Microbial and Amino Acid Changes in Pre- and Post- Fermentation Shrimp Paste from Cirebon, West Java

Nadhira P. Diani[1], Radestya Triwibowo[2]*, Rusky I. Pratama[1], and Novalia Rachmawati[2]

ABSTRACT

This study investigates the changes in microbial communities and amino acid profiles in shrimp paste (*terasi*) from Cirebon, West Java, before and after fermentation. Samples were collected from two traditional processors, representing different stages of shrimp paste processing. The microbial diversity was analyzed using 16S rRNA gene sequencing, revealing significant shifts in bacterial populations during fermentation. Processor A's shrimp paste showed a dominance of *Bacilli*, with *Staphylococcus* becoming more prevalent post-fermentation, while shrimp paste from processor B exhibited a more diverse microbial community, including *Empedobacter* and *Acinetobacter*. The study identified 60 and 64 genera in Processor A's samples before and after fermentation, respectively, and 26 and 41 genera in Processor B's samples. *Vibrio*, initially present in the raw material, was eliminated post-fermentation. Amino acid analysis indicated an increase in key amino acids such as glutamic acid and leucine post-fermentation, contributing to the enhanced flavor profile of the shrimp paste. Processor A's shrimp paste showed significant increases in amino acids, with glutamic acid reaching 64,249 mg/kg, Processor B's paste had a more moderate increase, with glutamic acid at 10,714 mg/kg post-fermentation. These findings highlight the significant influence of fermentation conditions on the microbial and biochemical composition of shrimp paste, emphasizing the importance of standardized processing methods to ensure consistent product quality and safety.

Keywords: *Acetes* sp., spontaneous fermentation, traditional, umami

[1] Fisheries Department, Faculty of Fishery and Marine Science, Padjadjaran University, Jl. Raya Bandung-Sumedang KM.21 Jatinangor, West Java, 45363, Indonesia

[2] Research Center for Veterinary Science, Research Organization for Health, National Research and Innovation Agency, Jl. Raya Jakarta-Bogor KM 46, Cibinong, Jawa Barat, 16911, Indonesia

*Corresponding Author:
radestya.triwibowo@gmail.com

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Introduction

Shrimp paste, known as *terasi* in Indonesia, is classified as a traditional condiment derived from fermentation processes utilizing shrimp or mixed shrimp-fish as primary ingredients, widely used to enhance the flavor of various traditional dishes such as chili sauce (known as *sambal*) (Murwani et al., 2015). According to the Directorate General of Strengthening the Competitiveness of Marine and Fishery Products, Indonesia exports several types of fermented seafood, including fish sauce, fish paste, and shrimp paste. In 2020, the export volume of shrimp paste reached 3,523 tons, reflecting an 88% increase (1,656 tons) compared to 2019 (Directorate General of Marine and Fisheries Product Competitiveness, 2022).

The production of *terasi* involves a fermentation process that varies in duration and conditions, significantly influencing its flavor and quality. Typically, this fermentation occurs spontaneously, without using a bacterial starter (inoculum) or controlled environmental conditions (Pongsetkul et al., 2014; Prihanto et al., 2021). The fermentation period generally ranges from several days to several months, although a period of 60 to 90 days is generally preferred to achieve a more robust flavor profile (Murwani et al., 2015; Prihanto et al., 2021). *Terasi* is distinguished by its complex aroma, derived from volatile compounds such as nitrogen-containing compounds, aldehydes, and esters, which are released when cooked (Surya et al., 2023). The distinct flavors and aromas of *terasi* vary by region in Indonesia, influenced by local raw materials and processing techniques. This regional variation contributes to the unique sensory qualities of *terasi*, making it a distinctive ingredient in Indonesian cuisine (Karim et al., 2014; Surya et al., 2024).

A diverse microbial community can enhance the fermentation process through microbial succession, which refers to the sequential changes in microbial populations during fermentation. These changes impact the final characteristics of the fish paste, as different microbial species contribute to unique sensory properties, such as flavor, aroma, and texture. Some types of bacteria, including *Lactobacillus*, *Lentibacillus*, *Tetragenoccocus*, and *Streptococcus,* may be responsible for the production of lactic acid, volatile compounds, free amino acids, and biogenic amines during fermentation (Che et al., 2021; Helmi et al., 2022; Ma et al., 2022). Furthermore, various factors influence the initial microbial diversity of pre-fermentation, including the type of raw materials, fermentation conditions, environmental parameters, and storage conditions. These may also result in discrepancies in the composition of the microbial community during fermentation. Incorporating starter cultures, defined as specific microbial inoculants, can also affect this diversity and the consequent flavor profile of the shrimp paste. A well-regulated microbial diversity not only enhances the sensory qualities of fermented fish paste but also guarantees a more stable fermentation process, avoiding pathogenic microbial contamination, thus producing a better quality and safer product (Li et al., 2023; Pongsetkul et al., 2023; Yu, Lu, Dong, et al., 2022; Yu, Lu, Zi, et al., 2022).

Considering that *terasi* fermentation occurs spontaneously and is also influenced by the environmental conditions during production, it is necessary to characterize the microbiome composition of the product to understand how bacterial diversity correlates with its distinctive flavors. This study aimed to investigate changes in microbial and amino acid composition before and after fermentation of shrimp paste from Cirebon, West Java.

Materials and Methods

1. Sample collection

Shrimp paste was collected from two traditional processors in Cirebon, West Java. Processing conditions of shrimp paste were recorded. This case study selected the two most representative processors based on production capacity, production methods, raw materials, and product types. These processors represent two main shrimp paste-producing villages in Cirebon (Jatimerta and Panjunan) that were actively operating/producing during the study. The samples represented two stages of shrimp paste processing: before and after fermentation. Water activity (a_w) and pH of each sample were also measured.

2. Bacterial community analysis

DNA of PAB was extracted from shrimp paste using DNAzol (Chomczynski et al., 1997). A total of 25-50 mg of shrimp paste was homogenized with 1 ml of DNAzol and centrifuged at 7,500 x*g* for 10 minutes at 4°C. The supernatant was then transferred to a new tube, and 500 µl of 100% ethanol was added and gently mixed by inverting the tube several times. DNA was precipitated by centrifugation at 4,000 x*g* for 1–2 minutes at 4°C. After discarding the supernatant, the resulting pellet was washed with 1 ml of 75% ethanol. The DNA pellet was suspended by gently inverting the tube. The ethanol was evaporated using a vacuum evaporator, and the DNA extract was finally redissolved in TE buffer. DNA extract was stored at - 20°C. Genomic quantification and DNA quality were analyzed using Nanodrop IMPLEN.

gDNA samples were amplified using primer pair 341-F (5'-CCT AYG GGR BGC ASC AG-3') and 806-R (5'-GGA CTA CNN GGG TAT CTA AT-3'), specifically targeting the V3-V4 region of 16S rRNA with a fragment length of approximately 470 bp. The PCR reaction was prepared using Phusion™ Plus PCR Master Mix (F631S) following the manufacturer's protocol. Each 20 µl of PCR mixture consists of 10 µl of 2X Phusion™ Plus PCR Master Mix, 1 µl of each primer with a final concentration of 0,5 µM, 2 µl of DNA template and free-nuclease water. PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s; and final elongation at 72°C for 5 min (Liu et al., 2019). DNA quantity and quality were analyzed using the Qubit dsDNA HS Assay Kit, and the PCR product was visualized with gel electrophoresis in 1% TBE agarose.

The bacterial community in shrimp paste was analyzed using the Illumina Mi-Seq Sequencing v3 (300 PE). Library preparation was carried out using the final PCR products, and the resulting library was sequenced on the Illumina platform to generate paired-end raw reads. Adapter and PCR primer sequences were trimmed from the paired-end reads using Cutadapt (Bellemain et al., 2010). DADA2 was employed to correct sequencing errors, filter out low-quality sequences, and remove chimeras (Martin, 2011). The resulting ASVs were used for taxonomic classification with the SILVA (silva_nr99_v138.1) 16S database. Further analysis and visualizations were conducted using various packages in RStudio (R version 4.2.3) (https://www.R-project.org/), along with

Krona Tools (https://github.com/marbl/Krona) and PICRUSt2 (https://github.com/picrust/picrust2).

3. Amino acid analysis

Approximately 0.1 - 1 g of sample was weighed into the microwave digestion vessel, and 10 mL of 6N hydrochloric acid was added for acid hydrolysis. The supernatant obtained was re-constituted with the aqueous solution to adjust it to a neutral pH value. The internal standard was added, and the sample was filtered by a 0.22 µm hydrophilic PTFE filter. Derivatization was performed using AccQ-Taq Ultra reagent. Waters Acquity UPLC with photodiode array (PDA) was used for analysis (Table 1).

Table 1. UPLC conditions for amino acid analysis

Parameter	Condition
UPLC System	Waters Acquity UPLC
Column	C18
Column temperature	49^{o}C
Mobile phase	Eluen Accq-Tag Ultra; ddH_2O
Injection mode	Gradient
Detector	PDA

Results and Discussions

1. Shrimp paste processing conditions

Cirebon is recognized as a traditional shrimp paste (*terasi*) production center in West Java, with processors spread across various regions, such as Sisingamangaraja, Kanci, and Jatimerta villages. *Terasi* is produced using spontaneous fermentation, which relies on the natural microbiota in *rebon* shrimp (*Acetes* sp.) as raw material, the added ingredients, and environmental conditions. Consequently, the quality and safety of the final product can be inconsistent.

The traditional process of making *terasi* in Cirebon generally involves sun-drying, pounding and fermentation, with the frequency of these steps depending on seasonal conditions, and performed up to three times if necessary. *Rebon* shrimp were obtained from the local capture of small fishermen, and fresh or dried *rebon* was used for *terasi* production. Additional ingredients, including palm sugar, salt, and simple seasonings, are incorporated according to processor preferences without artificial preservatives. Processor A added 350 grams of salt per kilogram of dried shrimp and 5% boiled palm sugar after the initial mixing. Processor B used less salt, adding only 150 grams per kilogram of raw shrimp, and did not include any sugar in the mixture. Fermentation begins immediately after the shrimp paste mixture is pounded and stored in sacks, where it continues to ferment and mature over time. The resulting shrimp paste is ready for sale or distribution, while the remaining stock is kept in storage for up to one or two years to enhance its flavor and quality further.

Table 2 presents the water activity (a_w) and pH values of shrimp paste. The pH of salted shrimp pastes typically ranged from 6.6 to 7.7, with a general trend of decreasing pH during the fermentation process (Jung et al., 2013; S. H. Lee et al., 2014; Se Hee Lee et al., 2014; Li et al., 2021; Lim et al., 2023). The pH of shrimp paste is influenced by the type of raw materials used (Lim et al., 2023). In this study, shrimp paste produced by Processor A consistently exhibited lower pH and a_w values than Processor B's. This variation is likely due to differences in raw material: Processor A uses dried shrimp, while Processor B utilizes fresh *rebon* shrimp. The higher a_w associated with fresh shrimp supports greater enzymatic and microbial activity, which can lead to the formation of alkaline compounds during fermentation, thereby influencing the pH.

Table 2. pH and a_w value of shrimp paste sample

Sample origin	Fermentation Process (sample code)	pH	a_w
Processor A	Before (N2)	5.93	0.7226
	After (N4)	5.99	0.7164
Processor B	Before (N8)	n.a.	n.a.
	After (N10)	7.44	0.8163

Note: n.a.: Not applicable due to the limited sample size; analysis was not conducted.

2. Microbial changes during fermentation of shrimp paste

The observed operational taxonomic units (OTUs) in this study ranged from 83 to 158 (Table 3). The species diversity was determined based on Shannon and Inverse Simpson indices. The sample from Processor B (N8 and N10) showed a higher value of these indices, showing a more diverse microbial community than the sample from Processor A (N2 and N4). Additionally, the fermented samples from Processor A and B showed greater microbial diversity than pre-fermentation samples.

Table 3. Diversity analysis of shrimp paste sample

Sample code	OTUs	Shannon index	Inverse Simpson index
N2	92	2.26	3.34
N4	83	2.86	8.87
N8	118	3.43	11.02
N10	158	3.68	17.14

For Processor A, 60 and 64 genera were identified from samples before and after fermentation. For Processor B, the number of genera identified was 26 and 41 for samples before and after fermentation, respectively. The relative abundance of each taxon is presented in Figure 1, while the shift in the microbial composition is presented in Figure 2. The most abundant bacterial class in shrimp paste from Processor A was Bacilli. The *Ceracibacillus* (65%) dominated the microbial community before fermentation, while *Staphylococcus* (58%) became dominant after fermentation. *Vibrio* comprised 17% of the total microflora in the raw material (before fermentation) and was eliminated in the fermented product. A similar finding is reported from other studies, where *Vibrio* is often dominant during the initial phase of shrimp fermentation but decreases as fermentation progresses (Helmi et al., 2022; Se Hee Lee et al., 2014). *Vibrio* spp. are significant bacterial pathogens in shrimp (Karunasagar et al., 2004), and their removal during food processing is recommended by reducing pH, adding salt at concentrations greater than 10%, and maintaining water activity below 0.94 (Codex Alimentarius, 2010).

After fermentation of shrimp paste from Processor A, several genera known for their involvement in fermentation become more abundant, including *Staphylococcus*, *Acinetobacter, Lentibacillus,* and *Virgibacillus*. *Staphylococcus* was reported in many traditional fermented fish products from Indonesia, including *bekasang*, *peda* and *jambal roti* (Huda, 2012). *Staphylococcus* was also found in a Chinese salt-fermented shrimp paste and was suggested to correlate with the formation of volatile compounds, such as aldehydes and acids (Li et al., 2021; Lv et al., 2020). *Acinetobacter* supported the fermentation of low-salt shrimp paste and correlates with physicochemical properties of the paste, including pH, amino acid, and TVB-N at 25°C (Yang et al., 2023). This genus was identified in some fermented products from Indonesia, such as *peda* and *wadi asin* (Huda, 2012). *Lentibacillus* was reported as the most abundant microorganism in Thai traditional shrimp paste after two months of fermentation and is believed to contribute to the good flavor quality of the product (Phewpan et al., 2020). Yu, Lu, Dong, et al. (2022) highlighted the potential use of *Virgibacillus* spp. and *Staphylococcus nepalensis* as starter cultures to improve the physicochemical, sensory, and taste properties of shrimp paste made from fresh *Acetes chinensis*. *Corynebacterium glutamicum* is well-known for its ability to produce amino acids and is frequently genetically modified for industrial-scale glutamic acid production (Sgobba et al., 2018). Numerous studies have demonstrated its capacity to enhance the flavor of fermented products and to shorten fermentation times, as seen in soy sauce production (Chen et al., 2024).

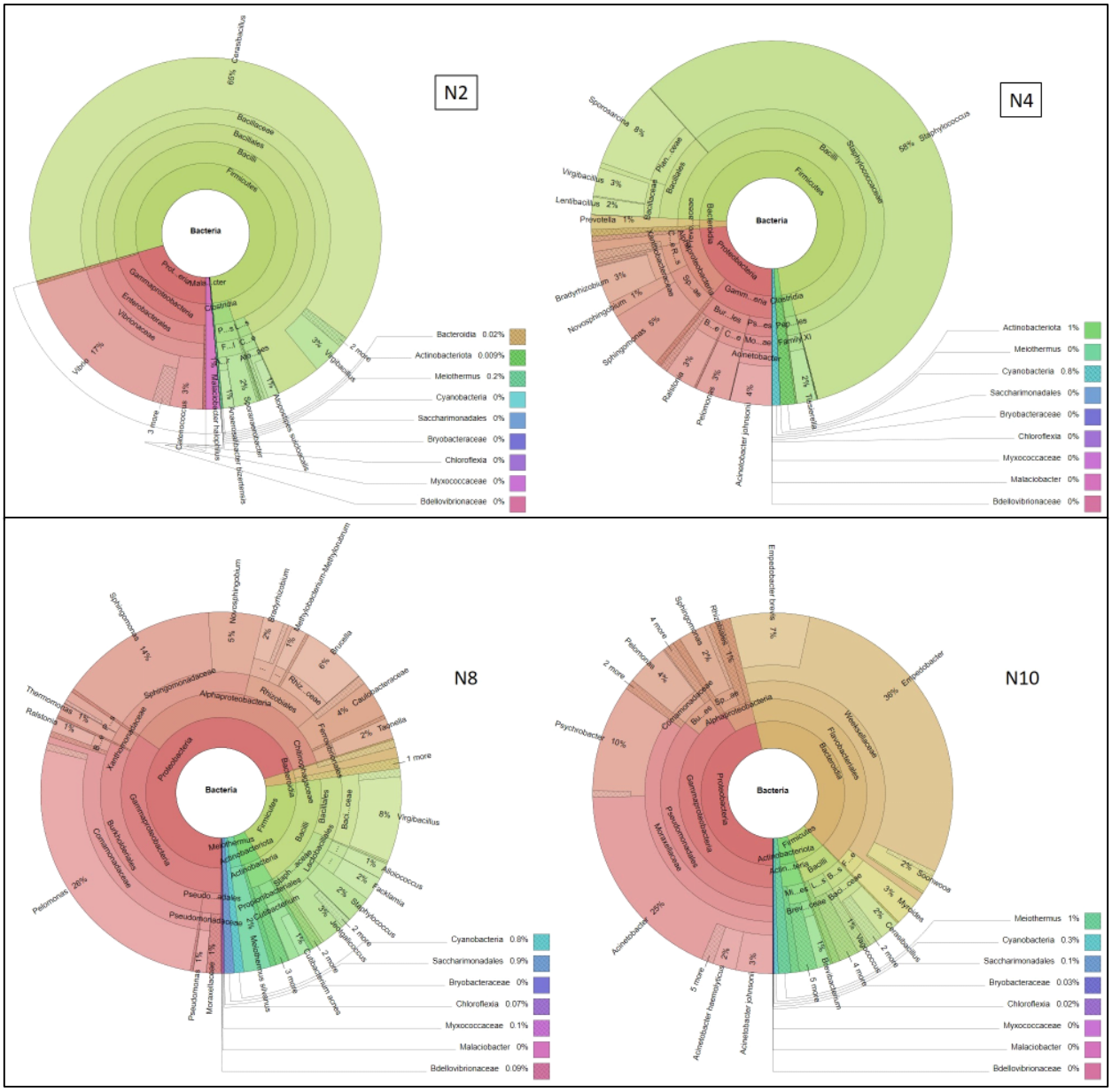

Figure 1. Krona diagram showing with taxonomic abundance and microbial diversity of shrimp paste.
Note: N2 and N4 represent pre- and post- fermentation samples from Processor A, N8 and N10 represent pre- and post- fermentation samples from Processor B

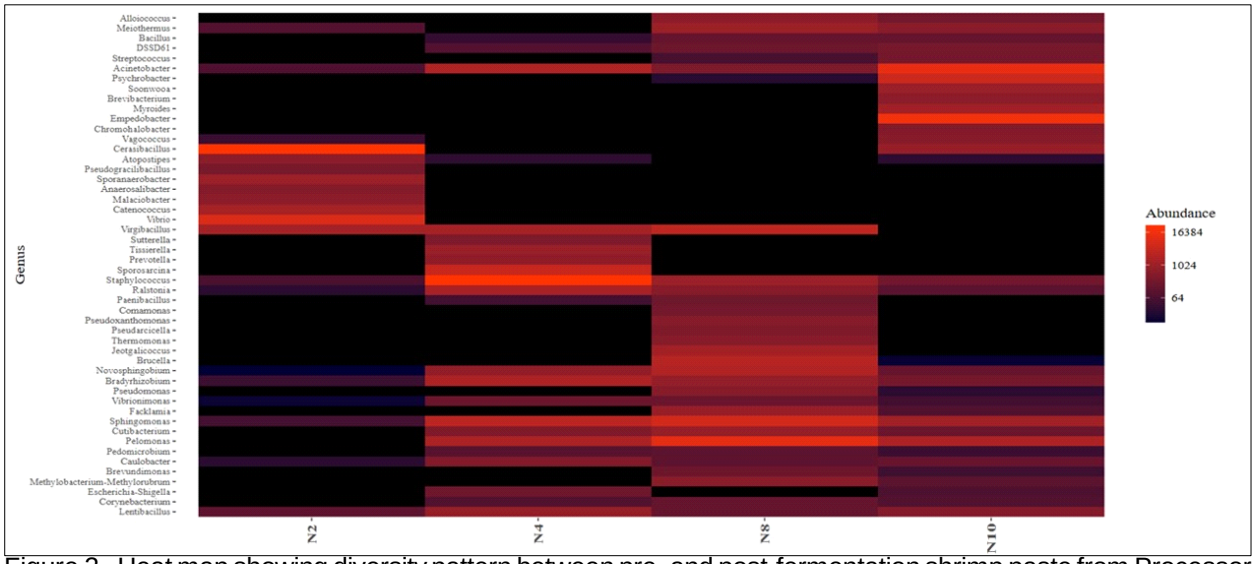

Figure 2. Heat map showing diversity pattern between pre- and post-fermentation shrimp paste from Processor A and B.

Note: N2 and N4 represent pre- and post- fermentation samples from Processor A, N8 and N10 represent pre- and post- fermentation samples from Processor B.

The pre-fermentation sample from Processor B was dominated by bacteria from Alphaproteobacteria and Gammaproteo bacteria classes, with *Pelomonas*, *Sphingomonas*, and *Brucella* comprising 26%, 14%, and 6% of the total microflora, respectively. In addition, *Virgibacillus*, *Jeotgalicoccus* and *Staphylococcus* were the dominant representatives from class Bacilli. The type of shrimp used as raw material likely affects the dominant microflora. For instance, a microbial community study of shrimp paste made from *Acetes japonicus* showed *Jeotgalicoccus* as a dominant microflora in raw shrimp, where asfermented shrimp from Thailand (*kapi*), made from *Acetes* sp., was dominated by *Vagococcus* sp. (Helmi et al., 2022; Phewpan et al., 2020). Proteobacteria, the dominant bacterial group in raw shrimp, are commonly found in the marine environments where shrimp are sourced or captured. These bacteria are closely associated with hygiene conditions and can negatively affect the freshness and quality of shrimp paste (Dai et al., 2018).

After fermentation, a significant shift in the bacterial community was observed, marked by the dominance of *Empedobacter*, *Acinetobacter* and *Psychrobacter,* accounting for 36%, 25% and 10% of the total microbial population, respectively. *Empedobacter* was known to have a significant correlation with the content of amino acids, including L-valine, L-tyrosine, L-leucine, L-tryptophan, L-phenylalanine, L- norleucine, and L-isoleucine and suggested that *Empedobacter* was strongly influenced the volatile aroma of the fermentation products. (Song et al., 2022; Wei et al., 2021).

The increased microbial diversity observed in the post-fermentation samples, particularly from Processor B, suggests a more complex fermentation process that could contribute to a richer flavor profile. The presence of different genera, known for their fermentative capabilities, indicates their potential role in improving the sensory qualities of shrimp paste. This microbial diversity enhances flavour and contributes to a more stable and safer fermentation process. However, several genera from the Proteobacteria group, particularly *Acinetobacter* species, were still detected in the post-fermentation shrimp paste from Processor B, indicating their potential to compromise the quality and safety of the final product.

3. Amino acid production during fermentation of shrimp paste

Amino acid production during fermentation is presented in Figure 3. For Processor A, the most abundant amino acids in pre- and post-fermentation shrimp paste were glutamic acid, leucine, aspartic acid, alanine, glycine, valine, and isoleucine. The concentration of these amino acids increased significantly after fermentation, with glutamic acid reaching the highest concentration of 64,249 mg/kg, followed by leucine and aspartic acid, each nearing 40,000 mg/kg. Histidine increased the most from 3,599 mg/kg before fermentation to 21,271 mg/kg after fermentation.

For Processor B, glutamic acid, glycine, leucine, and alanine were dominant before and after

fermentation, with glutamic acid being the most abundant amino acid post-fermentation at 10,714 mg/kg. Unlike Processor A, only a few amino acids in the shrimp paste from Processor B increased during fermentation, including lysine, threonine, and isoleucine. The concentrations of other amino acids decreased after fermentation, including a slight decrease in glutamic acid. Lysine increase might correlate with the base condition (pH 7.44) of the post-fermentation shrimp paste sample from Processor B. Tyrosine, and histamine were absent in the fermented shrimp paste from Processor B. A similar observation was reported for low-salt shrimp paste, where histamine concentration decreased during fermentation (Helmi et al., 2022). This reduction could be attributed to the activity of histamine oxidase or histamine dehydrogenase, which degrade histamine produced by species of lactic acid bacteria, such as *Lactobacillus plantarum* (Kung et al., 2017).

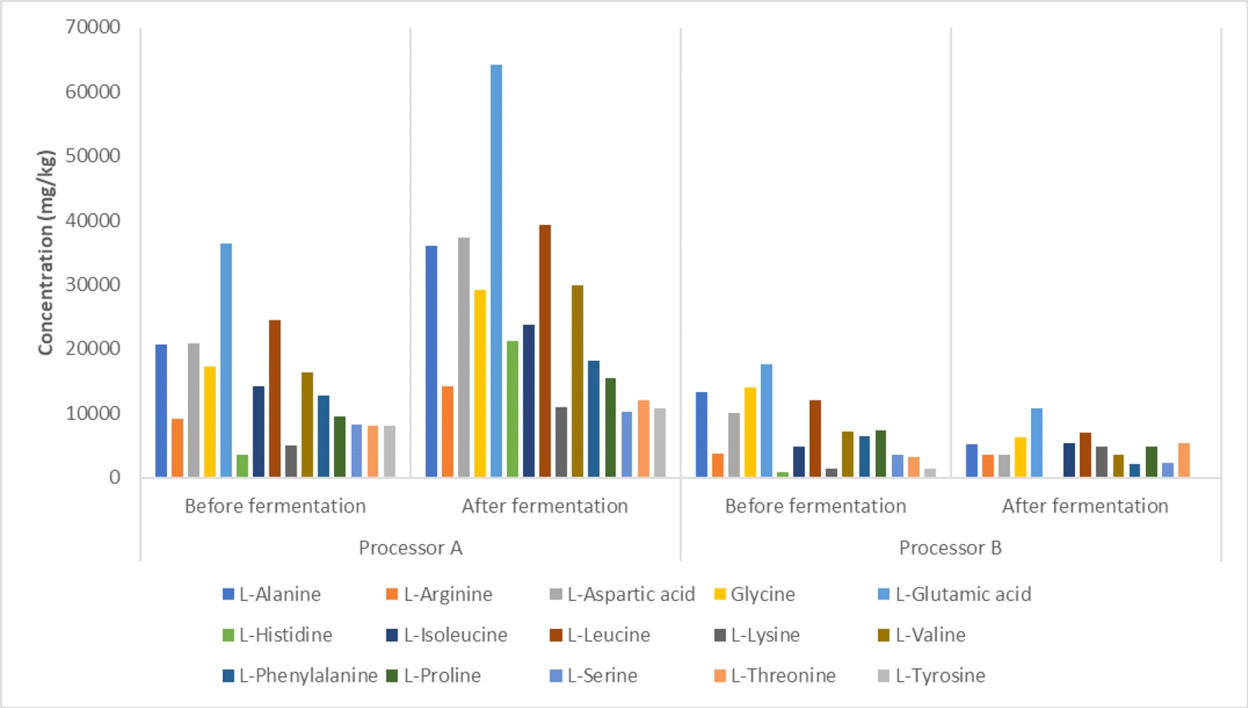

Figure 3. Amino acids production before and fermentation of shrimp paste from Processor A and B.

The amino acid composition of shrimp pastes from both processors followed similar trends observed in other studies, with glutamic acid, leucine, glycine, alanine, and aspartic acid accounting for more than 40% of the total amino acids (Cao et al., 2009; Lim et al., 2023). The significant increase in amino acids such as glutamic acid, leucine, and histidine post-fermentation underscores their role in flavor development. Glutamic acid, in particular, is known for its umami taste, which enhances the overall flavor profile of the shrimp paste. The differences in amino acid concentrations between the two processors highlight the impact of fermentation conditions on the biochemical composition of the product.

Conclusions

The variations in microbial communities and amino acid profiles between the two processors were observed and can be attributed to differences in processing methods and environmental conditions. Processor A's use of higher salt concentrations and palm sugar likely influenced microbial succession and amino acid production, resulting in a distinct flavor profile compared to Processor B. These findings emphasize the importance of standardized processing conditions to ensure consistent product quality.

While this study provides valuable insights, it is limited by the sample size and the scope of microbial analysis. Future research should explore the impact of different raw materials and further investigate the roles of specific microbes in flavor development. Additionally, studies on the long-term storage stability of fermented shrimp paste would be beneficial.

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