Physicochemical and microbiological attributes of dried anchovies (*Stolephorus commersonnii*) in the formation of histamine along the supply chain

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Abstract

Food safety along the supply chain must be safeguarded to protect the public's health from any foodborne disease. Despite the consumption of fish products, scombroid poisoning is not a well-known occurrence. This study was formulated to evaluate at which stage of the supply chain the increase in the histamine content of dried anchovies (*Stolephorus commersonnii*) occurs. The study showed that histamine substances and *Staphylococcus aureus* were detected at all stages of the supply chain. It was also found that the concentration of histamine generally increased from the fisherman to the processors and retailers. Post-hoc analysis using the Fisher test showed no significant difference in the histamine content of dried anchovies from the processing centers ($\bar{x}$=5 4.07 mg/kg) and retailers ($\bar{x}$= 67.63 mg/kg), but it was significantly higher than in the raw anchovies ($\bar{x}$= 8.29 mg/kg). *S. aureus* remains low at <10 colony-forming units (CFU/g). The aerobic plate count supports this conclusion with a 90.29% increase from the processing centers to the retailers. However, it is still significant as a source of histamine formation influenced by water activity and salt content. A correlation between salt content and water activity with histamine content was identified with a Pearson correlation coefficient of 0.8357. It is recommended to review the processing method to control the salt content and the handling and storage at the retailers, as they showed the highest potential for histamine-forming bacteria growth, leading to an increase in histamine levels in dried anchovies.

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Introduction

The supply chain is the network that the business develops with its suppliers and customers. Many micro, small, and medium enterprises have developed their own supply chains, contexts, and strategies. In the province of Samar, Philippines, the supply chain of dried fish products begins with the fishermen, who supply raw fish to processors. The processors then sell their products to the middlemen (buyers) and trade their products to the retailers, then to the consumers as end-users, as shown in Figure 1.

Consumer safety is of critical importance and fundamental to any stage in the food supply chain, regardless of the product category. Given the supply-chain complexities and the risks involved, the importance of food safety standards cannot be overstated. Ensuring food products are free from any contamination that causes foodborne disease must be safeguarded for healthy and high-quality food for consumers. Histamine fish poisoning, also known as scombroid poisoning, is the most common foodborne intoxication caused by the consumption of some species of marine fish containing high levels of histamine and other biogenic amines (Hungerford, 2010). These fish have high levels of free histidine in their tissue, which is converted into histamine by histamine-producing bacteria like *Staphylococcus aureus*, *Enterobacter* spp., and *Proteus* spp. (Huang *et al*., 2010).

In the Philippines, the Bureau of Food and Drug-Philippine National Standard established a regulatory limit for dried fish products at 200.0 mg/kg for histamine content, 1000 CFU/g for *S. aureus* and less than 500,000 CFU/g for aerobic plate count (APC). This regulation aligns with the provisions of Republic Act 10611, also known as the Food Safety Act of 2013, which serves to bolster

the country's food safety regulatory framework. Scombroid toxicity closely resembles the physiological processes associated with histamine release, resulting in symptoms like facial and neck flushing, diarrhea, urticarial rash, and headaches. In some cases, individuals with underlying medical conditions like asthma or significant heart problems may experience rare, severe bronchospasms or cardiac effects (Ferran and Yébenes, 2006). The symptoms affecting the gastrointestinal system are due to the contraction of smooth muscles, leading to abdominal cramps, diarrhea, nausea, and vomiting. The toxicity of histamine ingestion can rarely have severe bronchospasm or cardiac effects, usually in patients with predisposing medical conditions such as asthma or significant cardiac disease (Wilson *et al*., 2012). Without immediate and proper treatment, histamine poisoning is alleviated within 12 to 48 hours with no long-term sequelae (Harmelin *et al*., 2018).

Simora and Peralta (2018) also investigated the formation of histamine-forming bacteria (HFB) in dried-salted fish products sold at retail markets in the province of Iloilo (Philippines). *S. aureus* has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals than any other HFB (Tallent *et al.*, 2016). Thus, the presence of this HFB in processed foods or on food processing equipment is generally an indication of poor sanitation and handling. *S. aureus* causes very similar symptoms, with sudden onset nausea, vomiting, and abdominal pain within 2 hours after eating contaminated food. These patients, however, may develop a fever, while scombroid patients do not. Also, patients with scombroid typically have flushing or a rash, while *Staphylococcus* food poisoning does not (Traylor and Mathew, 2023). In the study conducted by Amascual *et al*. (2020), the formation of histamine in dried salted fish was detected in different local retailers in Samar and exceeded the regulatory limit of 200 mg/kg. The species with the highest concentration of histamine is *Stolephorus commersonnii*, compared with other varieties of dried fish such as *Rastrelliger commersonnii, Selar boops*, and *Sardinella* spp.

In the salting and drying processes, salt is used as a preservative because it reduces the water activity (A_w) of foods and extends their shelf life. The A_w of food is the amount of unbound water available for microbial growth and chemical reactions (Fennema, 1996). It has also been suggested that for some microorganisms, salt may limit oxygen solubility, interfere with cellular enzymes, or force cells to expend energy to exclude sodium ions from the cell, all of which can reduce the rate of growth (Shelef and Seiter, 2005).

This study aimed to investigate and correlate the HFB such as *S. aureus*, APC population, and level of histamine to physicochemical characteristics such as salt content and A_w at different stages in the supply chain.

Materials and Methods

Sample collection

A total of 18 batches of samples were obtained by random sampling from different stages in the supply chain (6 batches each from the fishermen, processors, and retailers). Each batch of samples is equivalent to 1 kg as a minimum requirement by the Bureau of Fisheries and Aquatic Resources. Samples were collected from six fishermen and dried anchovies from six processors and retailers, respectively.

Sample preparation

From each sample, 5 g were chopped into small pieces, followed by homogenization by adding 50 mL of analytical-grade methanol [(CH_3OH) Sigma, St. Louis, Missouri, USA] for 2 minutes. The sample was placed in a 100-mL volumetric flask, and the homogenizer was rinsed thoroughly with methanol. The rinse-off was added to the flask. The flask containing the homogenized samples was placed in a hot water bath at a temperature of 60°C for 15 minutes. The samples were allowed to cool to 25°C and diluted with methanol. The sample solution was shaken and filtered using Whatman filter no. 1 (Whatman, Maidstone, UK), and the filtrate was received into a new container and covered.

Histamine extraction and analysis

Histamines were extracted from the sample using methanol as a solvent, interfering with compounds such as histidine and other polyamines. Histamines were purified by the elution of sample filtrates. The column bed of resin was eluted with 4-5 mL of distilled water, and we discarded the eluate. The 50-mL volumetric flask containing 5.0 mL of 1.0 N hydrochloric acid [(HCl) Sigma, St. Louis, Missouri, USA] was placed at the column outlet. About 1.0 mL of the filtrate sample was pipetted onto the column and added to 4-5 mL of water. Eluates were collected using the 50 mL volumetric flask containing 5.0 mL of 1.0N HCl. The flowrate was maintained at approximately 1-2 drops using a prescribed column (Contess, Thermo Fisher Scientific, Waltham, USA). When the liquid level was approximately 2 mm above the resin, another 5.0 mL of water was added and allowed to flow through the resin. The addition of water was repeated by gradually increasing the volume increments until the eluate reached about 40 mL in the volumetric flasks and 50 mL with water.

In fluorophore formation, in separate 50-mL Erlenmeyer flasks, 5.0 mL of blank eluate were pipetted, each working as standard (totaling 4 of each sample eluate). 10 mL of 0.1N HCl and 3.0 mL of 1.0N sodium hydroxide (Sigma, St. Louis, Missouri, USA) were added to each flask and thoroughly mixed. After 5 minutes, the solution was added to the O-phthaldehyde (Sigma, St. Louis, Missouri, USA) reagent. After 4 minutes, 3.0 mL of 3.57N phos-

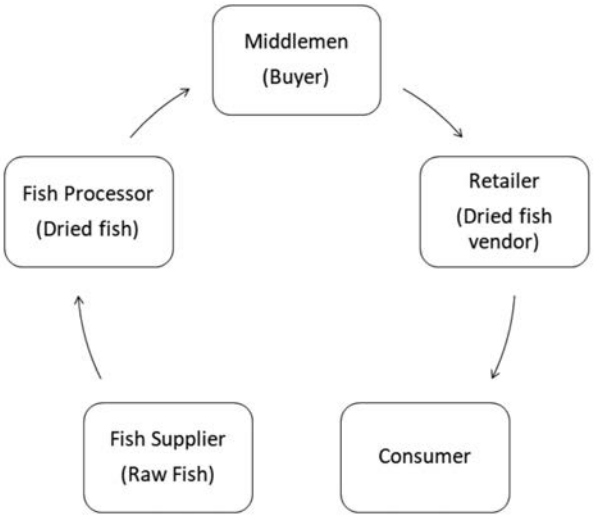

Figure 1. The stages of the supply chain of dried-salted fish products.

phoric acid (Sigma, St. Louis, Missouri, USA) were added and mixed immediately. After letting it stand for 15-20 minutes, the formation of fluorophore occurred in the samples. For blank and control samples, 1.0 mL of CH_3OH was passed through the column resin bed, and the eluate was collected as though it were a fish extract sample. Passed 1.0 mL of 10.0 µg/mL histamine [($C_5H_9N_3$.2HCl) Sigma, St. Louis, Missouri, USA] control sample solution through the same column resin bed after every elution of a blank sample and at every end of a set of eluate samples. The resins in the column were added to 10 mL of water before every elution of another filtrate sample. The fluorescence of fluorophores present in the sample was recorded by measuring the fluorescence intensity after 1.5 hours at an excitation wavelength of 450 nm using the Jenway model 6280 fluorimeter (Keison, Chelmsford, UK) and by linear equation/regression calculation. The sensitivity or gain setting on the fluorimeter, which will give approximately 80.0% full-scale reading with 0.3 µg/mL $C_5H_9N_3$.2HCl standards, was used.

Determination of pH value, salt content, water content, and water activity

Dried anchovy samples (10 g) were homogenized in sterile stainless blenders with 10 mL of distilled water to make a thick slurry and placed in a 50-mL glass tube. The pH of this slurry was measured using a Corning 145 pH meter probe (Corning Glass Works, Medfield, MA). Likewise, the salt content in each sample was determined following Bureau of Fisheries and Aquatic Resources-Association of Analytical Chemists procedures (Bureau of Fisheries and Aquatic Resources, 2001). 3 g of dried anchovies were homogenized with 18 mL of distilled water and then titrated with 0.1M silver nitrate ($AgNO_3$) using 10% w/v potassium chromate solution as the indicator. The water content was analyzed by the standard gravimetric method by drying 3 g of the test sample at 102.0°C±2.0°C under atmospheric pressure for 2 hours. The consistency of the mass was tested by additional 1-hour drying steps until the difference in the mass did not exceed 0.5 mg. The percentage of salt (NaCl) in the samples was calculated using Equation 1:

$$\text{Percent NaCl} = \frac{25.0\ \text{mL} - (\text{mL KSCN})(R)](N\ AgNO_3)(5.85)}{\text{sample weight}} \quad \text{[Eq.1]}$$

where R is the ratio of volume in mL of $AgNO_3$ over potassium thiocyanate (KSCN)

A_w was determined at 27°C maximum using an electric hygrometer (Hygrodynamics, Inc., Silver Spring, Maryland, USA). Water activity is equal to equilibrium relative humidity (ERH) divided by 100 (A_w=ERH/100).

Microbial analysis of the aerobic plate count and isolation of *Staphylococcus aureus* as histamine-forming bacteria

For APC, a 25 g portion of the dried anchovies sample was homogenized at 2,000 rpm for 2 minutes in a sterile blender with 225 mL of sterile potassium phosphate buffer (0.05M, pH 7.0). Before use, the stainless blade of the blender was sterilized by autoclaving for 15 minutes at 121°C. The homogenates were serially diluted up to the 8th dilution with a sterile phosphate buffer (1:9), and 1.0 mL aliquots of the dilute were poured onto Petri dishes (9 cm diameter). The highest dilution rate applied was 1/12.8. 15 to 20 milliliters of AP Cagar (Difco, Detroit, Michigan, USA), which contained 0.5% NaCl, were added and gently mixed at 45-50°C. The poured plates were allowed to solidify under a biologically clean bench. Bacterial colonies were counted after the plates were incubated at 35°C for 48 hours. Bacterial numbers in the samples were expressed as CFU/g.

Direct plate counts using coagulase tests were used to isolate strains of *S. aureus*. This method was used for the analysis of foods with more than 100 *S. aureus* CFU/g. For each dilution plated, aseptically 1 mL of sample suspension was transferred to 3 plates of Baird-Parker agar (Neogen, Ipswich, Australia), distributing 1 mL of inoculum equitably to the 3 plates. The inoculum was spread over the surface of the agar plate using a sterile bent glass streaking rod. The retained plates were placed in an upright position until the inoculum was absorbed by the agar (about 10 minutes on properly dried plates). The plates were inverted and incubated for 45-48 hours at 35-37°C. The suspected *S. aureus* colonies were placed into small tubes containing 0.2-0.3 mL of brain heart infusion (BHI) broth (Sigma, St. Louis, Missouri, USA) and emulsified thoroughly. Inoculate an agar slant containing an appropriate maintenance medium using tryptone yeast extract agar (Sigma, St. Louis, Missouri, USA) by transferring a loopful of BHI suspension. Then the BHI culture suspension and slants were incubated for 24 hours at 37°C. 0.5 mL of reconstituted coagulase plasma was added with ethylenediaminetetraacetic acid (Sigma, St. Louis, Missouri, USA) to the BHI culture and mixed thoroughly. The culture was incubated at 35-37°C and examined periodically over 6 hours for clot formation. The complete clot that formed and stayed in place in a tilted tube was positive for the *S. aureus* strain.

Statistical analysis

A statistical representation of the data was conducted using means and standard deviations. While one-way analysis of variance (ANOVA) at 95% confidence level was used to determine significant differences in parameter values across different stages in the supply chain, Fisher's test was used for confirmatory post-hoc analysis. Also, a box plot was used to summarize a set of data measured on an interval scale. Linear regression and Pearson's correlation were used to develop a model to determine the relationships between the salt content, water content, and histamine content of the samples. All statistical analyses were conducted using Minitab® statistical software 2018 (Minitab®, State College, Pennsylvania, USA).

Results and Discussion

Physicochemical characteristics and histamine content of dried-salted anchovies

Minitab® statistical software 2018 (Minitab®, State College, Pennsylvania, USA) was used to analyze and interpret the data. The range values of A_w, salt content, and histamine content of the dried anchovies from fishermen, processors, and retailers are shown in Table 1. The histamine content of raw anchovies was found to be 8.29±3.11 mg/kg, while the dried anchovies samples were found to be 54.07±23.98 mg/kg and 67.63±18.63 mg/kg from the processors and retailers, respectively, as presented in Figures 2-4. The comparative analysis of means using one-way ANOVA showed that there was a significant difference between the means of the histamine content ($p<0.001$). Post-hoc analysis using the Fisher test showed that while there is no significant difference in

the histamine content of dried anchovies from the processing centers ($\bar{x}$=54.07 mg/kg) and retailers ($\bar{x}$=67.63 mg/kg), these means are significantly higher than that of the raw anchovies ($\bar{x}$=8.29 mg/kg). The same results can be said about the A_w of the samples. One-way ANOVA showed that there is a significant difference between the means of A_w ($p<0.001$). The Fisher test also showed that, for raw anchovies, the A_w ($\bar{x}$=0.94) is significantly higher than that for the samples from the processing centers ($\bar{x}$=0.59) and retailers ($\bar{x}$=0.60). However, this is expected as the anchovies in the processing centers and retailers have undergone drying, which reduces A_w. There was also a significant difference between the means of salt content ($p<0.001$). The Fisher test revealed that the salt content in the processed anchovies ($\bar{x}$=9.89%) was higher than in the raw ones ($\bar{x}$=0.12%) and dried anchovies from the processing center ($\bar{x}$=1.71%). Though the A_w remains similar for the dried fish in the processing centers and retailers, the salt content from the retailers decreased from 9.89 mg/kg to 1.71 mg/kg. The reduction of salt content occurs when the salt crystals on the surface of dried fish dissolve or fall off during transportation, handling, or packaging, contributing to a perceived reduction in salt concentration. Reduction of salt content will lead to rapid growth of HFB such as *S. aureus* and other foodborne pathogens (D'Sa and Harrison, 2005). Salt causes microbial cells to undergo osmotic shock, resulting in the loss of water from the cell and thereby causing cell death or retarded growth.

Microbial analysis of dried-salted anchovies

The microbial content of raw and dried salted anchovies was analyzed through the APC and *S. aureus*. The results are summarized in Table 1. The mean APC in raw fish was found to be 420.000±56.569 CFU/g while it is 5.150±1.344 CFU/g at the processing centers and 9.800±10.182 at the retailers. However, the total count of *S. aureus* in all the samples remains low at <10 CFU/g.

The comparative analysis of means of APC using one-way ANOVA showed a significant difference (p=0.002). The Fisher test showed that the APC in raw anchovies is significantly higher compared to the dried anchovies in the processing centers and retailers. Although there is no significant difference in the dried anchovies in the processing centers and retailers, there is a notable increase of 90.29% in the two areas. The results of HFB found in the supply chain were supported by the study of Simora *et al.* (2016), in which the levels of histamine and other physicochemical parameters of dried-salted sardines (*Sardinella* spp.) along the supply chain showed that 66.7% of the samples exceeded the maximum allowable histamine level of 200 mg/kg for dried-salted fish set by the Bureau of Food and Drug.

In the study conducted by Feldhusen (2000), at least 10 genera of bacterial pathogens have been implicated in seafood-borne diseases. Kung *et al*. (2010) conducted another study on other varieties of dried fish: they isolated prolific HFB such as *Enterobacter*, *Klebsiella*, *Raoultella*, and *Citrobacter* spp. from sailfish fillets, dried milkfish, tuna dumplings, and tuna sandwiches in Taiwan.

Like many other HFBs, *S. aureus* has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals and family groups than any other HFB (Tallent *et al*., 2016). Among the 3 dried fish products, dried anchovies had the highest average *S. aureus* count of 5.03 CFU/g and a significantly higher APC of 6.37 CFU/g.

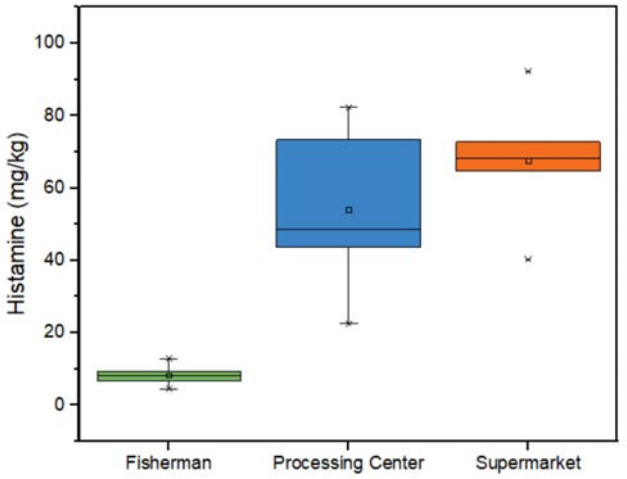

Figure 2. Box plot of the histamine content of raw and dried anchovies from different sources.

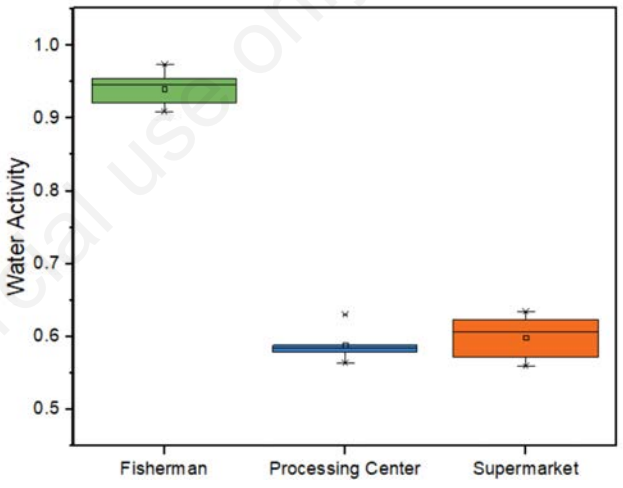

Figure 3. Box plot of the water activity of raw and dried anchovies from different sources.

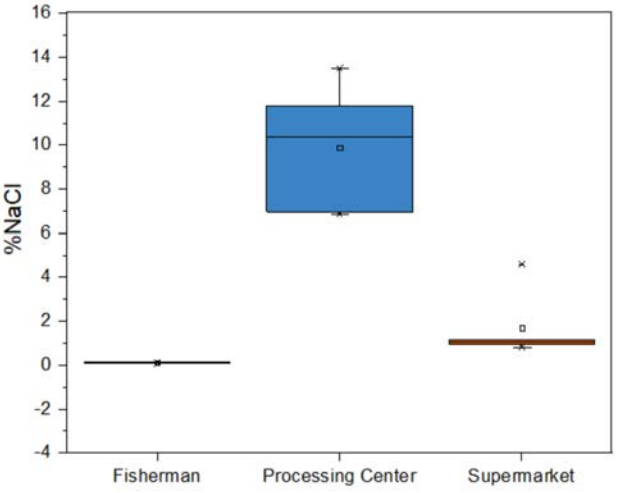

Figure 4. Box plot of the salt content of raw and dried anchovies from different sources. NaCl, salt.

Table 1. Microbial count of dried-salted anchovies from different sources in Samar.

Source	Statistics	AP CFU/g	*Staphylococcus aureus* CFU/g
Fisherman (Raw)	Mean±SD Range	420.000±56.569 380.000-460.000	<10
Processing (Dried)	Mean±SD Range	5.150±1.344 4.200-6.100	<10
Retailer (Dried)	Mean±SD Range	9.800±10.182 2.600-17.000	<10

APC, aerobic plate count.

Correlation of factors for histamine content in dried anchovies

The physicochemical properties of dried anchovies were correlated with the histamine content using linear regression. The results showed that A_w (p=0.000) and salt content (p=0.004) are significant predictors of it. The generalized linear model was calculated using Equation 2:

$$\ln(\text{histamine content}) = 7.753 - 6.012\ A_w - 0.0281\ \text{NaCl} \quad \text{[Eq.2]}$$

The Pearson correlation coefficient resulted in a value of r^2=0.8357 which means that there is a relatively strong correlation of histamine content between the A_w and salt concentration.

Conclusions

Histamine substances and *S. aureus* were detected at all stages along the supply chain. Although histamine content and the presence of *S. aureus* are below PNS/BAFPS 68:2008 (Philippine National Standard, 2008), there is an increase of 25.0% in histamine concentration from the fishermen to the processing centers and retailers. Results also showed that *S. aureus* remains low at <10 CFU/g and that the APC is below the regulatory limit of 500,000 CFU/g. However, it is still a significant source of histamine formation, with the influence of A_w and salt content in the process. Since sodium salts play a role in reducing the growth of HFB and other foodborne pathogens that spoil products and reduce their shelf life, it is recommended to maintain the optimum salt content requirements in processing centers and retail outlets to maintain quality and food safety and extend products' shelf life. Other potential sources that trigger the formation of histamine are decomposing organic matter, such as spoiled fish, which provides a ready source of nutrients for the bacteria to grow, the quality of raw fish, handling and storage, and unhygienic practices in all stages of the supply chain.

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