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Isolation and Identification of Histamine-Producing Bacteria from Fermented Fish-Based Paste and Shrimp Paste (*Belacan*)

(Pengasingan dan Pengenalpastian Bakteria Penghasil Histamin daripada Pes Berasaskan Ikan dan Pes Udang yang Difermentasi (Belacan))

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ABSTRACT

Shrimp paste (*belacan*), a traditional fermented food derived from *Acetes* spp., is an essential component of Southeast Asian cuisine, particularly in Malaysia. However, during fermentation, histamine-producing bacteria (HPB) in shrimp paste are produced by the decarboxylation of histidine through the enzyme histidine decarboxylase. This study aims to isolate and identify HPB from both fish-based and shrimp pastes. A total of 12 fish-based paste and 13 shrimp paste samples with varying salt concentrations (15%, 20%, 25%, and 30% *w/w)* and fermentation times (0, 2, and 7 days) were prepared. Samples were isolated and cultured on modified Niven's medium for the selective detection of HPB. Molecular identification was performed by 16S rRNA gene sequencing to screen bacterial profiles. The results showed that from the triplicate sampling batches, a total of 73 fish-based paste and 71 shrimp paste bacterial isolates were obtained, resulting in a total of 144 positive HPB isolates. The bacterial community in the fish-based paste comprises three dominant genera: *Staphylococcus* (75%), *Mammaliicoccus* (12.5%), and *Bacillus* (12.5%). Meanwhile, the dominant genera in the shrimp paste are *Staphylococcus* (70%), *Bacillus* (20%), and *Enterobacter* (10%). *Staphylococcus* and *Bacillus* were the most detected species in fish-based and shrimp pastes. *Staphylococcus* species, such as *Staphylococcus carnosus, Staphylococcus edaphicus,* and *Staphylococcus saprophyticus,* were detected. *Bacillus subtilis* and *Bacillus cereus* were found in fish-based and shrimp pastes, respectively. Likewise, less commonly reported species, like *Mammaliicoccus sciuri* and *Enterobacter quasiroggenkampii* were found in fish-based and shrimp pastes. The dominance of *Staphylococcus* and *Bacillus* suggests that some microbial communities survive fermentation conditions. This study investigates the presence of HPB in traditional fermented products, aiming to enhance food safety, quality control, and future research on microbial roles in biogenic amine formation.

Keywords: *Belacan*; fermentation; histamine; histamine-producing bacteria (HPB)

ABSTRAK

Belacan ialah makanan tradisi hasil daripada proses penapaian udang *Acetes* spp., merupakan salah satu komponen penting dalam masakan Asia Tenggara, khususnya di Malaysia. Walau bagaimanapun, proses penapaian ini turut menyumbang kepada penghasilan bakteria penghasil histamin (HPB) melalui proses dekarboksilasi histidin oleh enzim histidin dekarboksilase. Penyelidikan ini dijalankan untuk mengasing dan mengenal pasti HPB daripada belacan ikan dan belacan udang. Sebanyak 12 sampel belacan ikan dan 13 sampel belacan udang telah disediakan dengan kepekatan garam yang berbeza (15%, 20%, 25% dan 30% w/w) serta tempoh penapaian yang berbeza (0, 2 dan 7 hari). Proses pengasingan bakteria telah dijalankan menggunakan medium Niven yang diubah suai bagi pengesanan selektif HPB, manakala penentuan spesies bakteria dilakukan melalui penjujukan gen 16S rRNA. Hasil kajian menunjukkan bahawa koloni bakteria berjaya dikesan pada medium Niven daripada 73 sampel belacan ikan dan 68 sampel belacan udang. Hasil kajian menunjukkan bahawa daripada pensampelan triplikat ini, sejumlah 73 belacan ikan dan 68 belacan udang pengasingan bakteria telah diperoleh, menghasilkan sejumlah 144 pengasingan HPB positif. Komuniti bakteria dalam belacan ikan didominasi oleh tiga genus utama iaitu *Staphylococcus* (75%), *Mammaliicoccus* (12.5%) dan *Bacillus* (12.5%). Sementara itu, belacan udang didominasi oleh genus *Staphylococcus* (70%), diikuti *Bacillus* (20%) dan *Enterobacter* (10%). *Staphylococcus* dan *Bacillus* merupakan bakteria yang paling banyak dikesan dalam kedua-dua jenis belacan. Antara spesies *Staphylococcus* yang dikenal pasti termasuklah *Staphylococcus carnosus, Staphylococcus edaphicus* dan *Staphylococcus saprophyticus*. Selain itu, *Bacillus subtilis* ditemui dalam belacan ikan, manakala *Bacillus cereus* ditemui dalam belacan udang. Kajian ini juga berjaya mengesan kehadiran spesies yang jarang dilaporkan seperti *Mammaliicoccus sciuri* dalam belacan ikan dan *Enterobacter quasiroggenkampii* dalam belacan udang. Dominasi genus *Staphylococcus* dan *Bacillus* menunjukkan bahawa komuniti mikrob tertentu mampu bertahan dan menyesuaikan diri dalam keadaan penapaian yang mempunyai kandungan

garam yang tinggi. Secara keseluruhannya, kajian ini memberi sumbangan penting kepada pemahaman tentang kehadiran bakteria penghasil histamin dalam produk tradisional yang ditapai, serta dapat membantu dalam usaha meningkatkan keselamatan makanan, kawalan kualiti dan penyelidikan lanjut mengenai peranan mikrob dalam pembentukan amina biogen.

Kata kunci: Bakteria penghasil histamin (HPB); belacan; fermentasi; histamin

INTRODUCTION

Fermentation is a biological process that transforms food composition through microbial action involving fungi or bacteria, which convert carbohydrates into organic acids (Narzary et al. 2021). Across Asian countries, variants of shrimp-based products are known by several names: *belacan* in Malaysia and Brunei (Hj. Yaacob, Huda-Faujan & Md Yasin 2024; Kim et al. 2014), *terasi* in Indonesia (Herlina & Setiarto 2024), *nappi* in Bangladesh (Monwar et al. 2024), *kapi* in Thailand and Cambodia (Pongsetkul et al. 2017; Tamang 2016), *mam ruoc* or *mam tom* in Vietnam (Thanh & Anh 2016), *bagoong* or *alamang* in the Philippines (Stefanny & Pamungkaningtyas 2023), *ngapi* in Myanmar (Hlaing, Oo & Ni 2018), and *xiajiang* in China (Lv et al. 2020). Their aroma, color, and texture can vary widely due to factors regarding the specific ingredients used, production methods, food culture, consumer preferences, and regional climatic conditions (Zang et al. 2019).

In Malaysian cuisine, shrimp paste is used to enhance the taste and improve the nutritional value of numerous dishes (Abidin et al. 2020). Shrimp paste is made by fermenting shrimp with salt, and microorganisms play an essential role in the entire fermentation process. This process extends the shelf life of the food by breaking down protein in the raw materials into peptides, resulting in a unique flavor (Huang et al. 2024). The main microorganisms involved in the fermentation process are lactic acid bacteria (LAB), which are generally considered safe for human consumption (Sang et al. 2021). *Tetragenococcus muriaticus* is a member of LAB, which can tolerate high salt concentrations in fermented foods. Moreover, *T. muriaticus* has demonstrated the potential to reduce histamine levels when used as starter cultures (Li et al. 2023a). Fermentation of shrimp preserves the product by creating an environment that inhibits the growth of spoilage-causing microorganisms, allowing it to remain fresh and edible for longer periods (Speranza et al. 2021). Despite the high nutritional value and bioactivities, shrimp paste may potentially contain harmful compounds. These include sodium chloride, biogenic amines (BAs), and N-nitroso compounds, which may pose health risks (Cai et al. 2017; Wu et al. 2022). Although consumers do not commonly consume it in large quantities, it is essential to reduce or eliminate these undesirable compounds to minimize health risks, particularly excessive sodium chloride intake, which necessitates low-sodium production techniques (Campo, Rosato & Giagnacovo 2020).

BAs are nitrogenous and organic compounds that result from the microbial decarboxylation of amino acids (Doeun, Davaatseren & Chung 2017). The amino acids, classified by their chemical properties, include nonpolar aliphatic (valine, glycine, isoleucine, alanine, proline, and leucine), aromatic (phenylalanine and tyrosine), polar uncharged (serine, threonine, and cysteine), positively charged (histidine, lysine, and arginine), negatively charged (glutamic acid and aspartic acid), and sulfur-containing (methionine) amino acids. Histamine is specifically produced by bacterial strains that exhibit amino acid decarboxylase activity, converting the amino acid histidine into histamine through the enzyme histidine decarboxylase (Zaman et al. 2009). High histamine levels have been associated with foodborne intoxications, commonly referred to as scombroid poisoning, which can cause allergy-like symptoms in humans (Zhernov et al. 2023).

Regulatory agencies, such as the European Food Safety Authority (EFSA), set the maximum allowable level at 100 mg/kg in fishery products (EFSA 2015), while the U.S. Food and Drug Administration (FDA) considers fish to be a hazard if histamine levels exceed 50 ppm in multiple subsamples or 100 ppm in any single sample (FDA 2024) to prevent histamine poisoning. According to Regulation (EC) No. 2073/2005, the histamine level limit is at 400 ppm (DeBeeR et al. 2021). However, scombrotoxin food poisoning occurs in individuals when a dose of at least 50 ppm histamine is considered spoiled for consumption (Ginigaddarage et al. 2023). Several bacterial species have been isolated from fermented shrimp paste, including those that produce histamine, known as histamine-producing bacteria (HPB). The likes of HPB are *T. muriaticus*, *Bacillus,* and *Staphylococcus*, which are moderately halophilic LAB (Li et al. 2023b). Controlling the formation of BAs through effective microbial management is essential for ensuring the safety and quality of fermented shrimp-based products (Kannan et al. 2020). This study aimed to assess the microbiological safety specifically HPB diversity and of a newly developed fish-based paste relative to commercial shrimp paste during the primary fermentation stage.

MATERIALS AND METHODS

MATERIALS

Approximately 100 kg of shrimp (*Acetes* spp.) and 200 kg of fish (*Upeneus* spp.) were purchased from a local wet market in Terengganu, Malaysia. Coarse salt (Muslim Garam Kasar, Malaysia) was purchased from a supermarket in Terengganu, Malaysia. The samples and materials were transported to the Muscles Laboratory, Universiti Sultan Zainal Abidin, Besut Campus, Terengganu, Malaysia. Shrimp and fish samples were stored in the freezer (-20 °C) before further analysis. Trypticase soy broth (TSB) and trypticase soy agar (TSA) were purchased from Difco, USA. Tryptone, yeast extract, L-histidine monohydrochloride, sodium chloride (NaCl), calcium carbonate ($CaCO_3$), agar, and bromocresol purple (pH 5.3, $C_{21}H_{16}Br_2O_5S$) were obtained from Sigma Chemical Co., USA.

SAMPLE PREPARATION

The frozen shrimp and fish raw materials were thawed in a chiller for 48 h. Following thawing, excess exudate was removed by filtration using a stainless steel colander. The prepared samples were then mixed with salt at varying concentrations (15%, 20%, 25%, and 30% w/w) and transferred into airtight containers. The fermentation duration was established based on the current manufacturing practices of local Small and Medium Enterprises (SMEs) in Terengganu, Malaysia. A preliminary survey of these producers indicated that local 'fresh' shrimp paste variants typically undergo a short fermentation period ranging from 2 to 7 days. Consequently, the mixtures were allowed to ferment at room temperatures of 0, 2, and 7 days. Following the respective fermentation periods, the samples were oven-dried at 30 °C for 10 h using a food dehydrator (NBZ Food Dehydrator, Malaysia) to remove initial moisture. The semi-dried mixtures were moulded using a patty compressor (VEVOR Patty Press, China) and subjected to a secondary drying process at 30 °C for an additional 10 h.

ISOLATION OF HISTAMINE-PRODUCING BACTERIA (HPB)

A total of 12 fish-based paste and 13 shrimp paste samples with varying salt concentrations (15%, 20%, 25%, and 30% w/w) and fermentation times (0, 2, and 7 days) were prepared. The samples (1 g) were transferred aseptically to a stomacher bag containing 9 mL of sterile 0.85% (w/v) NaCl. The mixture was homogenized for 2 min using a stomacher (Stomacher, Malaysia) and then further diluted in saline at a 1:10 dilution. The pour plate method was applied. The diluted liquid sample (1 mL) was mixed with Niven's medium (Niven et al. 1981) with slight modification to qualitatively assess the histamine-producing capacity of the bacterial strains. Niven's medium contained 0.5% tryptone, 2.75% yeast extract, 2.75% L-histidine monohydrochloride, 0.10% sodium chloride (NaCl), 2% calcium carbonate ($CaCO_3$), 2% agar, and 0.006% bromocresol purple (pH 5.3, $C_{21}H_{16}Br_2O_5S$). The medium was autoclaved for 10 min to avoid excessive hydrolysis of the agar at low pH. The plates were incubated at 37 °C for 7 days.

PREPARATION OF TRYPTICASE SOY AGAR (TSA) AND TRYPTICASE SOY BROTH (TSB)

The TSA medium was prepared by dissolving 40 g of TSA powder in 1 L of distilled water. The mixture was heated and stirred until completely dissolved and then autoclaved at 121 °C for 15 min. The medium was chilled to 50 °C-55 °C before pouring into sterile Petri dishes. Once hardened, the plates were used to streak selected colonies to obtain pure bacterial isolates. To assess the ability of the purified isolates to HPB, each isolate was inoculated into TSB. The TSB was prepared by dissolving 30 g of TSB powder in 1 L of distilled water and heating it until completely dissolved. The solution was then dispensed into a sterile Schott bottle and sterilized under the same autoclaving conditions as TSA.

Positively pure colonies on the TSA were determined by the appearance of purple halos around colonies on Niven's medium within 7 days of incubation. The purple color was further streaked on TSA agar for pure cultures. Their ability to produce BAs (histamine) was determined by inoculating the isolates in TSB and incubating them without shaking at 37 °C for 24 h. Two mL of the culture broth were taken for quantitation and subsequent analysis.

DNA EXTRACTION AND SEQUENCING

The samples were collected under sterile conditions to prevent contamination and ensure the integrity of the sample. The isolates were cultured in TSB and streaked onto TSA to obtain distinct colonies. A single colony from each isolate was aseptically excised and stored under sterile conditions.

PCR amplification and sequencing of the bacterial 16S rRNA gene were performed to facilitate taxonomic identification. The bacterial full-length (~1500 bp) fragment of the 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Genomic DNA (gDNA) was extracted using the Bacterial DNA Barcoding Kit (1st BASE, KIT-1100-50) following the manufacturer's protocol. The extraction process involved bacterial cell lysis using the bacterial lysis buffer, ensuring efficient disruption of cell walls and the release of high-quality DNA suitable for downstream applications. The purified gDNA was subsequently quantified and assessed for quality before being used as a template for PCR. The PCR reaction comprised gDNA, 0.3 pmol of each primer, 400 μM of each deoxynucleotide triphosphate (dNTP), 0.5 U of thermostable DNA polymerase, and the supplied PCR buffer, with nuclease-free water added to

adjust the final volume to 25 µL. PCR amplification was conducted under the following thermal cycling conditions: an initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 53 °C for 30 s, and extension at 68 °C for 1 min. Upon completion of amplification, the PCR products were purified using a standard PCR clean-up method to remove excess primers, nucleotides, and enzymes. For sequencing, the purified PCR products were subjected to bidirectional Sanger sequencing using the universal primers 785F and 907R in conjunction with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). This approach ensured high-quality sequence data for downstream phylogenetic and taxonomic analyses.

PHYLOGENY AND POLYMORPHISMS STUDIES

The phylogenetic tree was constructed using MEGA 11, employing the neighbor-joining (NJ) tree statistical method with the maximum composite likelihood (MCL) model. The significance of the constructed branches was statistically tested using 500 bootstrap replicates.

RESULTS AND DISCUSSION

ISOLATION OF HISTAMINE-PRODUCING BACTERIA (HPB) USING NIVEN'S MEDIUM

Histamine is formed as a byproduct of the decarboxylation of free amino acids, especially histidine, by bacterial strains that exhibit decarboxylase activity. Figure 1 illustrates the identification of HPB by the presence of purple halos around the colonies formed. Figure 1(a) shows the Niven's medium without samples. The outcomes shown in Figure 1(b), 1(c), and 1(d) indicate the presence of histamine-producing strains. HPBs are often associated with foods. Raw ingredients used in food fermentation can naturally contain histidine, which can be converted into histamine during fermentation (Engevik et al. 2024). Therefore, adhering to proper hygiene during fermentation is essential to minimize food safety risks. Sanitary conditions are of great importance for the development of amine-positive microorganism strains (Wójcik, Łukasiewicz & Puppel 2021). HPB thrives under suboptimal hygiene conditions and temperature abuse, which enhances bacterial decarboxylase activity (Restuccia et al. 2015).

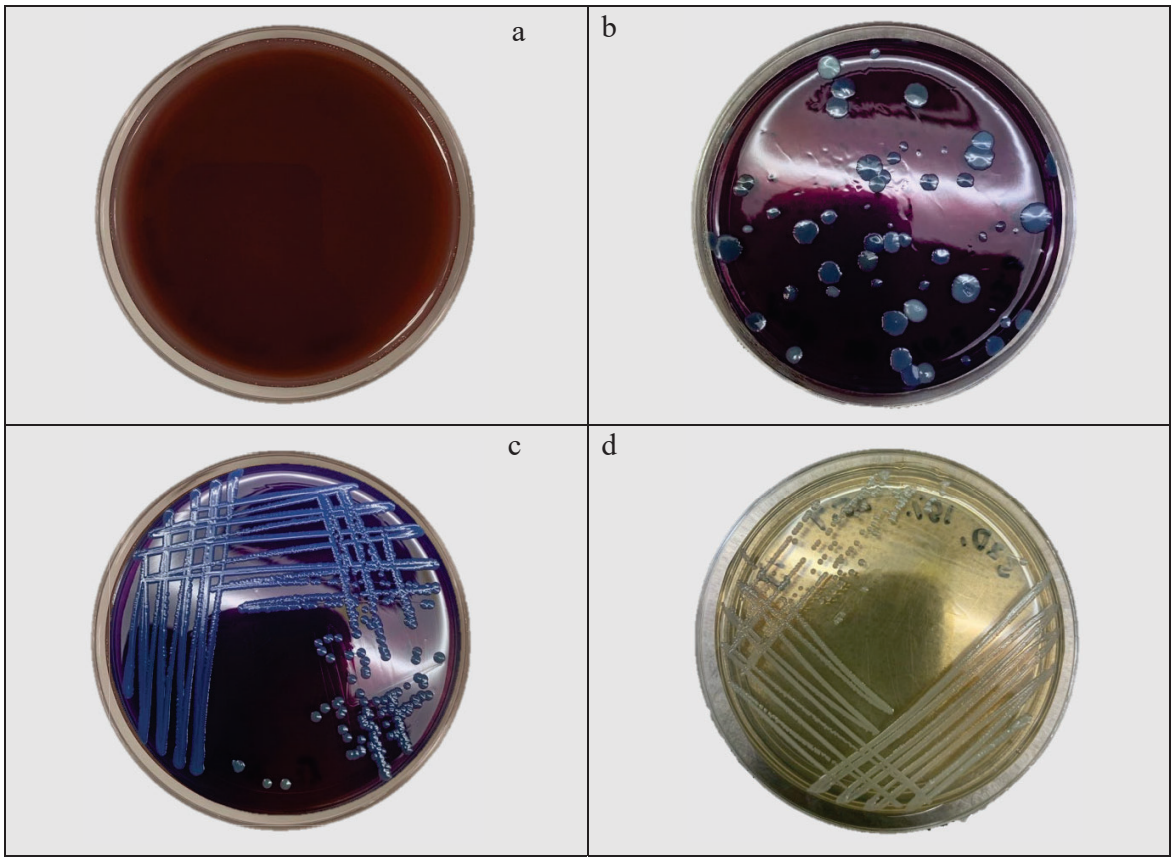

FIGURE 1. The different conditions of agar: (a) Niven's medium without samples, (b) Niven's medium: pour plate sample after incubation at 37 °C for 24 h, (c) Niven's medium: histamine-forming isolates after incubation at 37 °C for 24 h, and (d) TSA agar streaked with purple halos of c for pure cultures after incubation at 37 °C for 24 h

Table 1 summarizes the HPB strains isolated from fish-based paste and shrimp paste samples that were fermented for different durations (0, 2, and 7 days) and at varying salt concentrations (15%, 20%, 25%, and 30% w/w), including a commercial shrimp paste used as a control sample (labelled as sample C) purchased from local markets. In total, 144 presumptive HPB colonies were obtained from Niven's media. HPB was detected in 73 samples collected from the fish-based paste and 68 samples from the shrimp paste. HPB was most isolated from fish-based paste and shrimp paste samples at 2 and 7 days of fermentation. Salt concentration played a significant role in bacterial presence. HPB was most isolated (n = 8) from the fish-based paste sample (F2D30) and the shrimp paste sample (n = 7, S2D30) at 30% (w/w) salt concentration and 2 days of fermentation. However, at 25% and 30% (w/w) salt concentrations, the number of HPB isolated exhibited variable trends, suggesting that some strains could tolerate elevated salt levels due to endogenous enzymes in the samples and microbial enzymes surviving under high salt conditions (Pongsetkul et al. 2017).

TABLE 1. Isolated histamine-producing bacteria (HPB) from fish-based paste and shrimp paste using Niven's medium

Samples	Fermentation	Salt concentration (%)	Isolate code*	Batch 1	Batch 2	Batch 3
FISH	0-DAY	15	F0D15	2	2	1
		20	F0D20	2	2	2
		25	F0D25	2	1	1
		30	F0D30	2	-	2
	2-DAYS	15	F2D15	2	3	2
		20	F2D20	3	3	2
		25	F2D25	2	1	3
		30	F2D30	3	3	2
	7-DAYS	15	F7D15	2	3	3
		20	F7D20	2	2	3
		25	F7D25	3	-	3
		30	F7D30	2	-	2
			TOTAL	27	20	26
SHRIMP	CONTROL	20	C	3	-	-
	0-DAY	15	S0D15	2	2	3
		20	S0D20	-	1	1
		25	S0D25	-	2	1
		30	S0D30	-	3	2
	2-DAYS	15	S2D15	3	2	2
		20	S2D20	3	2	1
		25	S2D25	1	3	1
		30	S2D30	3	3	1
	7-DAYS	15	S7D15	2	2	3
		20	S7D20	1	2	3
		25	S7D25	1	2	3
		30	S7D30	2	2	3
			TOTAL	21	26	24
			GRAND TOTAL		144	

*Isolate code: F= fish-based paste, S= shrimp paste, C= control, 0/2/7D= sample fermentation duration, 15/20/25/30% (w/w) = salt concentration in sample

Note: Control data refers to commercially available samples used as a benchmark. No commercial control data is available for fish-based paste as it is a novel formulation developed in this study; the shrimp paste serves as the comparative standard.

The study demonstrates that HPB is prevalent in fermented seafood products and influenced by the fermentation period, salt concentration, and sample type. The ability of these bacteria to persist at varying salt levels highlights the need for crucial monitoring in seafood-based fermented foods, including the processing and packaging methods, hygiene conditions, and temperature requirements, to effectively control histamine accumulation and ensure food safety.

IDENTIFICATION OF BACTERIAL COMMUNITIES IN FISH-BASED PASTE AND SHRIMP PASTE

Table 2 lists the bacterial species identified from both fish-based paste and shrimp paste samples. From the total of 144 positive isolates, a representative subset of 18 isolates was selected for molecular identification. Selection was based on morphological distinctiveness and the intensity of the positive reaction on Niven's medium (indicative of high histamine potential). This dereplication

TABLE 2. The bacterial species identified from fish-based paste and shrimp paste

Sample name	Species	Order	Family	Domain	Accession number
F0D15	*Staphylococcus warneri*	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
F0D20	*Staphylococcus warneri*	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
F0D25	*Staphylococcus carnosus*	Bacillales	Staphylococcaceae	Bacteria	NR_116433.1
F2D20	*Staphylococcus warneri*	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
F2D25	*Staphylococcus warneri*	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
F2D30	*Bacillus subtilis*	Bacillales	Bacillaceae	Bacteria	NR_027552.1
F7D15	*Mammaliicoccus sciuri*	Caryophanales	Staphylococcaceae	Bacteria	NR_025520.1
F7D20	*Staphylococcus warneri*	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
S0D15	*Bacillus cereus*	Bacillales	Bacillaceae	Bacteria	NR_115714.1
	Staphylococcus edaphicus	Bacillales	Staphylococcaceae	Bacteria	NR_156818.1
	Staphylococcus saprophyticus	Bacillales	Staphylococcaceae	Bacteria	NR_115607.1
S0D20	*Staphylococcus saprophyticus*	Bacillales	Staphylococcaceae	Bacteria	NR_074999.2
S0D25	*Staphylococcus saprophyticus*	Bacillales	Staphylococcaceae	Bacteria	NR_074999.2
S0D30	*Staphylococcus saprophyticus*	Bacillales	Staphylococcaceae	Bacteria	NR_074999.2
S2D25	*Bacillus cereus*	Bacillales	Bacillaceae	Bacteria	NR_115714.1
	Staphylococcus saprophyticus	Bacillales	Staphylococcaceae	Bacteria	NR_074999.2
	Staphylococcus warneri	Bacillales	Staphylococcaceae	Bacteria	NR_025922.1
S7D15	*Enterobacter quasiroggenkampii*	Enterobacterales	Enterobacteriaceae	Bacteria	NR_179166.1

The sample name designated as F = fish-based paste, S = shrimp paste, 0/2/7D = sample fermentation duration, 15%/20%/25%/30% (w/w) = salt concentration in the sample

step was performed to eliminate clonal duplicates and focus sequencing efforts on distinct, high-activity strains distributed across the different fermentation treatments. Based on the single run, the bacterial identification showed a diverse microbial composition, predominantly consisting of species from the order Bacillales, with the most identified from the family *Staphylococcaceae*. The most detected species was *Staphylococcus warneri*, present in multiple samples of F0D15, F0D20, F2D20, F2D25, F7D20, and S2D25. *S. warneri* is commonly found in various environments and has been recognized for its opportunistic pathogenic potential (Alawad, Ali & Goravey 2022). Other *Staphylococcus* species identified include *S. carnosus, S. edaphicus,* and *S. saprophyticus*. *S. carnosus* was detected in the F0D25 sample and is primarily associated with fermented food products due to its enzymatic contributions to flavor development (Löfblom et al. 2017). Meanwhile, *S. saprophyticus,* detected in samples S0D20, S0D25, S0D30, and S2D25, is known for its role as a uropathogen, as well as its ability to persist in environmental reservoirs (Zhang et al. 2023).

In addition, *Bacillus* species from the order Bacillales were also detected, including *B. subtilis* in sample F2D30. *B. subtilis* is widely recognized for its probiotic properties and enzyme production (City, Sugata & Jan 2021). *B. subtilis* was detected in *ngapi,* or shrimp paste, originating from Myanmar (Kobayashi et al. 2003; Stefanny & Pamungkaningtyas 2023; Steinkraus 2004). *B. cereus,* which was identified in samples of S0D15 and S2D25, is of particular concern due to its potential to produce toxins associated with foodborne illness (Jovanovic et al. 2021). Few studies reported that *B. cereus* was also identified in various shrimp pastes such as *belacan* from Malaysia (Chuon et al. 2014; Huda 2012), *terasi* from Indonesia (Helmi et al. 2022; Huda 2012; Kobayashi et al. 2003), and *ngapi* from Myanmar (Kobayashi et al. 2003; Stefanny & Pamungkaningtyas 2023; Steinkraus 2004).

The presence of *M. sciuri* in sample F7D15 from the order *Caryophanales* suggests taxonomic diversity within the samples. *M. sciuri* lives on the skin and mucous membranes of many domestic, farm, and wild animals, as well as in animal-derived foods (Vechtomova et al. 2023). Additionally, the detection of *E. quasiroggenkampii* in sample S7D15, a member of the *Enterobacteriaceae* family, highlights the presence of Gram-negative bacteria, distinguishing it from the predominantly Gram-positive profile observed. *E. quasiroggenkampii* can be distinguished from all known Enterobacter species by its ability to ferment inositol, D-sorbitol, and melibiose but not potassium gluconate, L-fucose, and methyl-α-D-mannopyranoside. However, there is limited information regarding *M. sciuri* and *E. quasiroggenkampii* in the fermentation food industry.

The identification of *Staphylococcus* and *Bacillus* species suggests a microbial environment rich in facultative anaerobes and spore-forming bacteria, which may be shaped by environmental factors or selective pressures. Further analysis, such as functional gene profiling and metagenomic approaches, would provide deeper insights into the metabolic potential and ecological interactions of these microbial populations.

PHYLOGENY TREES

A phylogenetic tree was constructed based on the sequence alignment of the 16S rRNA gene. The phylogenetic tree showed the evolutionary relation of selected organisms, which can be grouped into three main clusters belonging to the orders Bacillales, Caryophanales, and Enterobacteriales. However, each order was further grouped into several sub-clusters. The phylogenetic trees of the isolated strains, based on the newly determined sequences of partial hdc genes and 16S rRNA sequences, are compared in Figure 2(a) and 2(b). The bootstrap scores observed for all the nodes are indicated. The isolates that cluster closely together with branch lengths of 0.0000 suggest nearly identical genetic sequences, likely indicating a common origin or adaptation to similar fermentation environments. The presence of multiple *Staphylococcus* species isolates with nearly identical sequences suggests that this species plays a key role in histamine formation. However, the dominant species differ, with *S. warneri* prevailing in the fish-based samples, as shown in Figure 2(a), and *S. saprophyticus* in the shrimp paste samples, as shown in Figure 2(b). Additionally, the presence of *Bacillus* and *Enterobacter* species suggests potential microbial interactions that may affect histamine accumulation. These differences highlight the variability in HPB communities based on environmental and fermentation conditions.

DOMINATION OF BACTERIOTA IN SAMPLES

The relative abundance of bacteria in the fish-based paste and shrimp paste samples is shown in Figure 3. The highest species in both samples was *Staphylococcus,* which accounted for 75% of the fish-based paste and 70% of the shrimp paste samples. The percentage of bacterial community abundance in fish-based paste was dominated by three genera of high relative abundance: *Staphylococcus* (75%), *Mammaliicoccus* (12.50%), and *Bacillus* (12.50%). The relative abundance of bacteria in the shrimp paste is dominated by the genera *Staphylococcus* (70%), *Bacillus* (20%), and *Enterobacter* (10%).

Staphylococcus, identified under the microscope by the formation of irregular grape-like clusters of cells, is a Gram-positive, facultatively anaerobic, catalase-positive, non-motile, and non-spore-forming bacterium with a high tolerance for salt (most strains survive in the presence of 10% NaCl) (Becker et al. 2014; Fayisa & Tuli 2023). However*, S. saprophyticus*, *S. warneri,* and *S. carnosus* were identified as coagulase-negative staphylococci (GNS) (Chen et al. 2022). GNS contribute to the sensory properties of fermented foods, including color, aroma, and

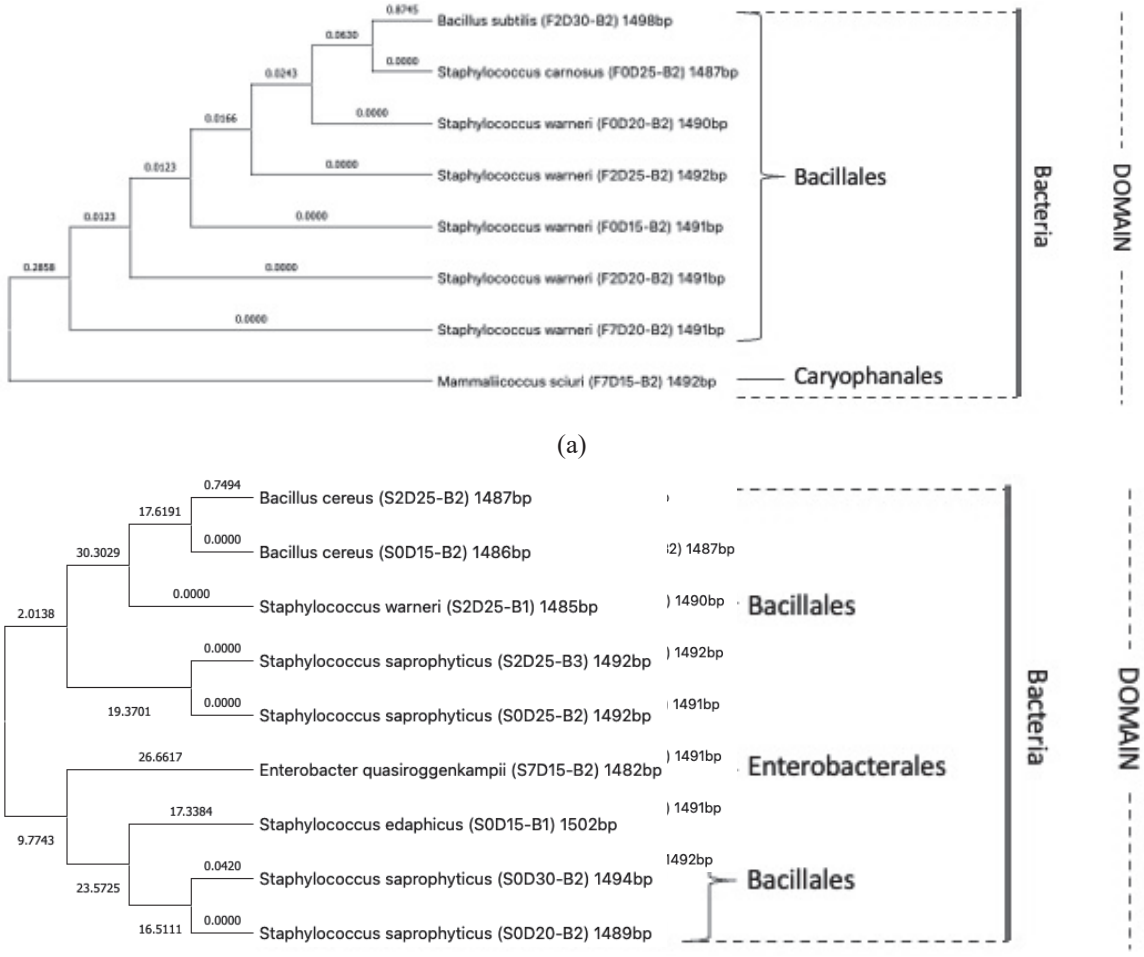

FIGURE 2. Phylogenetic analysis of the 16S rDNA sequences of major histamine-forming bacteria. Number above indicates bootstrap values from the maximum likelihood method. (a) is HPB from fish-based paste samples and (b) is HPB from shrimp paste samples

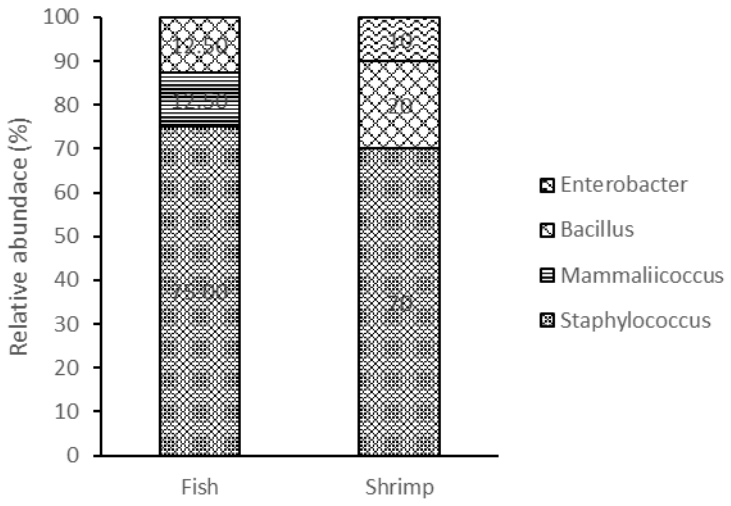

FIGURE 3. Taxonomic classification of sequences from bacterial community of the fish-based paste and shrimp paste

taste, and are considered non-pathogenic (Heo, Lee & Jeong 2020). *S. carnosus is* reported to have been used as a starter culture in the food industry and showed low rates of antibiotic resistance (Löfblom et al. 2017; Zell et al. 2008).

In food fermentation, the *Bacillus* species produce amylases and proteases, extracellular polysaccharides and polypeptides, as well as lipopeptides with antimicrobial activity. The metabolic traits of the *bacilli* also provide opportunities for use in food fermentations in which they do not traditionally occur. Most type strains of the *B. cereus* and *B. subtilis* groups produce multiple amylases (Li et al. 2023b). However, *B. cereus* is an etiological agent that causes foodborne illnesses. Its omnipresence in various environments, ability to form spores, adaptability to diverse environments, and production of harmful toxins contribute to its potential as a pathogen and a health hazard (Jovanovic et al. 2021). *B. cereus* can survive at different NaCl ranges from 5% to ≥ 10% and pH values as low as ≤ 4.3 and grow at high temperature ranges from 5 °C to 48 °C (Webb et al. 2019).

Enterobacteriaceae is considered the indicator bacteria for the microbiological quality of food and the hygiene status of a production process. Additionally, the food contaminated by *Enterobacteriaceae* poses a microbiological risk for consumers (Mladenović et al. 2021). *M. sciuri* is a non-pathogenic bacterium commonly found in fermented foods, where its presence is generally considered safe, with the potential as a probiotic strain due to its beneficial microbial properties (Naqqash et al. 2022). *M. sciuri* IMDO-S72 has been studied for its anticlostridial starter culture activity in meat fermentation, attributed to the production of micrococcin P1. Therefore, *M. sciuri* can be used as a bioprotective culture in food fermentation processes (Van der Veken et al. 2023). However, further studies are required to fully understand its functional role in the fermentation process, its impact on food quality and safety, and its potential applications in the food industry. Expanding knowledge of *M. sciuri* could provide valuable insights into its contributions to flavor development, preservation, and overall microbial balance in fermented food products.

CONCLUSION

This study successfully achieved the objective of isolating and identifying histamine-producing bacteria (HPB) from both the novel fish-based paste and traditional shrimp paste. The molecular identification showed a diverse bacterial profile, confirming the presence of *S. carnosus*, *S. saprophyticus*, *B. subtilis*, and the potentially pathogenic *B. cereus* in the fermented samples. Significantly, the isolation of *B. cereus* provides critical evidence regarding the safety profile of these pastes. The identification of this pathogenic species suggests that, despite the fermentation environment, histamine-producing contaminants can persist. Therefore, the study concludes that while both pastes harbour functional fermenting bacteria, they also act as reservoirs for HPB, necessitating rigorous microbial screening to prevent biogenic amine accumulation.

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