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Biogenic Amines and its Relation with Microbial Load in Some Fish Products

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Abstract: A total of 10 each of feseikh, molouha, smoked herring, frozen fish fillet and frozen fish fingers was collected from markets in Egypt and subjected to enumeration of aerobic mesophilic, psychrophilic, Enterobacteriaceae, Pseudomonas, coliforms (MPN) and fecal coliforms (MPN) counts in addition of determination of pH values and biogenic amines contents using HPLC. Mean values for histamine content (µg/100g) were 117.6, 110.8, 3.3, 2 &1.74 for feseikh, molouha, smoked herring, frozen fish fillet and frozen fish fingers respectively, meanwhile the mean values for cadaverine content were 803, 921.3, 895, 247&196.4 and 130.3, 208.4, 33, 25 & 22 for putrescine content respectively. Data of correlation coefficient revealed that not only Enterobacteriaceae and Pseudomonas counts but also the other bacterial groups were correlated to the formation of biogenic amines among examined fish products. Psychrophilic bacteria were not correlated with the formation of putrescine and had minimal contribution with the formation of histamine and cadaverine in the frozen fish products. Putrescine and cadaverine contents were inversely correlated with pH. Such results can proposed that cadaverine and putrescine are the most objective quality indicators among fish products.

Key words: Biogenic amines · Microbial load · Fish products · Molouha · Smoked herring

INTRODUCTION

Fish is an extremely perishable food commodity, its deterioration occurs as a result of enzymatic and microbial activities which lead to loss of quality and spoilage [1]. Deteriorative changes in fish are indicated by several physicochemical characteristics; among them is the production of biogenic amines due to microbial decarboxylation activity on free amino acids or by amintion and transamintion of aldhydes and ketones [2, 3].

Biogenic amines occur in a wide variety of foods, such as fish and fish products, meat and meat products, wine and other fermented foods. They have an important metabolic role in living cells; some of them are essential for growth and some of them involved in nervous system functions [2]. However, when high amounts of biogenic amines are consumed or normal pathways of amine catabolism are inhibited various physiological effects may occur including hypotension (in case of histamine, putrescine and cadaverine), nausea, headache, rash, dizziness, cardiac palpitation and even intracerebral hemorrhage and death in very severe cases [4].

High amounts of certain amines may be found in food as a consequence of the use of poor quality raw materials, contamination and inappropriate conditions during food processing and storage [5, 6]. In this respect, the quantity of biogenic amines is supposed to be a marker for microbiological contamination level in the food [7].

Factors affecting biogenic amines production are availability of free amino acids, presence of microorganisms that can decarboxylase amino acids and favorable conditions for the growth of such microorganisms and production of decarboxylase enzyme [8]. Clostridium species and Enterobacteriaceae especially *Proteus morganii*, *Klebsiella pneumoniae* and *Hafina alvei* are the most important histamine producing bacteria [9, 10].

Scombroid poisoning (scombrotoxicosis) is a worldwide problem of food borne intoxication caused by the consumption of seafood containing large quantities of histamine [11, 12]. It is mainly caused by consumption of scombroid fish including tuna, mackerel, saury and non-scombroid fish including bluefish, mahi-mahi, sardine, anchovy, herring and marlin [13].

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Histamine is usually taken as a chemical marker of hazardous decomposition; it is not always present in every decomposed sample and not correlated to organoleptic quality. Alkyl diamines, putrescine and cadaverine which give a decomposed piece of seafood its distinct putrid smell are better markers of decomposition and may even potentiate the deleterious health effects of consuming histamine spoiled seafood [14, 15]. Moreover putrescine and cadaverine may act as potentiators of histamine toxicity by their inhibition to histamine detoxification. Heat stable property of histamine and the lack of organoleptic indicators of histamine contamination are among the factors added to the difficulties of scombrotoxicosis prevention [16].

Food and Drug Administration (FDA) has set the maximum action level of histamine as 50 ppm for scombroid or scombroid like fish [17] however, a level of 200mg /Kg of food was ruled by the European Community (EC) and was established in Germany. Only 100mg /Kg of food was accepted in Canada, Finland and Switzerland. Similarly in E gypt histamine content must not exceed 200 mg/kg for salted, smoked and frozen fish products [18-20].

The present study aimed to give some additional information about biogenic amines in some traditionally salted, smoked and frozen fish products and its relation to microbial load.

MATERIALS AND METHODS

Samples: A total of 10 each of feseikh (salted *Mugil cephalus*), molouha (salted *Hydrocynus froskallii*), smoked herring, frozen fish fillet (Pangasius hypothalamus) and frozen fish fingers was collected from different local markets, transferred to laboratory in an ice box under complete aseptic condition without delay and subjected to bacteriological examination, measurement of pH-value and determination of biogenic amines.

Measurement of pH: The pH value of examined fish samples was measured directly on the muscles at room temperature using digital pH meter with a probe type combined electrode (Suntex TS-1), where three readings were recorded and the average was calculated [21].

Bacteriological Examination: Ten grams from each fish sample were aseptically homogenized in a sterile homogenizer flask with 90 ml of sterile 0.1% peptone (Oxoid CM9) for 2 minutes to provide a homogenate of 1/10 dilution. From the resulting dilution, ten-fold serial dilutions were prepared using the same diluent [22]. A 100 µl from the original and the subsequent decimal dilutions were inoculated into duplicate plates of plate count agar (Oxoid CM463) and incubated at 30°C for 2 days and 4.5°C for 14 days for enumeration of total aerobic mesophilic and psychrophilic bacterial counts, respectively [23]. Violet red bile glucose agar duplicate plates (Oxoid CM) were inoculated and incubated at 37°C for 24 hours for enumeration of Enterobacteriaceae count [22]. for Pseudomonas count duplicate plates of cetermide fucidin cephaloridine agar (Oxoid CM559, SR103) were inoculated with 100 µl and incubated at 30°C for 2days [24]. Coliform counts were conducted by the most probable number technique (MPN) 3-tubes method using lauryl sulphate tryptose broth (Oxoid CM451) and incubation at 35°C for 48 hours. Presumptive results were confirmed using brilliant green lactose bile broth (Oxoid CM31) [25]. MPN for fecal coliforms was recorded by sub-culturing positive coliform tubes into EC broth (Oxoid) tubes incubated at 45.5°C ±0.5 °C in a water bath for 24-48 hours.

Determination of Biogenic Amines: Fish samples were homogenized and 25 grams of homogenized fish were blended with 125ml of 5% trichloroacetic acid for 3 minutes using warring blender. The resulting homogenate was filtered using Whatmann No (1) filter paper. Ten milliliters of the filtrate was transferred into glass tube with 4g NaCl and 1 ml of 50% NaOH. The filtrate was extracted three times (2min each) using 5ml n-butanol: chloroform mixture (1:1v/v) and the upper layer was transferred to 100ml separating funnel. To the resultant organic extract, 15 ml of n- heptane was added in a separating funnel and extracted three times with 1.0 ml portions of 0.2NHCl,layers were collected in a glass stoppard tube. Solution was evaporated just to dryness using water bath at 95°C [26].

Dansylamines stock standard solutions of the tested amines were prepared by dissolving 25 mg of each standard: histamine-2HCl (Histamine dihydrochloride, CAS # 56-92-8), putrescine-2HCl (Putrescine dihydrochloride, CAS # 1476-39-7) and cadaverine-2HCl (Cadaverine dihydrochloride, CAS # 333-93-7) in 25 ml distilled water individually. One hundred micro liters of each stock standard solution was transferred to 50ml vial and dried under vacuum. Saturated $NaHCO_3$ solution (0.5ml) was added to the residue of the sample extract (or the standard), then vial was stoppard and its content was carefully mixed. One ml of dansyl chloride solution (500mg of Dansyl chloride: 100ml acetone) was added and mixed thoroughly using vortex mixer. Reaction mixture was incubated at 55°C for 45 minutes, then 10 ml of distilled water was added and the vial was stoppard and shacked vigorously. The extraction of dansylated biogenic amines was carried out using diethyl ether (5ml) for 3 times. The vial was stoppard again and shacked carefully for 11.0 minutes and the ether layer was collected in the culture tube. The combined ether extracts were carefully evaporated at 35°C in dry bath with aid of current air and the dry material was dissolved in 1ml methanol and 19µl was injected in HPLC (Agilent 1100 HPLC system, Agilent Technologies, Waldbronn, Germany, model G 1311A) equipped with UV detector (Model G 1314A) set at 254nm wavelength, auto sampler (model G 1329A VP-ODS) and Shimpack (150× 4.6 mm) column (Shimadzu, Kyoto, Japan) was used for biogenic amines separation.

Statistical Analysis: Data were analyzed using SPSS version 17.0. Results were recorded as mean ± SE. Pearson correlation test was used to find the correlation between histamine, cadaverine and putrescine and the investigated bacterial groups.

RESULTS AND DISCUSSION

Results of pH (Table 1) revealed that smoked herring had the lowest pH mean value followed by molouha and then feseikh (5.5, 5.7& 6.06, respectively). Such results fulfill Egyptian standard specifications [18, 19]. However, higher results were recorded for molouha and feseik [27, 28]. Meanwhile, frozen fish fillet had higher pH mean value then frozen fish fingers (7.5 & 6.15, respectively). These high results may be attributed to the type of frozen fish used in frozen fish finger production.

Data illustrated in Table (2) revealed that mean values of aerobic mesophilic count among feseikh, molouha and smoked herring were 6.11, 5.59 & 5.91 $\log_{10}$ CFU/g respectively. Such results are higher than that obtained by many authors for salted fish products [29-31]. Moreover these results exceeded the permissible limits set by Egyptian authorities [18, 19]. The mean values for frozen fish fillet and fish fingers were 6 $\log_{10}$CFU/g which are congruent to the maximum Egyptian permissible limits [20]. These results can be explained by the possibility of cross contamination during handling filleting and processing of such products. Concerning psychrophilic count, the values were 5 and 4.32 $\log_{10}$ CFU/g for frozen fish fillet and fish fingers respectively. As the presence of psychrophilic microorganisms in chilled seafood is important so a limit of 6 $\log_{10}$ CFU/g is suggested for acceptability in cold storage conditions [32].

Enterobacteriaceae count among frozen fish fingers was the highest followed by frozen fish fillet, smoked herring, feseikh and finally molouha with values of 5.26, 5.00, 4.91, 4.45 & 4.28 $\log_{10}$CFU/g respectively. Pseudomonas count among feseikh was the highest followed by frozen fish fillet, frozen fish fingers, smoked herring and finally molouha which was the lowest. The values were 5.65, 4.66, 4.41, 4.11 & 2.72 $\log_{10}$ CFU/g respectively. It is recommended that pseudomonas count in food be taken as an indicator for keeping quality and spoilage [33]. Slightly higher results for frozen fish fillet and fish fingers as compared to salted and smoked fish products may be attributed to the bad hygienic conditions during processing and post processing contamination.

Coliform count (MPN) among frozen fish fillet had the highest value followed by fish fingers, molouha then feseikh and finally smoked herring with mean values of 2.32, 2.28, 1.56, 1.44 and 1.24 $\log_{10}$ MPN/g respectively, while the highest fecal coliforms counts were recorded for frozen fish fillet followed by frozen fish fingers, molouha, smoked herring and then feseikh (2.15, 1.51, 1.28, 0.78 & 0.70 $\log_{10}$ MPN/g respectively). The obtained results are within the acceptable limits except for frozen products as Egyptian standards specifications permit only 100 organisms/g [18-20]. It is worthy to mention that the presence of coliforms in the frozen fish products indicates neglected sanitary measures during production and handling [22]. Fecal coliforms are useful as indicator of fecal contamination and the obtained results may be attributed to contamination and mishandling of raw materials during processing and also may be due to a defect in time / temperature abuse of the fish chilling immediately after caught.

Data of determination of biogenic amines contents declared that cadaverine were the highest followed by putrescine and finally histamine in all examined fish products. It is evident from the results recorded in Table (3) that the mean values of histamine among feseikh was the highest followed by molouha, smoked herring, frozen fish fillet and finally frozen fish fingers which was the lowest (117.6, 110.8, 3.3, 2 and 1.74 µg / 100g respectively. Food and drug Administration established a defect and hazard action level of histamine in fish at 5000 µg /100 g [34], while Egyptian Standards allows 2000 µg/100g as a permissible limit for histamine content in smoked, salted and frozen fish products [18-20]. It is clearly that histamine level in all the investigated fish products samples was much lower than the hazard level and this could be attributed to the types of examined fish which were non-scombroids. However, very low histamine level in frozen fish products may be attributed to the low

Table 1: pH values among examined fish products.

	Minimum	Maximum	Mean ± S.E
Feseikh	5.50	7.00	6.06 ± 0.14
Molouha	5.50	6.30	5.70 ± 0.1
Smoked herring	5.00	6.00	5.50 ± 0.09
Fish fillet	7.00	8.00	7.50 ± 0.12
Fish fingers	6.00	6.50	6.15 ± 0.07

Table 2: Bacterial counts (log_{10} cfu/g) among examined fish products.

	Aerobic mesophilic (log_{10} cfu/g)			Psychrophilic (log_{10} cfu/g)			Enterobacteriaceae (log_{10} cfu/g)		
	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE
Feseikh	3.00	6.79	6.11±5.8		NA[1]		2.49	5.08	4.45±4.0
Molouha	4.00	6.08	5.59±5.2		NA		<2.00	4.92	4.28±4.0
Smoked herring	4.80	6.40	5.91±5.4		NA		2.65	5.11	4.91±4.4
Fish fillet	4.94	6.58	6.00±5.6	<2.00	5.40	5.00±4.6	3.94	5.49	5.00±4.5
Fish fingers	4.96	6.60	6.00±5.6	<2.00	5.26	4.32±4.4	4.43	5.89	5.26±4.9
	Pseudomonas (log_{10}cfu/g)			Coliforms (log_{10}MPN/g)			Fecal coliforms (log_{10}MPN/g)		
Feseikh	<2.00	6.30	5.65±5.4	<0.48	1.63	1.44±0.7	<0.48	1.54	0.70±0.6
Molouha	<2.00	3.18	2.72±2.3	0.48	1.81	1.56±0.7	<0.48	1.63	1.28±0.6
Smoked herring	<2.00	5.32	4.11±3.4	<0.48	1.63	1.24±0. 8	<0.48	1.54	0.78±0.6
Fish fillet	<2.00	4.96	4.66±4.0	<0.48	2.66	2.32±1.7	<0.48	2.66	2.15±1.7
Fish fingers	3.78	4.73	4.41±3.7	0.56	2.66	2.28±1.8	<0.48	2.66	1.51±1.3

[1]NA= not applied

Table 3: Biogenic amines content (µg/100g) in examined fish products

	Histamine			Cadaverine			Putrescine		
	Min.	Max.	Mean ±SE	Min.	Max.	Mean ±SE	Min	Max	Mean ±SE
Smoked herring	ND[1]	14.8	3.30±1.7	28.0	4142	895±408.8	3.03	194	33±4.16
Feseikh	ND	493	117.6±60	170.5	1733.7	803±156	34.5	318	130.3±37.3
Molouha	ND	419	110.8±51	432.7	1666.6	921.3±172	69.0	445	208.4±43.2
Fish Fillet	ND	3.60	2.0±0.53	39.0	500.0	247±50	3.0	71	25±9.3
Fish Fingers	ND	12.0	1.74±10	48.0	618.0	196.4±52.2	6.5	69	22±8.8

[1]ND= not detected

Table 4: Correlation between Histamine with the other studied parameters

Fish Product	Aerobic mesophilic count	Enterobacteriaceae count	Pseudomonas count	Coliforms count	Fecal coliforms count	pH	Psychrophilic count
Smoked herring	0.7*	0.5	0.6*	0.8	0.9	-0.8	-
Feseikh	0.8*	0.7*	0.7*	0.66	0.9	-0.69*	-
Molouha	0.7*	0.75*	0.5	0.9	0.92	-0.56	-
Fish fillet	0.85	0.83	0.84	0.87	0.76*	-0.53	0.74*
Fish fingers	0.56	0.73*	0.6	0.76*	0.79	-0.34	0.59

* Means the correlation coefficient is significant at ($P < 0.05$)

Table 5: Correlation between cadaverine with the other studied parameters

Fish Product	Aerobic mesophilic count	Enterobacteriaceae count	Pseudomonas count	Coliforms count	Fecal coliforms count	pH	Psychrophilic count
Smoked herring	0.7*	0.5	0.6	0.7*	0.9	-0.79*	-
Feseikh	0.86	0.87	0.8	0.87	0.9	-0.78*	-
Molouha	0.83	0.86	0.6	0.9	0.8*	-0.55	-
Fish fillet	0.93	0. 9	0.86	0.95	0.94	-0.8*	0.8
Fish fingers	0.74*	0.78	0.77	0.8	0.84	-0.5*	0.72*

* Means the correlation coefficient is significant at ($P < 0.05$)

Table 6: Correlation between putrescine with the other studied parameters

Fish Product	Aerobic mesophilic count	Enterobacteriaceae count	Pseudomonas count	Coliforms count	Fecal coliforms count	pH	Psychrophilic count
Smoked herring	0.6	0.5	0.5	0.6	0.8	-0.79	-
Feseikh	0.84	0.78	0.8	0.84	0.93	-0.68*	-
Molouha	0.85	0.9	0.7	0.9	0.9	-0.8*	-
Fish fillet	0.8	0.79*	0.7*	0.97	0.96	-0.7*	0.6
Fish fingers	0.65*	0.78	0.79	0.84	0.89	-0.36*	0.78

* Means the correlation coefficient is significant at ($P< 0.05$)

temperature and short storage period which limits the capacity of histamine production [35-37]. Moreover, histamine formation decreases by salting and by the increase in brine concentration which reflects the effect of sodium chloride on the activities of decarboxylases of Enterobacteriaceae [2, 38, 39]. Heating step during smoking could eliminate bacteria which are able to convert the amino acid histidine to histamine [40-41] and that may explain the low level of histamine among smoked herring samples.

Histamine content was significantly correlated to APC and Enterobacteriaceae and Pseudomonas counts among feseikh and inversely correlated with pH. Meanwhile, in molouha, there was a significant correlation between histamine content and APC and Enterobacteriaceae counts but among smoked herring samples there was a significant correlation to APC and Pseudomonas counts. However, histamine content among frozen fish fillet was significantly correlated to psychrophilic and fecal Coliform counts but among frozen fish fingers it was correlated to Enterobacteriaceae and coliform counts (Table 4).

Results in Table 3 showed that cadaverine content among molouha was the highest followed by smoked herring, feseikh, frozen fish fillet and finally fish fingers with mean values of 921.3, 895, 803, 247and 196.4µg /100 g respectively. It is worthy to mention that cadaverine formation unlike histamine occurs in a wide range of fish species [39, 42-45] which could be explained by readily available free lysine in most fish species [46]. Cadaverine formation begins early, increase steadily and correlates well with histamine, muscle alteration, microbial activity and sensory odor, therefore it can be considered a good indicator of incipient and late spoilage of fish [47, 48].

Cadaverine content in smoked herring was significantly correlated to APC and coliforms count. However, cadaverine content was only significantly correlated to fecal coliforms among molouha. In frozen fish fingers, cadaverine content was significantly correlated to APC and psychrophilic count. Among all fish products except molouha, cadaverine content revealed an inverse correlation to pH (Table 5).

It is obvious from table (3) that mean values of putrescine among molouha was the highest followed by feseikh, smoked herring, frozen fish fillet and finally frozen fish fingers (208.4, 130.3, 33, 25 and 22 µg/100g respectively). Such results could be attributed to the higher proteolytic enzyme activity in whole fish products than in filleted fish products [49]. Higher levels of putrescine were reported in whole and gutted trout, however a level of 500 µg/100g of putrescine could be proposed as an early warning of autolytic degradation [47]. Putrescine content may be a good indicator of late spoilage, its formation require an acidic pH and a high enough concentration of ornithine [50, 51].

Only putrescine content in frozen fish fillet was significantly correlated to Enterobacteriaceae and Pseudomonas counts. Moreover there was an inverse correlation between putrescine content and pH values except in smoked herring (Table 6).

Cadaverine and putrescine are considered as potential indicators of fish quality as they show a steady rise once bacterial spoilage begins. Putrescine to be a useful biochemical quality parameter must be used in conjunction with cadaverine, or in a summation index of biogenic amines [42, 52-54].

Meanwhile salted fish products contained higher cadaverine and putrescine contents than histamine as cadaverine and putrescine producing bacteria can tolerate high salt concentrations than histamine forming bacteria [55]. Frozen fish products were the lowest products in histamine, cadaverine and putrescine contents as some bacterial populations responsible for biogenic amines production cannot withstand the low temperature of storage and the high pH value.

Correlation results revealed that not only Enterobacteriaceae and Pseudomonas counts were responsible for the formation of biogenic amines but also the other bacterial groups contributed in the formation of biogenic amines in the examined fish products. Moreover, it is obvious that psychrophilic count was not correlated to the formation of putrescine in frozen fish products and had a minimal contribution in the formation of histamine and cadaverine as biogenic amines formation is more related to mesophilic than psychrophilic bacteria [56], also many studies concluded that histamine formation in seafood by psychrotolerant bacteria was insignificant [57-59].

Putrescine and cadaverine contents were inversely correlated to pH since biogenic amines formation is enhanced in acidic pH [60, 61]. However in molouha histamine and cadaverine contents were not correlated to pH. Similar results were achieved in salted mackerel and salted fish sauce [62, 63].

It could be concluded that salted fish products were the worst examined fish products concerning its high biogenic amines content. Cadaverine and putrescine were the most objective quality indicators for fish products. Moreover hygienic handling of fish from the moment of capture to the point of consumption is crucial to reduce the formation of biogenic amines as their formation was clearly correlated to its bacterial counts.

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