African Journal of Microbiology Research Vol. 6(4) pp. 751-756, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.882
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Histamine-producing bacteria isolated from frozen longtail tuna (*Thunnus tonggoh*)

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Accepted 5 December, 2011

In this investigation, a series of samples were carried out to isolate and identify histamine-producing bacteria and analyze the histamine content for evaluation of harvesting and post harvesting procedures. The target fish was longtail tuna (*Thunnus tonggoh*), which were collected from Oman Sea waters harvested by gillnet or purse seine methods. Bacteriological isolates and the amount of histamine were obtained from the muscle around the gills. The obtained results indicated that the average of total and psychrophilic counts were 4.81 ± 0.26 and 4.66 ± 0.25 Log_{10}CFU/g, respectively. Diverse bacterial isolates were identified as histamine-forming bacteria. Amongst them, *Clostridium perfringens* with the highest abundance in samples contributed 24.4% followed by *Proteus* spp. (23.0%), *Klebsiella* spp. (13.9%), and *Enterobacter* spp. (11.1%). The examined samples of 20.0, 15.0 and 65.0% contained < 20, 20 to 50 and >50 ppm amount of histamine, respectively. Therefore, there are seafood safety risks in the current harvesting and post harvesting methods used in longtail tuna industry.

Key words: Longtail tuna, *Thunnus tonggoh*, histamine, bacteria, Oman Sea.

INTRODUCTION

Consumption of spoiled fish results in the outbreaks of food poisoning such as scombroid poisoning. Scombroid poisoning also called histamine poisoning is a worldwide intoxication, but since it is a rather mild illness it is usually incompletely recorded in most countries (Ahmed, 1991). It is known as a chemical intoxication related with some types of dark meat consumption belonging to Scombroidae and Scombrosocidae families which characteristically high level of free histidine generally in their muscle tissues (Mlcneryey et al., 1996). Histidine is converted to histamine by microbial histidine decarboxylase enzyme. Consumption of spoiled fish and fish products which contain unusually high levels of histamine result in the outbreaks of histamine fish poisoning (Çaklý and Kýþla, 2003; Choudhury et al., 2008). Scombroid poisoning is usually a mild illness with variety of symptoms including headache, giddiness, rash, stomachache, swallowing difficulties, low blood pressure, nausea, vomiting and diarrhea, flushing, tingling and itching of the skin (Taylor, 1986; Lehane and Olley, 2000).

The bacteria that possess large amounts of histamine mainly belong to Enterobacteriaceae family, although a large and diverse group of bacteria have been reported to be responsible for the histamine found in fish (Middlebrooks et al., 1988). In general, the amino acid decarboxylase enzymes, especially histidine decarboxylase, can be found in some species of Enterobacteriaceae, e.g. *Lactobacillus, Pseudomonas, Vibrio, Clostridium* and *Photobacterium* (Taylor, 1986; Ababouch, 1991; Lehane and Olley, 2000). Yoshinaga and Frank (1982) suggested that the diversity of bacteria with histidine decarboxylase activity observed in Scombroids can be attributed to the type of seafood, differences in fish species, duration and temperature storage condition, feeding habits, geographical position, fishing gear, season, water temperature and salinity, the way the product is handled after harvesting and market

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The longtail tuna is distributed in Persian Gulf and in all parts of the Oman Sea both Iranian and Omani waters (Assadi and Dehghani, 1997), and the main fishing methods in these area are drift gillnet, long-line and purse-seine. It is noteworthy to be mentioned that the caught fishes, especially in gill-net method, cannot immediately be collected after entangling and therefore remain inside the water for a while with considerable duration for further transferring on board to be cooled before being frozen and stored. If these delays are prolonged, some post mortem decomposition and accumulation of histamine can occur in the fish. Other factors such as unsuitable handling, post-caching contamination, inadequate chill-storage procedures, inadequate freezing and thawing procedures also affecting the probability of histamine accumulation occurring.

The main objectives of this study were to: (1) identify the type of histamine- producing bacteria and (2) determine the amount of histamine in frozen longtail tuna. This kind of experiments can be used for evaluation of current harvesting and post harvesting methods in longtail tuna industry.

MATERIALS AND METHODS

Twenty frozen samples of longtail tuna were randomly collected from coastal area of Oman Sea in the south of Iran and transferred ashore to be preserved in refrigerated condition for further laboratory examinations. Each specimen was gutted and then cut into small pieces. Since the tissues of gill and gut are considered as the major source of histamine-producing bacteria in fish (Lopez-Sabater et al., 1996), 0.5 to1 kg of the muscle near the gills were collected. Samples were covered with ice and immediately transported to the microbiology laboratory where they were preserved frozen until analysis.

Microbial examination

After thawing at room temperature, samples were skinned and deboned aseptically, and the flesh were homogenized and blended in Waring commercial blender without adding liquid. The homogenates were serially diluted in sterilized normal saline solution, and inoculated on duplicate plates of trypticase soy agar (TSA) for mesophilic and psychrophilic counts after incubation at temperature 37°C for 24 h and 20°C for 5 days, respectively. Enterobacteriaceae family was enumerated in violet red bile dextrose agar (VRBA) after incubation at 37°C for 24 h.

For the enumeration of histamine-producing bacteria, 6 plates were inoculated with 1 ml sample from each appropriate dilution; Pour plateing was used with Niven's medium (Niven et al., 1981) or modified Niven's medium (Yoshinaga and Frank, 1982). At each dilution, duplication plates were incubated at 37°C for 48 h, 2 other plates were set at 20°C for 5 days; for this measurement, the Niven's medium was used, and finally 2 plates containing the modified Niven's medium were set at 37°C for 48 h in anaerobic condition. The colonies with purple halo around Niven's medium and pink halo around in modified Niven's medium were enumerated. These positive colonies were aseptically isolated and streaked on trypticase soy agar slants supplemented with 0.1% L-histidine-Hcl with pH= 6.0 and incubated at 37°C for 24 h. Isolates were stored at temperature of 2°C until used for bacterial species identification.

Niven-positive isolates were Gram stained and examined under oil immersion. Gram-positive isolates were identified by catalase test, carbohydrate fermentation, colony morphology and various biochemical tests. While Gram-negative rods were identified by differential media and carbohydrate and biochemical properties as described by Berge's manual of determinative bacteriology (Holt et al., 1994). All isolated strains were tested for their ability to produce histamine by the methods described by Smith et al. (1982) and Yoshinaga and Frank (1982).

Analysis of histamine

For histamine content measurement, the muscles near the gills were used. The muscle samples were blended in Waring Commercial blender for microbiological examination, and analyzed for the amount of histamine by enzymic method. Enzyme linked immonosurbant assay (ELISA) method was used for this purpose, as proposed by Marcobal et al. (2005) and having a good correlation with high performance liquid chromatography (HPLC) analysis. Veratox® histamine test was from NEOGEN Corporation. It is commonly used for the quantitative analysis of histamine in scombroids.

Statistical analysis

For statistical analysis, the histamine content, the mean values of histamine-producing bacteria, and histamine-producing Enterobacteriaceae counts were compared using a one-way analysis of variance (ANOVA). Statistical analyses were done using the SPSS software (ver. 17), and all significant levels were considered at the level of $p<0.05$.

RESULTS

The mean counts of mesophilic and psychrophilic in 20 samples of longtail tuna were 4.81 ± 0.26 and 4.66 ± 0.25 Log_{10}CFU/g, respectively. The mean of histamine-producing bacterial count was 0.13% of total bacterial load (Figure 1). More also, the mean of Enterobacteriaceae count was 2.66 ± 0.13 Log_{10}CFU/g. Figure 2 shows histamine concentration and the mean values of histamine-producing enterobacteriaceae count in studied samples.

Fourteen bacterial strains with histidine decarboxylase activity were isolated and then tested for their ability to produce histamine, of which 8 strains (57.14%) of these tentative isolates showed positive results (Table 1). Four samples of a total of 20 frozen longtail tuna samples contained <20 ppm amount of histamine; but this amount was 20 to 50 ppm and >50 ppm in 3 and 13 samples, respectively. Table 2 shows histamine concentration and type of bacteria isolated in samples with histamine content > 20 ppm. There was a significant difference in histamine contents in samples with different numbers of histamine-producing bacteria; so that the samples with high quantities of histamine-producing bacteria had significantly higher levels of histamine than other samples

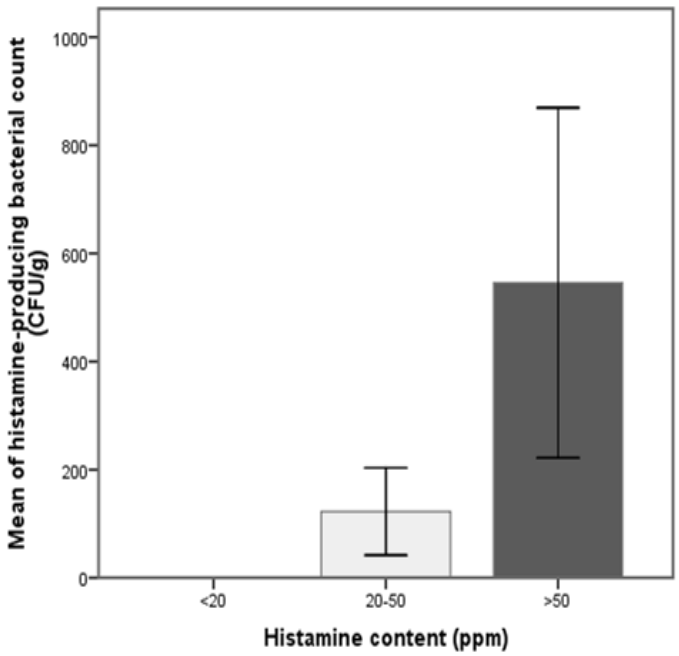

Figure 1. Histamine content and the mean values of histamine-producing bacterial count in longtail tuna.

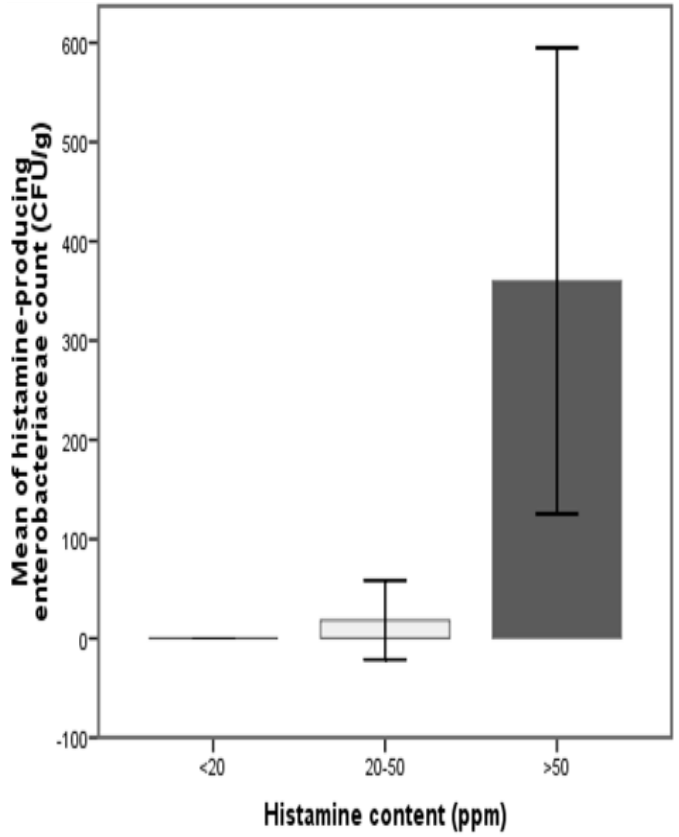

Figure 2. Histamine content and the mean values of histamine-producing Enterobacteriaceae counts in longtail tuna.

Table 1. Histamine-producing bacteria isolated from frozen longtail tuna.

Bacterial species	No. of tentative histamine-forming bacteria	Frequency (%)	Confirmed histamine-producing bacteria
Aeromonas hydrophila	190	2.6	n
Citrobacter freundii	425	5.7	n
Clostridium perfringens	1824	24.4	y
Enterobacter aerogenes	505	6.7	y
Enterobacter cloacae	330	4.4	y
Escherichia coli	105	1.4	n
Klebsiella oxytoca	190	2.6	y
Klebsiella pneumoniae	840	11.3	y
Morganella morganii	560	7.5	y
Proteus mirabilis	925	12.4	y
Proteus vulgaris	790	10.6	y
Pseudomonas aeruginosa	260	3.5	n
Pseudomonas fluorescens	450	6.0	n
Serratia marcescens	65	0.9	n
total	7459	100	-

y = Yes; n = no.

($p<0.05$). The same result was taken for samples with different numbers of histamine-producing Enterobacteriaceae ($p<0.05$).

DISCUSSION

Histamine content was significantly ($p<0.05$) higher for samples that had high histamine-producing bacterial count (Figure 1). The average of histamine-producing bacterial count was 0.13% of the average of total bacterial count, and this was similar to the results obtained by Lopez-Sabater et al. (1996) on the incidence of histamine-producing bacteria in which their estimation was < 0.1% of total bacterial load. Meanwhile, Ababouch et al. (1991) measured this value to be about 0.97% of total flora. Some other studies found higher values and it was reported that histamine-producing bacteria in skipjack and jack mackerel was about 31 and 13.4% of total bacterial load, respectively (Omura et al., 1978; Yoshinaga and Frank, 1982). However, it should be considered that these investigations were carried out with spoiled fish.

Significant relationship ($p<0.05$) was observed between histamine content and Enterobacteriaceae count (Figure 2). Enterobacteriaceae species are the most important histamine-producing bacteria in tuna fishes (Frank et al., 1985). In this investigation 63.5 and 24.4% of histamine-producing bacteria belonged to Enterobacteriaceae and *Clostridium perfringens*, respectively. Lopez-Sabater et al. (1996) reported that 83% of histamine-producing bacteria belonged to the Enterobacteriaceae family. Lopez-Sabater et al. (1994) also reported that all isolates with histidine-decarboxylase activity isolated in their investigation were Gram-negative, and from the 40 strains isolated from Niven's medium, 77.5% belonged to the Enterobacteriaceae family. However, it is noteworthy to mention that only aerobic histamine-producing bacteria could be isolated in their investigations. Meanwhile in present study, both aerobic and anaerobic were found. Based on Yoshinaga and Frank (1982) study, 50% of histamine-producing bacteria isolated were *C. perfringens*.

Furthermore, all the bacterial species with histidine decarboxylase activity isolated in this study (Table 1), have previously been reported by other researchers (Omura et al., 1978; Yoshinaga and Frank, 1982; Taylor and Speckhard, 1983; Frank et al., 1985; Middlebrooks et al., 1988; Lopez-Sabater et al., 1994; Kim, 2001; Tsai et al., 2004; Choudhury et al., 2008). Behling and Taylor (1982) indicated that the histamine-producing bacteria could be divided into two categories: a) those species capable of producing large quantities of histamine (>100 mg/100 ml) in tuna infusion broth (TFIB) during a short time (<24 h) incubation at temperature above 15°C and b) those capable of producing low histamine (<25 mg/100 ml) in TFIB with a long time incubation (≥48 h) at temperature ≥30°C. The prevalence frequency for different bacterial species indicated that *C. perfringens* (24.4%), *Proteus* spp. (23.0%), *Klebsiella* spp. (13.9%), *Enterobacter* spp. (11.1%), and *Morganella morganii* (7.5%) were the highest histamine-producing bacteria which belong to the category of prolific histamine producers, and samples with high concentrations of histamine contained various numbers of these organisms (Table 2). On the other hand, other species consisting of

Table 2. Bacterial isolates, histamine- producing bacterial count and histamine- producing Enterobacteriaceae count in longtail tuna samples with histamine concentration of >20 ppm.

Sample	Histamine- producing bacterial count (CFU/g)	Histamine- producing Enterobacteriaceae count (CFU/g)	Histamine content (ppm)	Bacterial isolates
1	920	500	153.3	*C. freundii, Cl. Perfringens, E. aerogenes, E. coli, K. pneumonia, M. morganii P. mirabilis,*
2	1260	980	115.6	*C. freundii, E. aerogenes Cl. Perfringens, P. mirabilis, P. vulgaris*
3	1820	1300	189.3	*E. aerogenes, E. coli, K. pneumoniae, K. oxytoca, P. mirabilis, P. vulgaris*
4	60	60	53.3	*E. aerogenes, K. pneumonia*
5	940	600	107.4	*Cl. Perfringens, P. mirabilis, P. vulgaris*
6	201	80	111.4	*C. freundii, Cl. Perfringens, E. aerogenes, K. pneumoniae, M. morganii, P. vulgaris*
7	226	160	102.6	*C. freundii, Cl. Perfringens, P. mirabilis, , M. morganii P. mirabilis*
8	530	150	76.8	*A. hydrophila, C. freundii, E. aerogenes, K. pneumoniae, P. fluorescens*
9	230	190	51.6	*A. hydrophila, E. aerogenes, K. pneumoniae,*
10	220	100	60.4	*Cl. Perfringens, E. aerogenes, M. morganii, P. mirabilis*
11	70	70	62.2	*C. freundii, M. morganii, P. vulgaris*
12	382	3000	103.8	*Cl. perfringens, E. aerogenes, K. pneumoniae, M. morganii, P. vulgaris*
13	232	190	89.3	*Cl. perfringens, E. aerogenes, K. pneumoniae, P. fluorescens, P. vulgaris, S. marcescens*
14	0	0	23.7	-
15	260	30	22.0	*A. hydrophila, P. aeruginosa, P. fluorescens, S. marcescens*
16	108	25	47.5	*Cl. perfringens, E. aeruginosa, k. pneumoniae*

Citrobacter freundii with 5.7% prevalence, *Pseudomonas* spp. (9.5%), *Escherichia coli* (1.4%), *Seratia marcescens* (0.9%) and *Aeromonas hydrophila* (2.6%) can be categorized as slow-producer of histamine group.

Histamine intake ranging within 8 to 40, 40 to

100 and >100 mg/100 g may cause slight, intermediate and intensive poisoning, respectively (Parente et al., 2001; Önal, 2007). The USFDA (1995) recommends that histamine amount in fresh and/or frozen raw fish should not exceed 20 mg/100 g and in tinned fish should not exceed 50 mg/100 g. The obtained results showed that of 20 examined samples of 20.0, 15.0, and 65.0% contained <20, 20 to 50 and >50 ppm amount of histamine, respectively. Therefore, it can be concluded that there are sea food safety risks in the usual fishing method in the Oman Sea and post-fishing procedures used in longtail tuna canning industry. Since the prolific histamine producing bacteria were mesophilic and typically occur as a result of post-fishing contamination, good hygienic practices and proper cooling of tuna after catching and during transportation is recommended.

ACKNOWLEDGEMENT

The authors appreciate Dr. Motalebi for his generous cooperation and facilities rendered during this research.

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