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Assessment of Histamine Levels and Histamine-producing Bacteria in *Euthynnus affinis* Marketed in Tanga and Mtwara Coastal Areas, Tanzania

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Abstract: Histamine (scombrotoxin) food poisoning is a community health risk associated with consumption of some marine fish species and it has been proposed that some anaerobic bacteria contribute to this form of food poisoning. However, histamine levels and histamine producing bacteria in fish marketed in Tanzania have not been appraised. This study was conducted to determine histamine levels and histamine-producing bacteria in the *E. affinis* (tuna) value chain in Tanga and Mtwara regions. High-performance thin-layer chromatography (HPTLC) was used to determine histamine levels, Bacteriology and biochemical tests were employed to determine histamine-producing bacteria. A total of 64 tuna fish samples were collected from deep sea fish market and Sahare Tanga and ferry market, Mtwara coasts. The mean level of histamine was found to be 86.4±43.9 in Tanga and 64.8±47.5 in Mtwara and the overall mean was 77.1±46.4. All fish 64 samples were positive for histamine; however, the mean level was within the recommended limit for consumed fishery products according to European Union regulation of 200 mg/kg (m) to 400 mg/kg (m) (EC, 2005). *Klebsiella spp, pseudomonas spp,* and *proteus spp* were isolated and confirmed as histamine-producing bacteria based on bacteriology and biochemical characteristics. It concluded that although within the recommended levels, all fish samples were positive to histamine and three bacterial species associated with its production isolated. To prevent raising histamine to concern levels, maintenance of hygiene and low temperature along the production chain should be observed.

Keywords: Colony-Forming Unit, *histamine*, Scrombotoxin, Tuna, High-Performance Thin-Layer Chromatography, Food Poisoning

1. Introduction

Tuna fish like *Euthynnus affinis* (Kawakawa) has low-fat contented and important source of protein to a human being essential in improving body immunity and prevention of heart diseases [21]. Tuna fish and tuna-related species (kawakawa) is the saltwater fish which is broadly but sparingly disseminated all over the ocean and highly consumed along Indian coastal areas like Tanga, Mtwara, Dar es Salaam, Lindi, and Zanzibar Islands in Tanzania [19]. Consumption of tuna and tuna-related fishes has been associated with histamine fish poisoning and have been incriminated to be the cause [3].

Histamine food intoxication is a most important distress in the fish and fishery industry globally [12]. Fish putrefaction can lead to the accretion of histamine and other biogenic amines to a toxic level [1]. During decomposition, the histidine in muscle is converted to histamine [2]. Histamine levels intensification indicates tuna fish deterioration [17]. The presence of biogenic amines at high levels may cause foodborne intoxications which develop allergy-like symptoms such as headache, fatigue, nausea, diarrhea, vomiting, oral burning sensation, hives, itching, red rash, and hypotension. The symptoms happen within limited minutes, limited hours to 24 hours, or a few days after ingestion of histamine at toxic levels [3].

Sanitary and quality built-up are required to prevent histamine contamination in fishery industries and thus the possible health hazards to the consumer. The natural level in fresh fish is less than 5mg per 100g and the higher value shows spoilage [9]. Histamine is formed during the storage of pelagic fish species example tuna, mackerels, and Carangidae species at high temperatures. In deceased fish, histamine-forming bacteria in muscle tissue progress and produce histamine through decarboxylation of amino acids the originator of histidine formation [13]. The formation of histamine in tuna and tuna-related species has been recognized to microbial accomplishment rather than endogenous histidine decarboxylase actions [17].

Euthynnus affinis (Kawakawa) is a species of ray-finned bony fish in the family Scombridae, known as tunas [7]. Also are normally named little tuna and mackerel tuna. Kawakawa is a species of tuna that exists in the tropical and sub-tropical waters of the indo-west pacific [14]. The histidine in tuna muscles can be transformed to histamine by the decarboxylation process by the act of histamine-producing bacteria which produces histidine decarboxylase enzymes [11]. Temperature and time are the main influences inducing histamine production [5]. The produced histamine cannot be incapacitated by freezing, heating, or drying.

Histamine producing bacteria in fish include enteric bacteria like Proteus vulgaris, Proteus mirabilis, Enterobacteraerogenes, Enterobacter cloacae, Serratiafonticola, Serratialiquefacience and Citrobacterfreundii [6, 16]. Others include Clostridium spp, Proteus spp, Vibrio aliginoliticus, Acinetobacterlowfii, Plesiomonasshigelloides, Pseudomonas putida, pseudomonas fluorescent, Aeromonasspp, and Photobacterium spp. [15]. In tuna and tuna-related species categorized as scombroid fish the commonly isolated bacteria, includes Morganella psychrotolerans, and Morganellamorganii [10].

Histamine food poisoning is similarly recognized as scrombotoxin poisoning. The scrombotoxin food poisoning problem was once very debated because its symptoms are similar to several other food-borne illnesses [4]. Since 1980 the cases of scrombotoxin fish poisoning declined in developed countries like Japanese following institution of low-temperature storage of fish [8]. Nevertheless, in Tanzania, limited information is available about histamine food poisoning [20].

In Tanzania particularly Tanga and Mtwara, food poisoning as an outcome of tuna fish consumption is a collective problem. Community knowledge concerning histamine is significant for the avoidance of the problem. To protect the consumer from histamine fish poisoning it is important to study and monitor levels and related risk issues. Furthermore, post-harvest losses including physical losses, quality losses, and financial losses are inevitable if not permitted. This study pointed to determine the community awareness and the levels of histamine in tuna and tuna-related species marketed in the Tanga and Mtwara regions. The outcomes from this study will form the basis for forecasting the prevention of histamine fish poisoning hence protecting fish consumers.

2. Material and Methods

2.1. Study Area

The study was conducted in Tanga and Mtwara coast areas where consumption kawakawa and other tuna related species is common. Samples were collected from March to August 2020 in Tanga deep sea and Sahare market and Mtwara ferry market.

2.2. Study Design

A cross-sectional study design was employed to determine histamine levels using a high-performance thin-layer chromatography technique as described by Taylor *et al*., (1978) [20]. On the other hand, bacterial isolation and biochemical tests were carried to determine histamine-producing bacteria.

2.3. Sample Collection and Treatment

Samples from Tanga and Mtwara coast were collected in aseptic and sterile conditions then packed in water-tight bags to avoid cross-contamination. The samples were stored in the cool box then transferred to the refrigerator and kept at -4°C overnight. All samples were transported to microbiology laboratory in the College of Veterinary Medicine and Biomedical Science at Sokoine University of Agriculture for analysis. The samples were maintained in cold chain packaging and storage of the sample in the cool box prior to analysis. The analysis included determination of total viable count, macro morphology, and micromorphology characteristics (Gram stain), confirmation of histamine producing bacteria by biochemical test.

2.4. Analysis of Histamine Levels

The histamine level in fish samples was determined using a High-performance thin-layer chromatography (HPTLC). The plates were visualized using Vision CATS-licensed to pharm PAL with SN 103056. The standard was designed with low concentration to increase the chance of detecting relatively low concentrations of histamine present in the fish sample. The standard was used as a positive control. Samples were tested with their own blank to allow for any non-specific effects caused by the chemical of interest. The results were obtained and converted to mg/Kg. Each sample data was triplicated (XA, XB, and XC) and then the mean was calculated and recorded.

2.4.1. Analysis Procedure

10 gm of fish samples was homogenized with 50 ml methanol and transferred with methanol risings to a 100 ml volumetric flask. The stoppered flask was immersed in a water bath at 60°C for 15 minutes then cooled. 50mls of methanol 100 mls which was then centrifuged to obtain a clear extract for TLC.

The extract was spotted together with histamine standard

solution onto TLC plate. A useful spotting regime were 1, 5, 10 microliters of extract and 0.05, 0.07, 0.08, 0.1, and 0.12 microliter standards and the spotting procedure was automated by computer commands.

The spotted plates were immersed into a solvent mixture to enter the mobile phase. The plates were then thoroughly dried with a hair drier then sprayed with 0.2g ninhydrin reagent in 100ml methanol and then heated to 110°C until the spot appeared. The plates were then transferred to the CAMAG LINOMAT visualizer whose output was read in form of peak area implying histamine concentrations. (Vision CATS-licensed to pharm PAL with SN 103056). As described by Taylor, Lieber, and Leatherwood (1978) [20].

2.4.2. Preparation of Standards

The standards were prepared based on international conference harmonization guidelines (ICH). Calibration curves of standard 50%, 70%, 80%, 100%, and 120% same as 0.05, 0.07, 0.08, 0.10, 0.12 mg per ml respectively. Graphical using equation of straight line (y=mx+c) Calibration curve of the standard proved to r squared equal to 0.99996.

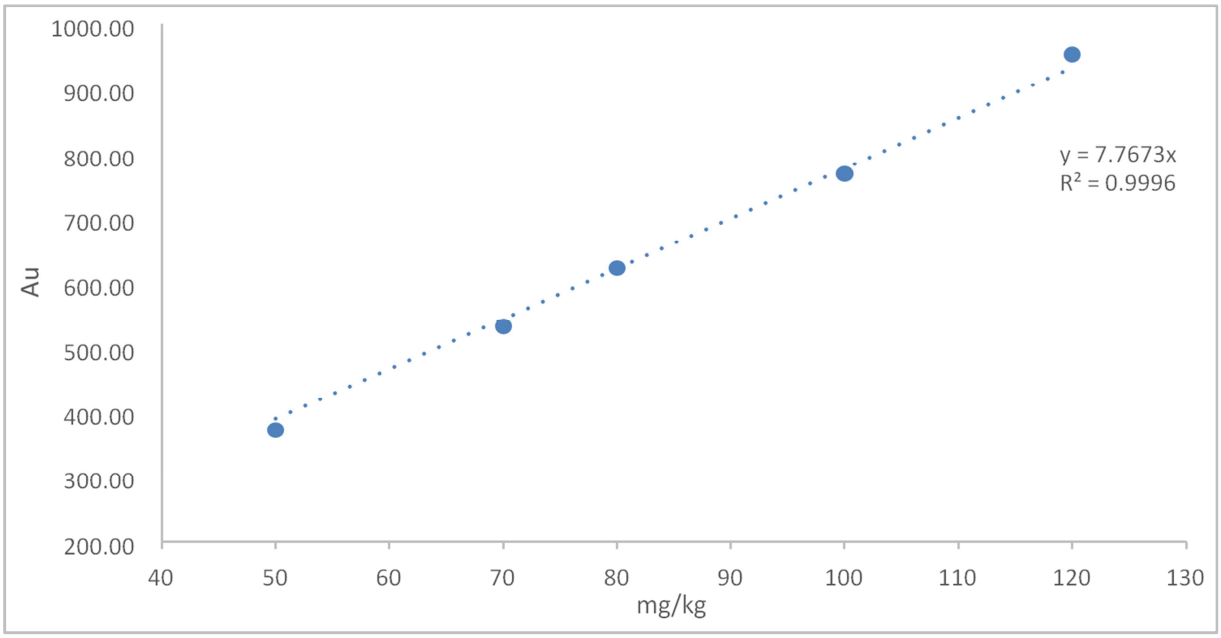


Figure 1. *Calibration curve for determination of histamine in fish samples.*

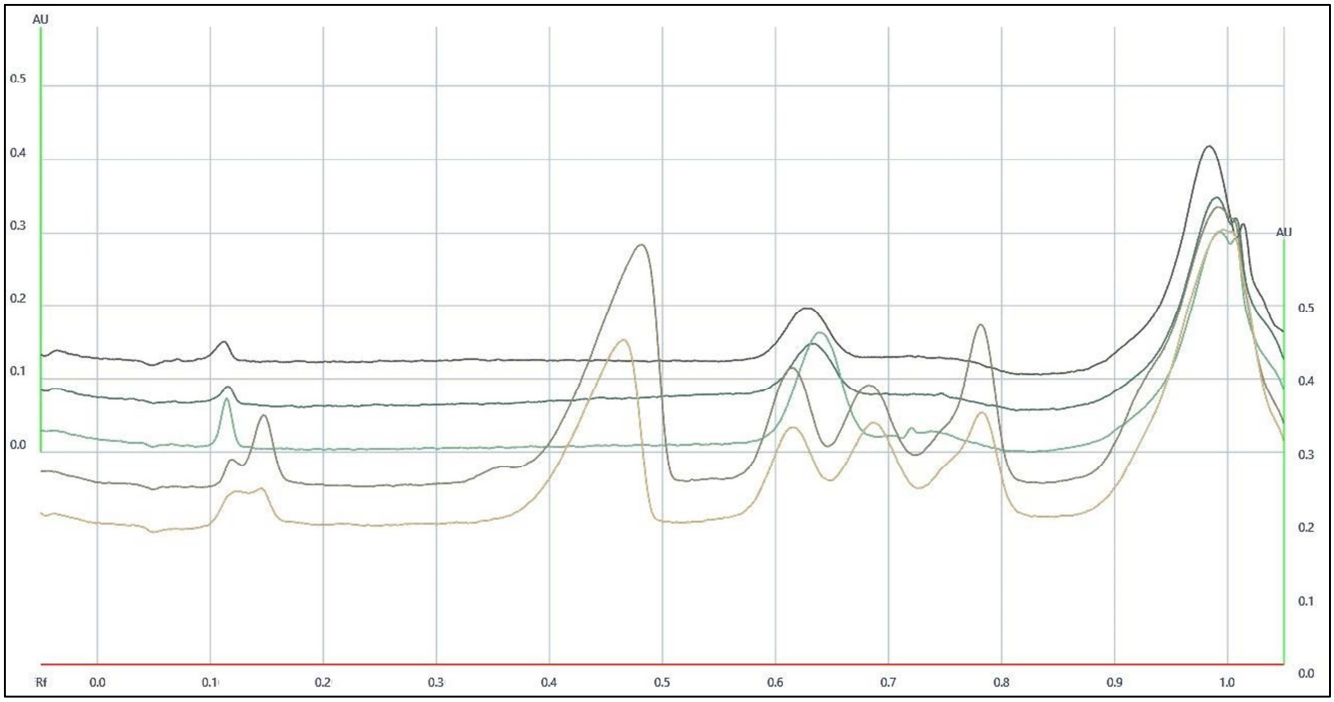


Figure 2. *HTPLC densitogram at UV 214 nm for determination of histamine in fish samples.*

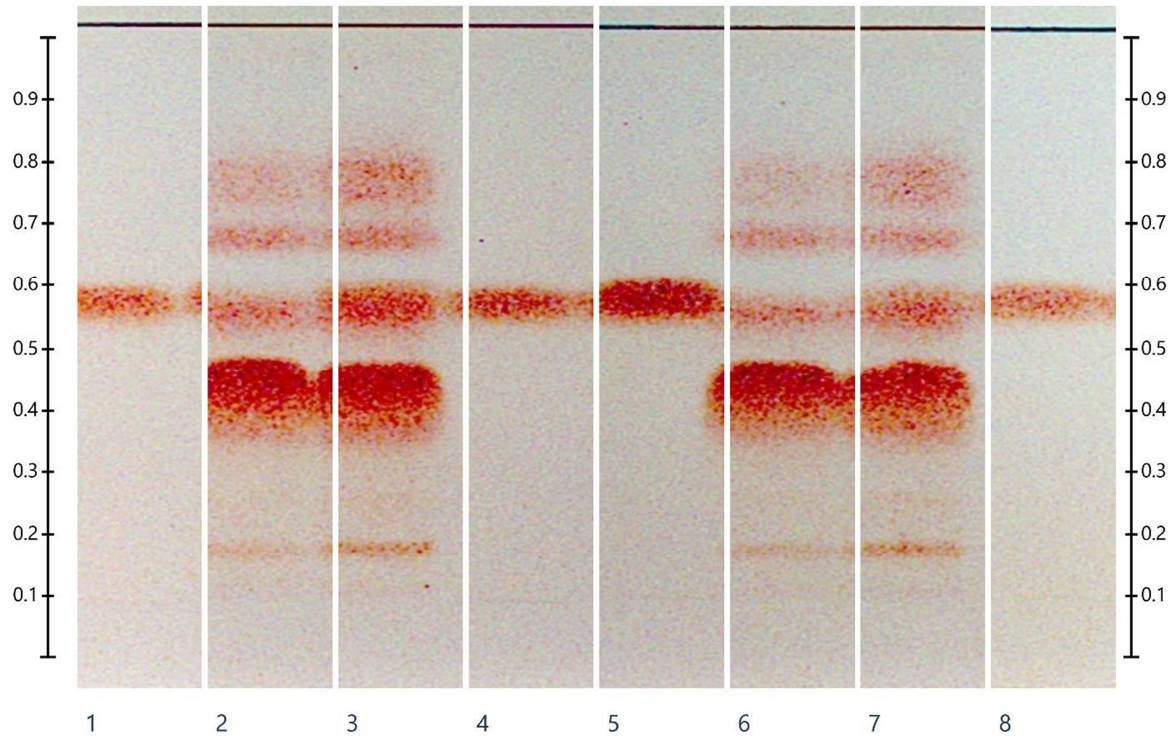


Figure 3. *HPTLC profile under white light 366 nm of histamine in fish samples, tracks 1, 4, 5, and 8 are standards while tracks 2, 3, 6, and 7 are samples.*

2.5. Identification of Histamine Producing Bacteria

2.5.1. Isolation of Histamine Producing Bacteria

Samples were thawed under the normal condition at room temperature (25-27°C). 10 grams of each sample was chopped under aseptic conditions then transferred to an enrichment media (tryptic soy broth (TSB) then incubated at 37°C for 24 hours. Then inoculated by streaking method in nutrient agar (NA), tryptic soy agar (TSA), and McConkey agar (MCA) and incubated at 37°C for 24 hours. The suspected bacteria colonies by macromorphology characteristics were isolated and subcultured to obtain pure colonies for micromorphology and biochemical tests.

Biochemical Test

Suspected bacteria based on colonies macromorphology and gram stain, were subjected to biochemical test to confirm histamine producing bacteria based on their ability to utilize biomolecule resulting to useful compound. Indole test, oxidase test, urease test, triple sugar iron (TSI), and other biological characteristics were confirmed including shape and motility.

2.5.2. Determination of Total Viable Counts

Samples to be used for bacterial counting were thawed under the normal condition at room temperature 25-27°C. Normal saline was then prepared to be utilized for the tenfold method of bacteria enumeration in the samples.

Bacterial enumeration

10 grams of the fish sample was chopped under aseptic condition and then transferred to an enrichment media (tryptic soy broth (TSB)) then incubated at 37°C for 24 hours. 1ml of each sample was added in 9ml normal saline and serially diluted into each 9ml normal saline along ten test tubes. The last 1ml in each serial dilution was discarded to reduce the number of bacteria. 1ml from each diluted test tube, (10^{-1}to10^{-10}) was then transferred to nutrient agar and incubated for 24 hours at 37°C. Each sample was counted in form of CFU/ml depending on the growth of bacteria in each of their dilution factors.

The total number of bacteria was calculated using the formulas; (cfu/ml) = *log of number of counted colony + dilution factors.*

3. Data Analysis

Data were analyzed using "Statistical Package for Social Sciences" (SPSS) version 20. Results were summarized using descriptive statistics such as frequency distribution table, mean, and cross-tabulation whenever required.

4. Results

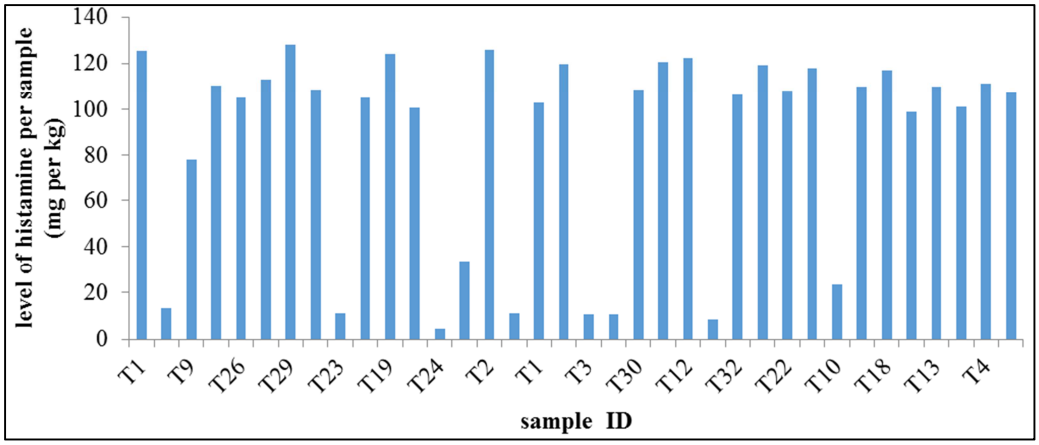
4.1. Status of the Sampled Fish

64 samples of kawakawa (*Euthynnus affinis*), 28 from Mtwara and 36 from Tanga coast were collected in aseptic and sterile conditions then packed in water-tight bags to avoid cross-contamination between the samples. The samples were later stored in the cool box then transferred to the refrigerator to cool at -4°C overnight. All samples were in good condition and later transported in a cold chain maintained by the packaging and storage of the sample together with ice blocks in the cool box prior to further analysis. The total viable count, macro

morphology, and micromorphology characteristics (Gram staining technic), confirmation of histamine producing bacteria by biochemical test for the isolated histamine formers was conducted at the College of Veterinary Medicine and Biomedical Science microbiology laboratory in the Sokoine University of Agriculture. On the other hand, histamine analysis was conducted in the laboratory at the College of Pharmacy at Muhimbili University of Health and Allied Sciences.

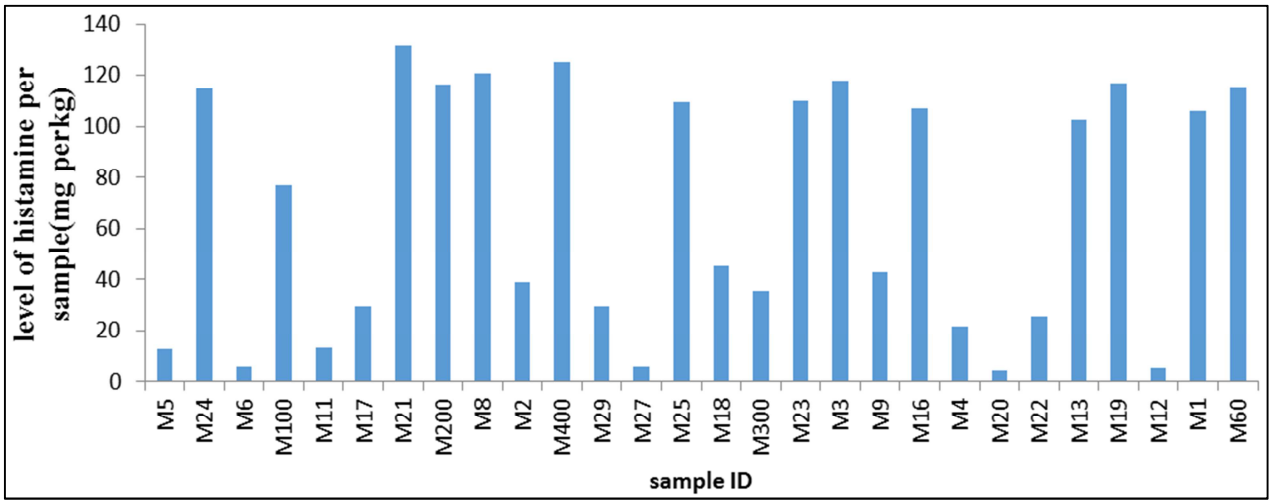
4.2. Histamine Levels

The mean and standard deviation levels of histamine were 86.4±43.9 in Tanga and 64.8±47.5 in Mtwara with minimum values and maximum of (3.3 -128.21) and (2.9-138.86) in Tanga and Mtwara, respectively (Tables 1 and 2). Overall mean and standard deviation for both regions were found to be 77.1±46.4 in all tuna fish samples. All 64 samples were positive for histamine.



KEY: T is Tanga

Figure 4. *The mean level of histamine per sample from Tanga coast.*



Key: M is Mtwara

Figure 5. *Shows the mean level of histamine per sample from the Mtwara coast.*

Table 1. *Histamine means levels and standard deviations in kawakawa (Euthynnus affinis) sample from Tanga and Mtwara coasts.*

	Tanga Histamine Level	Mtwara Histamine Level	Overall
Mean	86.28	64.84	77.09
Standard deviation	43.97	47.59	46.43
Standard error	7.33	9.16	5.85
Minimum value	3.3	2.9	2.9
Maximum value	128.21	138.86	138.86

4.3. Histamine Producing Bacteria

Three bacterial genera were isolated and confirmed as histamine-producing bacteria based on bacteriology and biochemical characteristics. The genera include *Klebsiella, Pseudomonas* and *Proteus*. The bacteria belonging to the

genera known to be responsible for histamine production were identified by observing macro- and micro-morphological characteristics, Gram staining techniques and biochemical tests. The results elaborating this identification are outlined below:

Table 2. *Histamine producing bacteria and total viable count of each kawakawa (Euthynnus affinis) samples in Tanga and Mtwara regions.*

Region	Sample ID	Histamine Producing Bacteria Genus			Total viable count per each screened sample		
		Pseudomonas	*Klebsiella*	*Proteus*	No. of colony	Dillution factor	Cfu/ml
Mtwara	M5	☑	☐	☑	36	6	36 × 10^6
	M24	☐	☐	☐			
	M6	☐	☐	☐			
	M100	☐	☐	☐			
	M11	☐	☑	☑	66	8	66 × 10^8
	M17	☐	☑	☐	43	7	43 × 10^7
	M21	☐	☐	☐			
	M200	☑	☑	☐	56	8	56 × 10^8
	M8	☐	☐	☐			
	M2	☑	☑	☑	68	9	68 × 10^9
	M400	☐	☐	☐			
	M29	☑	☑	☑	70	6	70 × 10^6
	M27	☐	☐	☐			
	M25	☐	☐	☐			
	M18	☐	☑	☐	12	4	12 × 10^4
	M300	☐	☐	☐			
	M23	☐	☐	☐			
	M3	☑	☐	☐	42	9	42 × 10^9
	M9	☐	☐	☐			
	M16	☐	☐	☑	48	7	48 × 10^7
	M4	☐	☐	☑	41	8	41 × 10^8
	M20	☐	☐	☑	40	7	40 × 10^7
	M22	☐	☐	☐			
	M13	☑	☑	☐	37	7	37 × 10^7
	M19	☐	☑	☐	34	8	34 × 10^8
	M12	☑	☑	☑	88	5	88 × 10^5
	M1	☐	☐	☐			
	M60	☐	☐	☐			
	T1	☑	☑	☑	76	4	76 × 10^4
	T6	☐	☑	☐			
	T9	☑	☐	☐	48	9	40 × 10^9
	T14	☐	☐	☐			
	T26	☐	☐	☐			
	T28	☐	☑	☑	39	7	39 × 10^7
	T29	☐	☐	☑	37	8	37 × 10^8
	T15	☐	☐	☐			
	T23	☐	☐	☑	46	7	46× 10^7
	T16	☐	☑	☑	49	6	49 × 10^6
	T19	☐	☑	☑	68	8	68 × 10^8
	T100	☑	☐	☑	58	6	58× 10^6
	T24	☐	☐	☐			
	T34	☐	☐	☐			
Tanga	T2	☐	☐	☐			
	T11	☑	☐	☐	35	8	35 × 10^8
	T1	☑	☐	☑	38	6	38 × 10^6
	T27	☐	☐	☐			
	T3	☐	☐	☐			
	T28	☐	☑	☑	52	6	52 × 10^6
	T30	☐	☐	☐			
	T17	☐	☐	☐			
	T12	☐	☐	☑	45	9	45 × 10^9
	T25	☐	☐	☐			
	T32	☐	☐	☐			
	T33	☐	☐	☑	41	7	40 × 10^7
	T22	☐	☑	☐			
	T31	☐	☐	☐			
	T10	☐	☐	☐			

Region	Sample ID	Histamine Producing Bacteria Genus			Total viable count per each screened sample		
		Pseudomonas	*Klebsiella*	*Proteus*	No. of colony	Dillution factor	Cfu/ml
	T5	☐	☐	☑	44	6	44 × 10^6
	T18	☐	☑	☑	72	5	72 × 10^5
	T35	☐	☑	☑	56	7	56 × 10^7
	T13	☐	☐	☐			
	T20	☐	☐	☑	50	5	50 × 10^5
	T4	☐	☐	☐			
	T7	☐	☐	☐			

5. Discussions

Histamine was detected in all fish (*Euthynnus affinis*) samples although at low levels. The mean histamine level in all samples was within recommended limit value for fishery products which are 200 mg/kg (m) to 400 mg/kg (m) European Union regulation (EC, 2005). The mean levels of histamine in fish samples (±SD) were found to be 86.4±43.9 mg kg and 64.8±47.5mg kg from fish in Tanga and Mtwara respectively. The histamine in all samples was below the recommended values although samples collected from the Tanga coast had their mean levels higher than those Mtwara coast. The observed level of histamine in the samples might be influenced by poor storage circumstances of fish from the catchment area to the coast and unhygienic conditions of the vessels. In addition the lack of maintenance of the cold chain from the catchment area at Mtwara coast might be the source of the observed differences.

The results indicated that fish species samples collected directly from the catchment area (Tanga and Mtwara) had low levels of histamine compared to frozen fish samples collected from fish selling points. This variation might be influenced by poor hygienic condition of the handlers and the vessels used for fish storage. Furthermore, the hygienic conditions at the market are unsatisfactory to support the conduct of food supply business. Similar observations have been reported by earlier studies on histamine levels in fish in different areas [22], [23]. [24] Conveyed mean levels of histamine as 10-178mg/100g in a total of 80 canned fish samples studied in Iran. [22] Have recognized that the levels of histamine in four canned tuna fish ranged between 0-2.4 ppm in South Africa. [23] Examined 23 samples of canned fishing in Bosnia and Herzegovina and found less than 100 ppm of histamine levels.

Temperature control is the, most operative method for safeguarding food safety for fish species likely to produce histamine [18]. Cross-contamination can introduce histamine producing bacteria elevating the level in tuna fish.

The bacteria associated with histamine production in kawakawa fish from Tanga and Mtwara region fish samples include proteus spp, pseudomonas spp and klebsiella spp. More species of klebsiella and followed pseudomonas spp found to be most histamine producers and fish samples with this kind of bacteria shows the higher level of numeration with higher colony forming unit compare to that of proteus spp. Also, presence of single kind of bacteria species proves to have low level of histamine production compared to those samples with mixed colony.

6. Conclusion and Recommendation

6.1. Conclusion

In this study, histamine has been detected in all fish samples at the means levels (±SD) of 86.4±43.9 mg kg in Tanga and 64.8±47.5mg kg in Mtwara. In the present study *Klebsiella, pseudomonas* and *Proteus species* were found to be associated with histamine production in kawakawa fish.

6.2. Recommendation

To prevent histamine formation in tuna fish low-temperature storage of fish throughout the food chain and good hygienic practices in stores retails, markets, and catering centers is recommended.

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