in Figure 3. Salt addition lowers water activity and selectively inhibits spoilage and pathogenic bacteria, allowing halotolerant and lactic acid bacteria to dominate in the fermentation medium. Endogenous fish enzymes and microbial proteases hydrolyze muscle proteins into peptides and free amino acids, which serve as substrates for fermentative microbes and contribute to umami flavor. Lactic acid bacteria and halophilic bacteria produce organic acids, causing a pH decrease that further suppresses undesirable microbes and stabilizes the fermented product. As fermentation progresses, microbial succession adapts to increasing salt and lower pH leading to product maturation. Overall, physicochemical factors regulate microbial growth, while microbial metabolism continuously modifies the chemical environment of the substrate, determining flavor, texture, color, safety and shelf life of the fermented viscera.

Scaling and quality standardization can be considered in *dayok* production, but this involves a transition from traditional, variable practices to strictly controlled commercial protocols to ensure safety and consistent sensory profiles of the product. Scaling up necessitates accelerated fermentation using starter cultures, exogenous enzymes, or novel processing technologies to achieve rapid and consistent turnover (**Li *et al.*, 2024**). Moreover, key implications of quality standardization in *dayok* production include improved food safety and risk reduction, consistency in sensory attributes, process optimization and market expansion (**Belleggia and Osimani, 2023**).

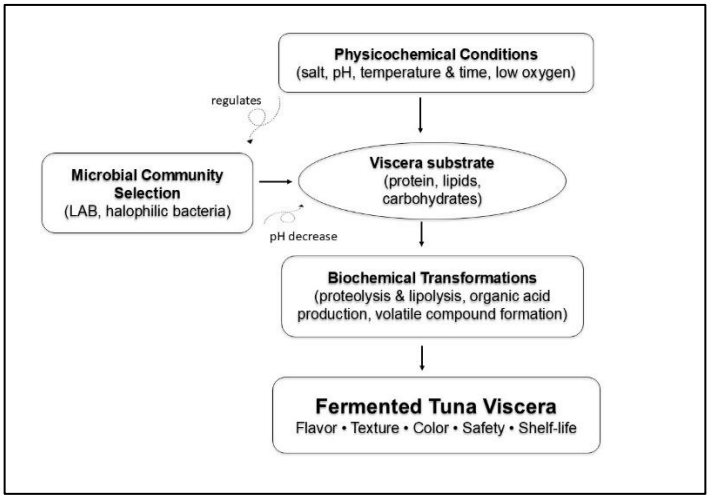

Figure 3 Summary of the physicochemical and microbial interactions during fermentation of skipjack tuna viscera

CONCLUSION

Skipjack tuna viscera is a potential raw material for fish paste and fish sauce production due to its high protein and low lipid content. The physicochemical properties of the salt-fermented skipjack tuna viscera changed significantly over the 150-day fermentation period. Three important physicochemical properties of fish-based fermented food were observed during the fermentation process: a consistent decrease in pH along with a corresponding increase in titratable acidity facilitated the production of lactic acid by LAB, and salt content which remained relatively stable throughout the fermentation period acting as a preservative agent. Moreover, microbial population analysis revealed a decline in the total viable count and an increase in lactic acid bacteria during the early stages of fermentation, suggesting microbial succession. The bacterial genera *Bacillus*, *Staphylococcus*, and *Enterococcus* were the dominant bacterial species during the fermentation of tuna viscera, contributing to the enhancement of flavor and odor throughout the fermentation process. These findings align with previous studies on fermented fish, highlighting the complex relationship of factors influencing the fermented product characteristics. These variations can impact the flavor, texture, color and safety of fermented fish products. Further research is necessary to describe the specific mechanisms underlying these changes and to optimize the fermentation process for the desired product attributes of *dayok* from tuna viscera.

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