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RESEARCH ARTICLE

Quantification of Histamine Concentration, Identification, and Antibiotic Resistance of Potential Histamine-Forming Bacteria in Bullet Tuna

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ABSTRACT

Consuming scombroid fish containing high histamine levels can lead to histamine fish poisoning caused by the proliferation of histamine-forming bacteria (HFB). Interestingly, some HFB strains have developed resistance to antibiotics. This complicates efforts to control their populations in fish and aquatic environments. As these bacteria become harder to eliminate, the risk of histamine fish poisoning may increase, highlighting a concerning link between antibiotic resistance and food safety. In this study, the histamine concentration levels and the presence and antibiotic resistance of isolated HFB in bullet tuna were investigated. The fluorometric method (AOAC 977.13) showed that the average amount of histamine was 39 ppm, which was below the local regulatory limit (< 200 ppm). Ten (10) presumptive HFBs were isolated using modified Niven's agar. However, only five of the 10 isolates were identified at the species level by molecular methods. These isolates were then identified as *Proteus mirabilis* (Hfb_5, Hfb_8, and Hfb_13) and *Klebsiella pneumoniae* (Hfb_6, and Hfb_10). These potential HFBs were subjected to antibiotic susceptibility testing. Results showed that all potential HFBs displayed multidrug resistance to the antibiotic used. Despite the occurrence of low histamine levels, it is still recommended that the proper handling and storage of fish products should be strictly practiced to mitigate the proliferation of HFB. Moreover, the presence of antibiotic-resistant bacteria on fish products should be sufficient to raise awareness about the extent of such phenomena in the aquatic environment, therefore encouraging future researchers to delve into this interesting yet concerning environmental problem.

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1. INTRODUCTION

Histamine fish poisoning (HFP), also known as scombroid or scombrotoxin-poisoning, is one of the common illnesses related to fish consumption (Jantschitsch et al. 2011; Hungerford 2010). Humans are susceptible to histamine, and its effect on health is based on intoxication and intolerance (Visciano et al. 2014). Symptoms of HFP vary, according to Jantschitsch et al. (2011), which may include urticaria, nausea, diarrhea, sweating, and a burning sensation in the mouth. It primarily results from consuming histamine-contaminated fishes commonly found in the Scombridae family (e.g., tuna, mackerel, and bonito). This family of fish is known to be rich in free histidine, which is converted to histamine upon contamination of bacteria that have histidine decarboxylase (Hungerford 2010). For instance, *Auxis* spp., such as *A. rochei* and *A. thazard*, has been reported to contain free histidine levels of 4,330 to 10,100 mg/kg (FAW/WHO 2013). Moreover, other studies have shown that non-scombroid fishes also have the opportunity to form histamine (Lunestad et al. 2011).

Histamine, a biogenic amine that can be generated in fish muscle via enzymatic decarboxylation of free histidine, is catalyzed by bacteria-released exogenous decarboxylase. This capacity has been

documented in various gram-positive and gram-negative bacterial taxa, species, and strains (Ladero et al. 2010). Furthermore, the gills and skin of fish tend to be the reservoir for these bacteria (Kim et al. 2001), which also continue to thrive after the death of the fish because the defense mechanisms are inactive. Histamine-forming bacteria (HFB) can proliferate quicker at high temperatures of abuse (≥ 21.1°C) than at moderate temperatures of abuse (7.2°C), consequently leading to a dangerous increase in histamine synthesis at 32.2°C (Food and Drug Administration [FDA] 2022b). In this aspect, histidine plays a crucial catalytic role in the enzymes of various classes of biological processes (Kessler and Raja 2023). The metabolic process of histidine follows several routes, one of which is the enzymatic conversion of free histidine to histamine (Özogul and Özogul 2019).

Antibiotic resistance in seafood represents a critical issue in food safety, posing significant health risks to consumers worldwide. As the demand for seafood continues to rise, so does the use of antibiotics in aquaculture to prevent and treat bacterial infections (Hossain et al. 2022; Guillen et al. 2019). However, this practice has led to the emergence of antibiotic-resistant bacteria (ARB), which can be transferred to humans through the consumption of contaminated seafood (Hossain et al. 2022). This transfer poses a serious threat as it can lead to infections that are difficult to treat with conventional antibiotics. Furthermore, the aquatic environments–often recipients of antibiotic runoff–serve as reservoirs for resistance genes, which may spread across different bacterial communities (González-Plaza et al. 2019). Antibiotic-resistant HFB was identified in seafood products (Marijani 2022; Ghosh and Mandal 2010).

Studies and recorded data regarding HFP in the Philippines have recently been lacking. In a study by Azanza (2006), only 124 morbidity cases of HFP were recorded from 1995 to 2004. This small number of reported cases may be due to HFP being misdiagnosed. Hence, it is also known to be a fish-related disease that is vastly underreported (Feng et al. 2016). HFP could become a food-borne chemical hazard and can impose a public safety concern (Simora et al. 2016). There have been no reports on the occurrence of histamine concentration and HFB in raw fish obtained from local wet markets in the Philippines. Despite the growing concern over antibiotic resistance, there is a limited understanding of HFB and their resistance profiles in bullet tuna. This study investigated the antibiotic resistance characteristics of HFB in bullet tuna (*Auxis rochei*, Risso 1810) to address this gap. Therefore, the present study investigated the level of histamine concentration as well as the identification and antibiotic susceptibility of HFB in bullet tuna samples.

2. MATERIALS AND METHODS

2.1 Preparation of bullet tuna homogenates

Eight (8) bullet tuna were obtained from Cubao Farmers Market (14° 37′ 02″ N, 121° 03′ 04″ E), a major fish and seafood distributor in Metropolitan Manila. Of these, seven were designated for experimental analysis and one for species verification. Samples were transported to the laboratory in a cooler maintained at 5°C and stored at –20°C to prevent spoilage and contamination. Samples were then carefully dissected using a scalpel. The flesh of the fish were collected separately and weighed prior to homogenization. The homogenized samples were transferred to sterile 250 mL beakers, and microbiological and histamine analyses were performed. Species identification of the fish was conducted through morphometric analysis, utilizing FishBase (https://www.fishbase.se) (Supplementary Material), and confirmed by the Corals and Marine Fishes Zoological and Wildlife Museum, University of the Philippines-Los Baños.

2.2 Microbiological analysis

Modified Niven's agar medium was prepared from 0.5% tryptone (HiMedia®), 0.5% yeast extract(HiMedia®), 2.7% L-histidine 2HCL (Sigma-Aldrich®), 0.5% NaCl, 0.1% calcium carbonate, 6.0% bacteriological agar (HiMedia®), and 0.006% bromocresol purple (Techno Pharmchem™) with pH adjusted to 5.3. The medium was autoclaved at 121°C for 10 minutes (Mavromatis and Quantick 2002). Following the aseptic technique, 1 ml of the flesh homogenates was added to a sterile test tube containing trypticase soy broth (TSB; HiMedia®) and incubated for 24 h at 25°C. Then, the cultures were serial diluted to 10^{-4}, and 0.1 mL of the sample was pipetted and spread onto modified Niven's agar plates using a spreader. After 48 h of incubation at 37°C, plates were checked for typical, purple-colored colonies. A typical colony was randomly picked from each plate for pure isolation, inoculated into trypticase soy agar (TSA; Neogen®) plates, and incubated for 24 h at 25°C. Initially, 14 isolates were subjected to purification, but four later exhibited atypical characteristics

upon restreaking on modified Niven's agar. A total of 10 presumptive HFBs were characterized based on colony and cell morphology. After incubation, colonies were observed macroscopically for size, shape, surface, elevation, edge, opacity, color, and texture. Subsequently, microscopic examination of bacterial cells was conducted using Gram staining. Stock cultures were prepared by inoculating pure isolates into trypticase soy broth (TSB; HiMedia®) with 10% glycerol and then stored in the freezer at –20°C. A subsequent test was conducted to measure the ability of presumptive HFBs to decarboxylate histidine. A 6 mm sterile filter paper disk was soaked in 1 µL of inoculum and placed on Niven's agar plate. After incubation at 25°C for 24 h, the sizes of the purple halo surrounding the disks were measured using ImageJ version 1.53t and calculated to obtain the mean sizes.

2.3 Histamine analysis

Standard solutions were prepared prior to histamine quantification (Supplementary Material). The homogenized flesh samples (n = 7) were subjected to histamine analysis according to AOAC Official Method 977.13. Briefly, 10 g of homogenate from each sample was blended with 50 mL 75% (v/v) methanol, filtered using filter paper (Whatman® Grade 1), and the filtrate was transferred into a 250 mL beaker. Before histamine extraction, 4-5 mL of distilled water was passed through the column containing ion-exchange resin (Amberlite® CG50), and the eluate was discarded. Then, 1 mL filtrate was pipetted onto the column with resin, and 4-5 mL distilled water was added. When the liquid level was approximately 2 mm above the resin, 5 mL of distilled water was added and allowed to elute. Next, 5 mL of eluate was collected into a 50 mL Erlenmeyer flask; 10 mL of 0.1 M HCl and 3 mL of 1M NaOH were then added and mixed. Within 5 min, 1 mL of 0.02% (w/v) *o*-phthalaldehyde solution (Sigma-Aldrich®) was pipetted and immediately mixed. The extracted histamine was quantified using a fluorometer (Shimadzu RF-6000). The readings were corrected against the blank control, and the histamine was calculated using the formula: histamine per g = [histamine in the solution as read by the fluorometer (*I*)/wt. of the sample (g)]*10. The analysis was done in duplicates, and results were expressed as parts per million (ppm).

2.4 Molecular identification of potential HFB

2.4.1 DNA extraction

The HFBs were inoculated in TSA supplemented with 20 mg L-histidine and incubated for 24 h. Following the manufacturer's protocol, the bacterial DNAs were extracted using the G-spinTMgenomic DNA extraction kit (iNTRON Biotechnology, Inc.). Briefly, bacterial cells were harvested by centrifugation for 1 min at 13,000 rpm. The supernatant was removed, and 300 µL of buffer solution was added. The mixtures were incubated in a heat block at 65°C for 15 minutes with gentle inverted mixing every 5 minutes. Then, 250 µL of binding buffer was added and gently vortexed. Cell lysates were loaded into spin columns, centrifuged at 13,000 rpm for one minute, and washed twice with 500 µL of washing buffer. The columns were placed in new microcentrifuge tubes, and 50 µL elution buffer was added directly onto the membrane. The tubes were then incubated at 25°C for 1 minute, followed by centrifugation at 13,000 rpm for 60 sec. The DNA concentrations were measured using a NanoDrop 2000 spectrophotometer. Only five of 10 presumptive HFBs have good DNA concentrations (Supplementary Material); therefore, the researchers decided not to subject them to further analysis.

2.4.2 PCR amplification

Extracted DNA was amplified using a PCR amplification kit (TaKaRa Bio Inc.). The primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTTACGA-3') were purchased from Gene LinkTM, USA. PCR reaction mixtures were prepared by combining 39.5 µL sterile ultrapure water, 2 µL 10X PCR buffer, 3 µL MgCl2, 4 µL dNTP mixture, 1 µL 27f primer, 1 µL 1492r primer, 1 µL DNA sample, and 0.5 µL of *TaKaRA* Taq. Then, a touchdown PCR was performed using a T-100TM thermal cycler (Bio-Rad Laboratories, Inc.) under the following conditions: initial denaturation at 95°C for 3 minutes followed by 29 cycles of denaturation at 95°C for 30 sec, amplification at 68°C for 30 sec, and elongation at 72°C for 30 sec. Then, another 29 cycles of denaturation at 95°C for 30 sec, amplification at 60°C for 30 sec, and elongation at 72°C. The termination step was at 72°C for 5 minutes and stored

in the thermal cycler at 4°C until use. Amplified products were separated in 1% agarose gel in 1X Tris-acetate-EDTA buffer (Hi-Media®) for 45 min at 90V. DNA marker 1 kb plus ladder (New England Biolabs®) was used as a size marker (Supplementary Material).

2.4.3 DNA sequencing

A capillary sequencing technique was employed for DNA sequence analysis. The PCR mixture of each HFB isolate was composed of amplicon and primers, which were incorporated with fluorescently labeled chain terminator ddNTPs (ABI BigDye® Terminator v3.1 Cycle Sequencing Kit). Ethanol precipitation was carried out to remove unincorporated ddNTPs, excess primers, and primer dimers. The purified PCR products from the remaining five presumptive HFBs were loaded into the ABI 3730 xl DNA analyzer (Applied BiosystemsTM). The obtained sequences were subjected to base calling using Sequencing Analysis Software v5.4 (Applied BiosystemsTM). FASTA sequences were analyzed and cleaned using 4Peaks software version 1.8. The cleaned sequences were run through the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD). Comparing the measured sequence with the existing data in the DNA library helps identify the strain with the highest degree of matching, with a minimum threshold of 98% identity.

2.5 Phylogenetic analysis

The phylogenetic relationships among potential HFBs and selected known HFBs were investigated using Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Supplementary Material). Initially, the sequences were aligned using the Multiple Sequence Comparison by Log Expectation (MUSCLE) algorithm within MEGA 11, ensuring an alignment crucial for accurate phylogenetic inference. The Maximum-Likelihood (ML) method was employed for the phylogenetic analysis, incorporating the Tamura-Nei model, which assumes unequal nucleotide frequencies and different substitution rates among pairs of nucleotides. The robustness of the phylogenetic tree was assessed via a bootstrap analysis with 1000 replicates to provide confidence estimates on the observed branching patterns. Bootstrap values of > 75% consider a clade well-supported, while < 75% are viewed as weak and should not be considered reliable.

2.6 Antibiotic susceptibility tests of potential HFB strains

The potential HFB strains were subjected to Kirby-Bauer disk diffusion assay. The *E. coli* ATCC 25922 was used as the control bacterium. The cultures were streaked on TSA plates and incubated at 25°C for 24 hr. Colonies were resuspended in test tubes containing 0.8% saline solution and compared to 0.5 McFarland standard. A sterile cotton swab was immersed in the suspension, and excess liquid was removed by swirling the swab against the sides of the tube. Then, the swab was passed over the entire surface of the Mueller Hinton Agar (MHA; HiMedia®) plate three times. The swab was rotated between streaks at approximately 60° angle to ensure even distribution. Antibiotic discs (Bioanalyse®) were then placed on the MHA plates using a tweezer, and the plates were incubated for 18–24 hr at 37° C. The zone of inhibition surrounding each disk was measured using a caliper and interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (Supplementary Material).

3. RESULTS

3.1 Histamine concentration

In this study, only two of the samples (n = 7) had the highest histamine extracted from the flesh of bullet tuna samples (Table 1). The sample AR-02 has a histamine level of 68.82 ± 0.71 ppm, followed by AR-05 with 62.74 ± 0.05 ppm. The mean histamine concentration was 39.22 ± 19.88 ppm. However, four of the samples (n = 7) have histamine levels < 35 ppm. According to the FDA (2022a), a seafood product containing > 35 ppm of histamine is already considered

Table 1. Histamine concentration (ppm/g) in samples from *A. rochei* (Risso 1810).

	Samples						
	AR-01	AR-02	AR-03	AR-04	AR-05	AR-06	AR-07
Histamine (ppm/g)	31.82 31.49	68.32 69.32	44.11 43.74	20.90 20.30	62.78 62.70	25.77 26.49	20.49 20.79
Mean ± SD	31.66 ± 0.23	68.82 ± 0.71	43.93 ± 0.26	20.60 ± 0.42	62.74 ± 0.05	26.13 ± 0.51	20.64 ± 0.21

poor quality. Therefore, HFBs have proliferated in some bullet tuna samples, causing the production of > 35 ppm of histamine.

3.2 Isolation and characterization of potential HFB

Typical HFB, which appeared purple-colored colonies, were successfully isolated on modified Niven's agar plates (Figure 1). The colony and cell morphologies of presumptive HFB are shown in Table 2. Most colonies have sizes ranging from 0.3 cm to 0.5 cm and were described as circular, with smooth surfaces, raised elevation, and an entire edge. Regarding the staining morphology, nine of the 10 isolates were gram-negative rods. Moreover, the halo coloration of the 10 presumptive HFB isolates varied from light to dark purple (Figure 1). The largest halo size was observed in Hfb_4, measuring 3.32 ± 0.91 mm, whereas the smallest halo was found in Hfb_12 with 0.37 ± 0.07 mm. Thus, results suggest that the presumptive HFBs isolated from the flesh of bullet tuna samples exhibit varying abilities to convert histidine to histamine.

All presumptive HFBs were subjected to molecular identification. Five of 10 isolates were identified at the species level (Table 3). Specifically, three isolates (Hfb_5, Hfb_8, and Hfb_13) were identified as *Proteus mirabilis* species, while two were *Klebsiella pneumoniae* (Hfb_6, and Hfb_10). The phylogenetic tree provides a detailed analysis of the genetic relationships among various HFB strains (Figure 2). Sequences of seven known HFB strains

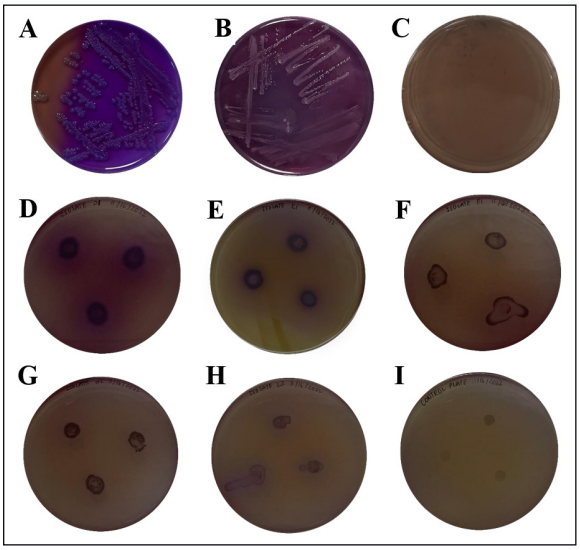

Figure 1. Presumptive HFB isolates on Niven's agar plates. Bacteria appear in purple-colored colonies on modified Niven's agar plates, like A) Hfb_06 and B) Hfb_5. C) Control (uninoculated) plate. Further analysis was made to check their abilities to decarboxylate histidine by using Niven's agar plates. D-H) Purple halo was observed on each plate. I) Uninoculated (with paper disks) control plate.

Table 2. Colony and cell morphologies of presumptive HFB isolates.

Characteristics	Histamine-forming bacteria isolates									
	Hfb_4	Hfb_5	Hfb_6	Hfb_7	Hfb_8	Hfb_9	Hfb_10	Hfb_11	Hfb_12	Hfb_13
Colony morphology										
Size (cm)	0.4	0.5	0.3	0.3	0.5	0.4	0.4	0.4	0.3	0.4
Shape	Circular	Circular	Circular	Circular	Circular	Irregular	Circular	Circular	Circular	Circular
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised	Raised	Raised	Raised	Convex	Raised	Raised	Raised
Edge	Entire	Entire	Entire	Entire	Undulate	Undulate	Curled	Undulate	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Color on modified Niven's agar	Purple	Purple	Purple	Purple	Purple	Purple	Purple	Purple	Purple	Purple
Texture	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid	Mucoid
Halo surrounds the colony	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Halo size on Niven's agar (mm)	3.32 ± 0.91	1.92 ± 0.56	2.29 ± 0.83	1.50 ± 0.43	1.49 ± 0.33	2.50 ± 0.53	2.12 ± 0.50	2.03 ± 0.80	0.37 ± 0.07	1.60 ± 0.60
Cell morphology										
Gram stain	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Arrangement	Single	Single	Single	Single	Single	Single	Single	Single	Single	Single

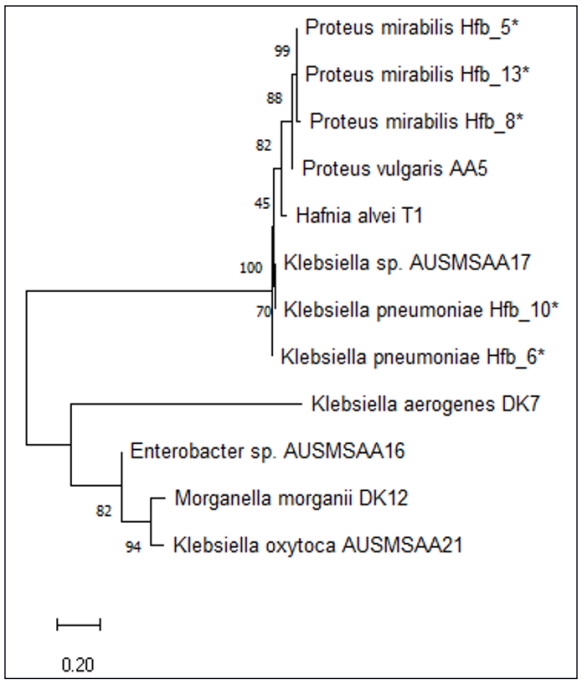

Figure 2. The phylogenetic tree shows close genetic relationships among potential HFB *P. mirabilis* strains and a robust clustering of *Klebsiella* species, indicating strong genetic links supported by high bootstrap values. (*) potential HFB strains identified in this study. The phylogenetic tree was constructed using MEGA version 11.

were retrieved from GenBank (Supplementary Material). It revealed that the potential HFB *P. mirabilis* strains are closely related, as indicated by high bootstrap values (99, 99, and 88, respectively). On the other hand, *Klebsiella*species formed a robust cluster with potential HFB *K. pneumoniae* strains and a *Klebsiella* sp. (AUSMSAA17) showing a bootstrap support of 100, suggesting a strong genetic link.

3.3 Antibiotic resistance of potential HFB

A disk diffusion assay was conducted to assess the extent of antibiotic resistance or sensitivity among the potential HFB strains. The zones of inhibition were measured after 24 h of incubation. Results indicated that antibiotic resistance was prevalent among the potential HFB strains (Table 4). For instance, *K. pneumoniae* Hfb_10 exhibited resistance to erythromycin (Figure 3). Most of them were resistant to erythromycin (n = 5) and tetracycline (n = 4) but sensitive to cefepime and meropenem (n = 5), ciprofloxacin, gentamicin, and sulfamethoxazole-trimethoprim (n = 4). Consequently, different patterns of antibiotic resistance were identified (Table 5). It was also found that all potential HFB were multidrug-

Table 3. Identification and genomic comparison of potential HFB strains with high sequence identity.

Strain	Organism identified	Percentage identity (%)	GenBank accession number
Hfb_5	*Proteus mirabilis*	99.83	NR_113344.1
Hfb_6	*Klebsiella pneumoniae*	99.81	NR_117686.1
Hfb_8	*P. mirabilis*	100.00	NR_114419.1
Hfb_10	*K. pneumoniae*	99.83	NR_117683.1
Hfb_13	*P. mirabilis*	99.71	NR_043997.1

Table 4. Antimicrobial susceptibility test results of potential HFB strains. Nine antibiotics were used as follows: Ciprofloxacin (CIP), Tetracycline (TE), Erythromycin (E), Gentamicin (CN), Cefepime (FEP), Meropenem (MEM), Sulfamethoxazole-trimethoprim (SXT), Ampicillin (AM), Chloramphenicol (C). Zones of inhibition were measured and interpreted as follows: resistant (R), susceptible (S), or intermediate (I).

Antibiotics	CIP	TE	E	CN	FEP	MEM	SXT	AM	C
Potency	5 µg	30 µg	15 µg	10 µg	30 µg	10 µg	25 µg	10 µg	30 µg
P. mirabilis Hfb_5	38 mm	32 mm	9 mm	18 mm	31 mm	31 mm	27 mm	24 mm	0 mm
	(S)	(S)	(R)	(S)	(S)	(S)	(S)	(S)	(R)
K. pneumoniae Hfb_6	32 mm	9 mm	10 mm	17 mm	35 mm	34 mm	23 mm	0 mm	0 mm
	(S)	(R)	(R)	(S)	(S)	(S)	(S)	(R)	(R)
P. mirabilis Hfb_8	37 mm	10 mm	7 mm	17 mm	33 mm	32 mm	22 mm	26 mm	24 mm
	(S)	(R)	(R)	(S)	(S)	(S)	(S)	(S)	(S)
K. pneumoniae Hfb_10	20 mm	12 mm	0 mm	14 mm	31 mm	30 mm	0 mm	0 mm	4 mm
	(I)	(R)	(R)	(I)	(S)	(S)	(R)	(R)	(R)
P. mirabilis Hfb_13	29 mm	10 mm	0 mm	15 mm	30 mm	31 mm	21 mm	7 mm	20 mm
	(S)	(R)	(R)	(S)	(S)	(S)	(S)	(R)	(S)

Note: Mean (n = 3) measurements.

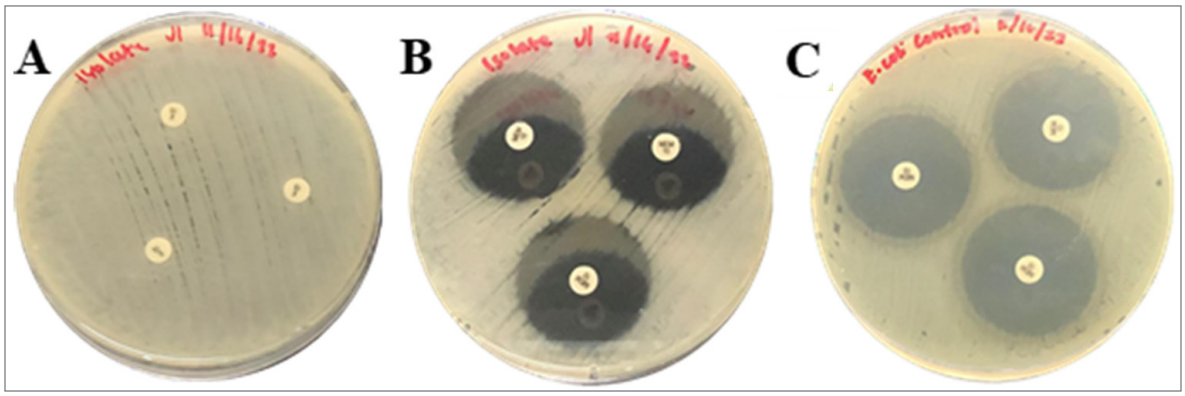

Figure 3. Antibiotic susceptibility test results. A) *K. pneumoniae* Hfb_10 exhibits resistance to E (15 µg) with confluent growth around the disks. B) However, it presents susceptibility to MEM (10 µg) with large inhibition zones. C) The *E. coli* ATCC 25922 control bacterium shows susceptibility to MEM indicated by clear inhibition zones.

resistant organisms (MDROs), demonstrating resistance to two or more antimicrobial drugs (Table 5). Notably, *K. pneumoniae* strains exhibited more significant resistance patterns, with some showing resistance to four and five antibiotics.

4. DISCUSSION

4.1 On histamine concentration

In order to ensure the quality and safety of fresh fish for consumption, regulatory limits on histamine concentration levels are implemented. Various countries, such as New Zealand, Australia, and the European Union, have set their maximum histamine levels for raw fish at 200 ppm (Visciano et al. 2020). In the Philippines, the same regulatory limits as those of the countries mentioned above are issued by the Bureau of Fisheries and Aquatic Resources (BFAR-DA 2001). However, the FDA has updated its Compliance Policy Guide regarding the surveillance of decomposition and histamine formation in scombrotoxin-forming fish and fishery products (FDA 2022a). Under stricter guidelines, fish containing more than 35 ppm of histamine are considered adulterated, as they are decomposed or produced in unsanitary conditions, and those with histamine levels over 500 ppm are a health hazard. Furthermore, these updated regulations require facilities processing histamine-prone fish to implement detailed HACCP plans, focusing on critical control points such as time and temperature.

4.2 On HFB present in bullet tuna

Bacteria are present in all natural environments, including fish tissue. In small numbers, they are not dangerous and can even benefit the fish. However, this can also indicate poor hygiene and mishandling during the processing and storage of the fish (Simora and Peralta 2018; Feng et al. 2016; Satomi 2016; Guillén-Velasco et al. 2004). In this case, the proliferation of HFB in fish could harm consumers, as it may increase histamine concentrations, potentially causing HFB when ingested. In this study, most of the isolated presumptive HFB from the bullet tuna samples were gram-negative bacilli. The source of the bacteria and its characteristics usually belong to *Enterobacteriaceae*. This is corroborated by a study by Refai et al. (2020), wherein *Enterobacteriaceae* was the predominant HFB found among the examined fish and fish products, including smoked herring, salted mullet, mackerel, and sardines. Furthermore, Klausen and Huss (1987) also noted that most HFBs are characterized as gram-negative rods within the Enterobacteriaceae family, emphasizing that only strains of *K. aerogenes, Morganella morganii*, and selected strains of *K. pneumoniae*are responsible for the high histamine yields in Scombridae fish.

Table 5. Phenotypic antibiotic resistance patterns of potential HFB.

Potential HFB strains	Pattern of Resistance	No. of Antimicrobial Agent
Proteus mirabilis Hfb_5	E-C	2
P. mirabilis Hfb_8	TE-E	2
P. mirabilis Hfb_13	TE-E-AM	3
Klebsiella pneumoniae Hfb_6	TE-E-AM-C	4
K. pneumoniae Hfb_10	CIP-E-SXT-AM-C	5

Normal fish skin, gills, and intestine microflora contain HFB (Refai et al. 2020; Koohdar and Shoaibi-Omrani 2016; Bjornsdottir-Butler et al. 2015). The quantity of these organisms in these microenvironments is influenced by the abuse of temperature and contaminants. According to studies, the intestinal tract of scombridae fish is a key source of bacteria that produce histamine (Tembhurne et al. 2013; Gu et al. 2022). Consequently, species such as *Enterobacteriaceae, Lactobacillus, Morganella, Photobacterium, Pseudomonas, Raoultella, Staphylococcus, Tetragenococcus,* and *Enterococcus* are reported to be common bacteria capable of producing biogenic amine compounds, making the intestinal tract their reservoir (Dalgaard et al. 2008; Satomi et al. 2016). Psychrophilic HFB, which can produce high histamine levels at low temperatures, was isolated from the swordfish meat (Torido et al. 2012). These HFBs were identified as *Phb phosphoreum* and *Phb iliopiscarium*. In this study, the potential HFB strains isolated from bullet tuna samples were not as diverse as expected. This could be due to different factors, such as sample size, physico-chemical properties of the fish, and, most probably, the absence of severe deterioration in the fish. Nonetheless, the potential HFBs identified in this study are commonly found in fish products (Oktariani et al. 2022; Hu et al. 2015; Emborg ad Dalgaard 2006).

The results obtained from the bacterial identification coincide with the results of Tembhurne et al. (2013), in which potential HFB strains identified were *P. mirabilis* and *K. pneumoniae*. These bacteria are characterized as common histamine-forming gram-negative rod bacteria belonging to the Enterobacteriaceae family. Several studies investigating the presence of HFB in fish have isolated *Proteus* and *Klebsiella* strains, along with *Morganella*, *Pseudomonas*, and *Vibrio* species (Refai et al. 2020; Joshi and Bhoir 2011; Comas-Basté et al. 2019). These findings indicate that these bacteria are commonly occurring histamine-formers and suggest the dominance of histamine-forming Enterobacteriaceae in fish. Notably, genetic analyses were not performed to identify the histidine decarboxylase (*hdc*) gene responsible for histamine production in bacteria. This genetic marker is crucial for confirming the specific pathways through which histamine is produced (Ferrario et al. 2014). Nevertheless, the phylogenetic tree showed that the potential HFB strains are closely related to known HFB, such as *P. vulgaris* AA5 (Genbank accession number KJ459008.1) and *Klebsiella*sp. AUSMSAA17 (Genbank accession number KP689584.1). Therefore, further studies must include molecular techniques such as PCR amplification of the *hdc* gene to definitively identify and characterize the histamine-producing capabilities of the bacterial strains isolated in this study.

4.3 On antibiotic resistance of HFB

Antibiotic resistance occurs when a bacterium develops the ability to tolerate antibiotics. This can happen through intrinsic resistance, which is a bacterium's natural or innate ability to withstand the effects of antibiotics due to mutations generated by physiological changes (Zhang 2007). Intrinsic chromosomal β-lactamases are responsible for the resistance of *Enterobacteriaceae* to β-lactam antibiotics (Susić 2004). In this study, different β-lactamases were used to test the resistance of potential HFB. All potential HFB strains were characterized as MDROs as they exhibited resistance to two or more antibiotics. It has been reported that enteric bacterial pathogens, including *P. mirabilis* and *K. pneumoniae*, were reported to resist the action of β-lactamase antibiotics (Bush and Jacoby 2010). Furthermore, the World Health Organization (WHO) highlighted the threat of gram-negative bacteria that are resistant to multiple antibiotics, which include *Proteus* and *Klebsiella* (WHO 2017). It has been reported that *P. mirabilis* contains multi-drug resistance (MDR) genes, such as *blaTEM*, *aac(6')-lb*, *Intl1* and *Intl2*, responsible for multi-drug resistance (Gumar et al 2022). On the other hand, *Klebsiella pnuemoniae* has MDR genes responsible for resistance against ampicillin (*SHV-1*penicillinase) (Wyres and Holt 2018), tetracycline (*tetA*, *B*, *D*, and *G*) (Taitt et al. 2017), and chloramphenicol (*cmlA1*) (Kumar et al. 2011).

Despite limited attention to assessing the marine environment for antibiotic resistance genes (ARGs) and ARB (Allison 2005), studies show the prevalence of ARGs in fish samples. In the study by Ghosh and Mandal (2010), bacterial isolates from commercial marine fish showed resistance to ampicillin, posing a health risk to consumers as these fish might be a reservoir of ARB. Additionally, a study conducted in Tanzania found that bacterial isolates from marine fish samples were more resistant to antibiotics, including tetracycline and erythromycin (Marijani 2022), compared to those isolated from freshwater fish. This corresponds with the results of the study, in which bacterial isolates from bullet tuna were resistant to some antibiotics.

Antibiotic-resistant HFB presents a significant threat to food safety, primarily due to its role in exacerbating the risk of HFP. Such

bacteria become difficult to control with standard antimicrobial treatments due to their resistance. This resistance not only complicates the management of bacterial populations in aquatic environments and food products but also challenges traditional food preservation techniques that depend on reducing microbial activity. Furthermore, ARB can transfer their resistance genes to other pathogens, increasing the prevalence of resistance across different bacterial species (Jian et al. 2021). The presence of these resistant bacteria can lead to widespread foodborne illnesses, food recalls, economic losses in the seafood industry, and public health risks.

5. CONCLUSION

In this study, the histamine concentration of bullet tuna samples had an average of 39 ppm, below the regulatory limit of the Bureau of Fisheries and Aquatic Resources. However, adopting a stricter approach, this amount is considered to be adulterated by the Food and Drug Administration and should strictly follow proper handling and storage. Potential histamine-forming bacteria were also found in the fish samples and were identified as *Proteus mirabilis* and *Klebsiella pneumoniae*. The further proliferation of these bacteria could lead to the toxic accumulation of histamine in the fish. Finally, *P. mirabilis* and *K. pneumoniae* both exhibit multi-drug resistance. Most of the potential HFBs were resistant to erythromycin, tetracycline, ampicillin, and chloramphenicol but were sensitive to cefepime and meropenem. This indicates how widespread antibiotic resistance is in wild-caught fish, which is a cause for food safety concerns. It also highlights the need for a deeper understanding of the various factors influencing the antibiotic resistance of these bacteria in fish.

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SUPPLEMENTARY MATERIAL

Link to the electronic supplementary material. Supplementary file.

AUTHOR CONTRIBUTIONS

Delos Santos ST: Conceptualization; Methodology, Formal analysis, Investigation, Data curation, Writing - original draft preparation, Writing - Reviewing & editing, Visualization. **Bastian GM:** Formal analysis, Investigation, Data curation, Writing - Original draft preparation, Writing - reviewing & editing, Visualization. **Deyta J:** Data curation, Writing - original draft preparation. **Fortaleza JAG:** Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft preparation, Writing - Reviewing & editing, Visualization. **Managuelod EM:** Formal analysis, Investigation, Data curation, Writing - Original draft preparation, Writing - Reviewing & editing, Visualization. **Nevado DLR:** Methodology, Formal analysis, Investigation, Data curation, Writing - Original draft preparation, Writing - Reviewing & editing, Visualization. **Salazar RA:** Supervision, Funding acquisition. **De Jesus RS:** Methodology, Software, Validation, Formal analysis, Writing - Reviewing & editing, Supervision.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ETHICS STATEMENT

No animal or human studies were carried out by the authors.

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