major bacterial species reported in fish, which can form histamine, are: *Klebsiella pneumonia, Staphylococcus xylosus, Escherichia soli, Morganella morganii, Hafnia alvei, Enterobacter aerogenes, Enterobacter cloacae, Proteus mirabilis, Serratia liquefaciens, Serratic fonticola, Citrobacter freundii* and *Raoultella ornithinolytica* (4). These types of bacteria naturally present on the gills, external surfaces and in the gut of live saltwater fish with no harm to the fish. Up on death, the defense mechanism of the fish no longer inhibits bacterial growth in the muscle tissue and histamine forming bacteria may start to grow resulting in the formation of biogenic amines (5). Inadequate cooling following harvest promotes bacterial histamine production and can result in outbreaks of scombroid poisoning (6) Specific conditions are needed for preservation of fish and avoid the chemical intoxication hazardous (7). Freshly caught fish does not contain histamine however, once fish starts to decay or is subjected to temperature abuse; histamine can start to accumulate. The production of histamine in fish is generally believed to result from the growth of Gram negative, rod shaped bacteria capable of producing pyridoxal phosphate dependent decarboxylase enzymes (8). This process can start as soon as the fish dies and kept at temperature above 4°C for an extended period (FAO/WHO, 2012). Histamine forming bacteria produce histidine decarboxylase (HDC) enzyme, once the HDC enzyme is synthesized, it continues to produce histamine even if the bacteria get inactivated (EFSA, 2011; FDA, 2011). Cooking can inhibit the HPB and the HDC, while histamine cannot be degraded by cooking, smoking, or canning of fish, indicating that both raw and cooked fish might cause HPB (2). Fish is very important source of protein especially in Egypt. Fish and fish products are one of the most important food stuffs as they are one of the cheapest sources of animal protein (9). So far, little information is available about HPB in Egypt. Therefore, this study was conducted for evaluation of the histamine content in addition to bacteriological quality of sardine fish samples.

Materials and methods

Samples collection: A total of 50 sardine samples were collected from various commercial fish retails and markets at Ismailia city, Egypt. Samples were kept in icebox during transportation to the laboratory and stored at −20 °C until further analysis. bacteriological evaluation was determined in the all samples.

Determination of pH value, salt content, water activity and total volatile nitrogenous compound (TVN): The muscles of sardine fish samples (15 g) were blended in mixer for 10 min at 6000 rpm with 30 ml distilled water to make thick slurry. The salt content of the fish muscle was determined by the method of AOAC procedures (1995)(10. 10 ml of the filtered extract (D: dilution coefficient) were pipetted into 100ml Erlenmeyer and 1ml of K_2CrO_4 (IN) was added. The samples were titrated with 0.1NAgNO3 standard solution (f: factor) until the first perceptible pale red brown appeared (T: titration volume).

Calculation: Concentration of sodium chloride based on sodium can calculated by the formula: NaCl (%)= 0.00585× T × D × ---------- × F. Water content was detected by Hygrometer electric instrument at 27 °C. The pH was measured using pH meter. The (TVN) was measured by dissolving 100g of the sardine fish sample in 200ml (7.5%) aqueous solution of trichloro-acetic acid, then filter the mixture and mixing 25ml of the extract in a distillation flask with 6ml 10%NaOH. The TVN condensed is mixed with 10ml (4%) boric acid and 0.04ml of methyl-red and bromocresol green indicator which turns green when alkalinized by the TVN (11).

Calculation: The TVN content was calculated by the formula: TVB-N = (14 × a × b × 300) / 25 (mg/100g). Where: (a) is a volume of sulphuric acid (ml) and (b) is the normality of sulphuric acid (%).

Microbiological analysis: The following Bacterial counts and Isolation of histamine-forming bacteria were carried out according to (12), the flesh of fish sample transferred to a homogenizer to mince the sample using Moulinex Food Processor (La Kiloulinette, type D56, Paris, France), 25g of homogenized sample was added to 225 mL of 0.1% peptone water using plastic bag, put in a Seward stomacher (4001R/LIK) for 2 min. prepared serial dilutions a final dilution of (10-7) by peptone water.

Total aerobic bacterial count: Standard plate count agar by spread plate method were used for the determination of aerobic plate counts, and then incubated at 30±1°C/72±3 hrs.

Total anaerobic colony counts: The same procedures as for the previous sample preparation were incorporated using Reinforced Closterdial

medium agar (Oxoid; CM151), incubated in an anaerobic jar (Gaspak plus anaerobic system) at 37°C/48 hours.

Staphylococci spp.: Staphylococci spp. were determined on Baird Parker Agar (Oxoid CM 275) supplemented by egg yolk tellurite at 37 °C for 24–48 h. Typical black colonies with zones around and atypical black colonies were considered as Staphylococcus spp.

Total Enterobacteriaceae Counts: Enterobacteriaceae were detected using violet red bile glucose agar (VRBGA) which were incubated at an inverted position at 30-35°C/24 hrs.

Measurement of Biogenic amine content in fish muscles using HPLC: Fish samples were subjected to HPLC to analyses the contents of histamine, putrescine, cadaverine, serotonin, phenylethylamine, spermidine and tyramine. In brief, 5g muscle from each sample was homogenized separately using l0mL trichloroacetic acid extracting solution (Thermo Fischer Scientific, Waltham, MA) and centrifugation. The supernatant was filtered, mixed with lmL of 1M NaOH (Thermo Fischer Scientific), and incubated at RT for 5 min. One milliliter of ophthalaldehyde (Acros Organics, Geel, Belgium) and 3nnL ethyl acetate (Thermo Fischer Scientific) were then added, and the homogenate was precipitated by centrifugation. The pellets were dried on a rotatory evaporator, then re-suspended in 1 ml acetonitrile (Thermo Fischer Scientific). A histamine dihydrochloride standard solution (Sigma- Aldrich, St. Louis, MO) was serially diluted in 0.1M HCI (Thermo Fischer Scientific) to obtain the following concentrations: 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/L. The standard and the processed samples were injected into intersil C18 columns (Agilent Technologies, Waldbron, Germany); each determination was injected twice. The chromatographic separation was performed using 1050 HPLC (Agilent Technologies) according to (13).

Determination of total histamine-forming bacterial counts: Spread 0.1ml aliquot of diluted sample on histamine-forming bacterium isolation agar (HBI agar) mixed with L¬histidine. Incubation of agar plates for 4 days/35°C, counted the purple and blue colonies, picked on trypticase soy agar (Difco) to identification by pure cultures (14).

Identification histamine-forming bacteria: Bacterial isolates were identified using the Vitek instrument (bio-Merieux Vitek Inc.,Hazelwood, Mich., U.S.A.). Additional biochemical tests were carried out for definitive species identification by screening on (TSI) triple sugar iron agar, Vogs-proskauer (VP) test, Oxidase test, Fermentation test, Methyl-Red test Citrate-utilization test, Urease test, Indole production test (15, 16).

PCR Identification: DNA extraction was done using marketed kit for DNA extraction (Presto Mini-DNA Bacteria Kit. Ltd. USA). Preparation of DNA: using 0.05 N NaOH and 0.25% sodium dodecyl sulfate. PCR product analysis performed using a 1% agarose substance by electrophoresis apparatus and observed under the UV trans-illuminator. Amplification and sequence determination of 16S rDNA.

Statistical analysis; Data are presented as mean±SEM. Analysis was performed using GraphPad Prism 7.03 software (GraphPad Software, San Diego, CA, USA). Statistical differences over two groups were verified by Student's *t*-test

Results

The obtained results in Table 1 represented the values of the salt content, pH, total volatile nitrogen (TVN), water activity. The salt content, pH value, TVN and water activity in all samples ranged from 0.01 to 9.6%, 6.1 to 7.6, 6 to 7.8 and 21.4 to 35.7 mg/100 g respectively. The average TVN level (24.3 mg/100 g) of frozen samples were significantly ($P < 0.05$) lower than those of salted samples. The levels of TVN in most fish samples were below the Egyptian regulatory standard of 25 mg/100 g, 6 (Table 1).

TVN, including NH3, dimethylamine (DMA) and trimethylamine (TMA) is used as indicators for the fish spoilage and quality. In this study, the TVN values in salted sardines were significantly higher than those obtained in frozen samples. Bacteriological profiles of total coliforms, Aerobic, anaerobic, *Enterobacteriacea spp. E. coli* and *Staphylococcus spp.* counts were detected in table 2. Statistical analysis for Different Types of Microorganisms (Log CFU/g) in the Sardine samples were; the total aerobic counts reported about; 8×10^3, 6×10^4, $4\times10^4 \pm 2.1\times10^4$ as minimum, maximum, mean ±SE values respectively while, about; 5×10^3, 6×10^4, $5\times10^4 \pm 1.2\times10^3$ Log CFU/g were detected as minimum, maximum, mean ±SE values respectively in case of total anaerobic count. *Enterobacteriacea spp. s*howed about; 4×10^3,

6.0×10^5, $5\times10^4 \pm 2.5\times10^4$ Log CFU/g as minimum, maximum, mean ±SE values respectively while, about; 3×10^3, 4×10^5, 3×10^4 $\pm 3.7\times10^4$ Log CFU/g were detected as minimum, maximum, mean ±SE values respectively in case of *Staphylococcus spp.* counts. The lower water content (<0.92) and higher salt concentration (>4.0%) in these salted sardine samples apparently had some inhibitory effect on coliform bacterial growth.

Table 3 reported the contents of biogenic amines in the frozen and salted sardine fish. The average content of each of the seven biogenic amines in all samples was less than 10 mg/100 g. Based on the results, it is cleared that higher levels of histamine were found in salted and frozen sardine fish was 8.25 and 0.64 mg/100g. Among them, higher level of biogenic amines in salted sardine samples as follow, the average cadaverine (2.82 mg/100 g), tyramine (4.17 mg/100 g), putrescine (4.72 mg/100 g) and agmentine (4.57 mg/100 g) this results of salted sardine samples were significantly ($p < 0.05$) higher than those of frozen samples.

Figure 1 and 2 show the identity and Frequency distribution of histamine-forming bacterial isolates of frozen sardines' samples as determined by 16S rDNA sequences. They were identified as *Citrobacter freundii* (thirteen strain), *Enterobacter aerogenes* (ten strain), *Enterobacter cloacae* (twelve strain), *Klebsiella pneumoniae* (eleven strain), *Micrococcus spp* (fourteen strain), *Proteus vulgaris* (thirty strain), *Pseudomonas aeruginosa* (seventeen strain), *Staphylococcus xylosus* (sixteen strain), In this study, that the highest incidence was recorded by *Proteus vulgaris* spp. which represented 12 (20%) and 18 (29%) in both frozen and salted samples respectively. On the other side, the lowest incidence in frozen samples was recorded by *Micrococcus spp.* and *Staphylococcus xylosus* spp. which represented 4 (7%). Although, the lowest incidence in salted samples was recorded by *Enterobacter aerogenes* spp. which represented 4 (5%) then *Citrobacter freundii* and *Enterobacter cloacae* which represented 4 (6%).

Table 1: Values of the salt content, pH, water content, total volatile nitrogen (TVN) in Frozen and salted Sardine sam-ples

Samples	Salt content (%)	pH	Water content	TVN(mg/100 g)
Frozen Sardine	0.01-0.12 (0.05 ± 0.01) A	6.2-7.2(6.6 ± 0.1) A	48–78 (63.4 ± 2,9)A	21.4-30.4 (24.3 ± 0.93)B
Salted Sardine	4.5-9.6(7.6 ± 0.5)A	6.1-7.6 (6.75 ± 02.)A	6-17 (13.6 ± 1.3)A	23.1–35.7 29.9 ± 1,59)A

Mean ± SD. Values in the same column with different letters are statistically different ($P < 0.05$).

Table 2: Microbial content and percentage of evidence of aerobic and Anaerobic plate count (APC), total coliform (TC), *Escherichia coli* (*E. coli*), *Enter. cloacae* and *Staph. xylosus* in Frozen and salted Sardine samples

Sam-ples	APC		TC		E. coli(		Anaerobic plate count		Enter. cloacae		Staph. xylosus	
	Percent %	(log CFU/g)	Percent %	(MPN/g)	Percent %	(MPN/g)	Percent %	(log CFU/g)	Percent %	(log CFU/g)	Percent %	(log CFU/g)
Frozen Sardine	25	$2X10^3 \pm$ 0.9 B	5	<3	25	<2	75	$2X10^2 \pm$ 0.9 B	50	$3X10^3 \pm$ 1.2 B	25	$3X10^3 \pm$ 2.2 B
Salted Sardine	100	$8X10^3$-6 $X10^4$(4X $10^4 \pm$ 1.8)A	20	<3	50	<3	100	$5X10^3$-6 $X10^4$(5X $10^4 \pm$ 1.2)A	100	$4X10^3$-6 $X10^5$(5X $10^4 \pm$ 2.5)A	50	$3X10^3$-$4X10^5$(3X $10^4 \pm$ 3.7)A

Table 3: Contents of biogenic amines in in Frozen and salted Sardine samples

Samples	No. of samples	Cad	Tyr	His	Agm	Put	Phe	Spd
Frozen Sardine	50	0.26 (0.01 ± 0.05)A	0.97 (0.37 ± 0.22)A	1.64 (0.13 ± 0.1)A	ND-0.15 (0.03 ± 0.01)A	0.52 (0.05 ± 0.14)A	ND	ND
Salted Sardine	50	2.82 (0.69 ± 0.48)A	4.17 (1.72 ± 1.21)B	8.25 (0.89 ± 1.62)A	4.57 (5.13 ± 4.79)B	4.72 (2.18 ± 2.00)B	ND	ND

Cad: cadaverine; Tyr: tyramine; His: histamine; Agm: agmatine; Put: putrescine; Phe: 2-phenylethylamine; Spd: spermidine.

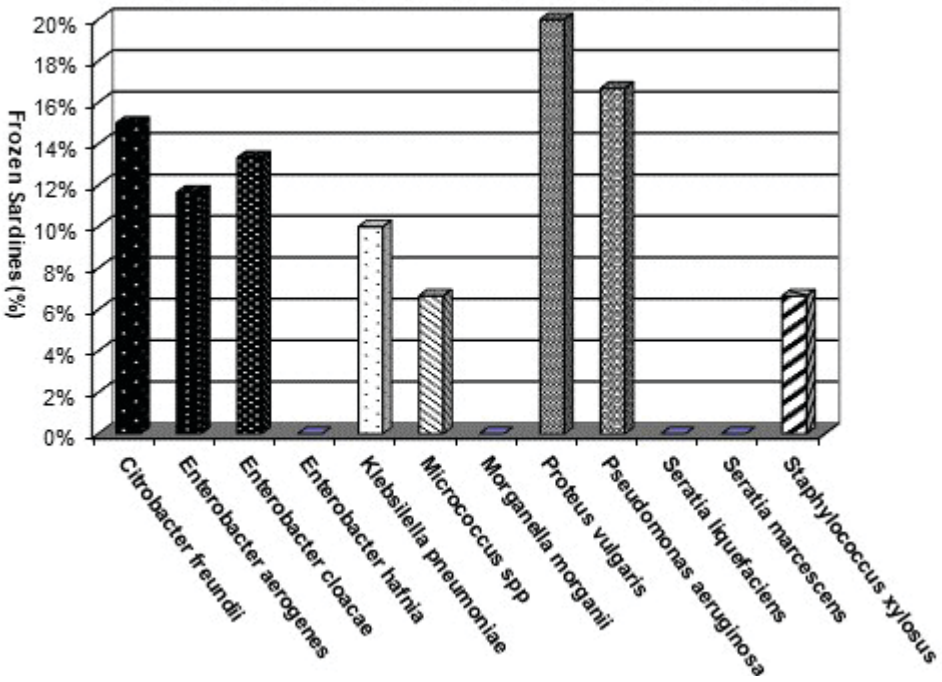

Figure 1: Frequency distribution of histamine-forming bacterial isolates of frozen sardines' samples

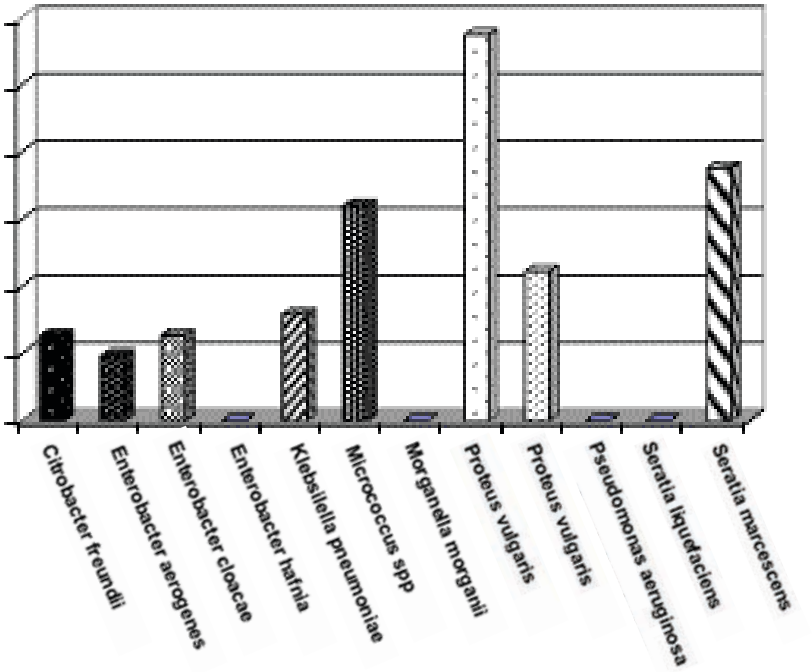

Figure 2: Frequency distribution of histamine-forming bacterial isolates of salted sardines' samples

Discussion

Scombroid is a type of food poisoning experienced around the world, with a higher reported prevalence in Japan, England and the United States. This could be attributed to the high consumption of scombroid fish (17). Histamine presence in food items reflect the food quality, bad storage conditions one of the major inducers of histamine formation and aggregation as the histamine-forming bacteria can produce the enzyme "histamine–decarboxylase" which converts histidine amino acid in fish tissues in histamine even at low temperatures as 5°C. formation of

histamine not degraded by freezing or heating (18). Based on the finding that the higher levels of TVN noticed in dried salted fish samples, we explained that these samples have been seriously contaminated during food preparation and processing (19). The obtained results of TVN in frozen sardine samples were nearly like those were submitted by (19). Although, lower TVN results were obtained by (20) confirmed that the TVN values of frozen sardine samples were ranged from 15 to 17mg/100g. El-Dengawy et al. (21) studied that mean TVN values of frozen sardine samples were 13.34±0.00 and 16.23±0.00mg/100g. (22) reported that the TVN values of frozen sardines were ranged from 11.2 to19.9mg/100g. Similar results with TVN (43 mg/100 g) and higher salt concentration (5.6%) in salted fish product were reported (23). The total aerobic, anaerobic and *Enterobacteriacea spp.* were approximately 100% of the total sample count, while only 50% of tested samples had *Staphylococcus spp.* Similar results in the total aerobic count were detected by (24,25) during their assessment of the bacteriological quality of the bacteriological quality of some meat products in the Egyptian retail markets. Also, nearly similar results observed by (26) during their bacteriological evaluation of different fish samples of Dharan markets as; (54%) in case of *staphylococcus sp.* Lower results reported by (27) during their microbiological quality survallaince in Istanbul as (96.67%). While, only about (24%) of *Enterobacteracea sp.* and (8%) of *staphylococcus sp.* was estimated by (28). Similar results found by Salem, *et al.* (28) whome reocrded about $5.61x10^5$ Log CFU/g of total aerobic counts. Ragab, *et al.* (24) detected about ($6.6x\ 10^8$ Log CFU/g) of total aerobic counts. While, (29) also recorded about (4.27×10^3 Log CFU/g) of *Enterobacteracea sp.* and 5.6×10^5 Log CFU/g of *staphylococcus sp.* was detected by (30). Lower result observed by Shaltout, *et al.* (20) who obtained about 8.03×10^4 Log CFU/g of total aerobic count while, in case of *Enterobacteracea sp.* they recorded about (2.02×10^2 Log CFU/g) and isolated about (2.67×10^2 Log CFU/g) of *staphylococcus*. Most aerobic of these bacteria would die or at least stop growing as the salt content of the fish was increased to 6–8%. (26) also reported similar findings with the unclean market and storage temperatures could have contributed to the high contents of TVN in samples (26). The observed results of histamine in frozen *sardine* samples were parallel to those were pointed out by Lee *et al.* (31). Biji *et al.* (32) confirmed that histamine levels in *sardines* were ranged from 1.0 to 2.6 mg/100g. Also, Martin *et al.* (33) studied that mean values of histamine were ranged from 0.21±0.37 to 1.04±0.82 mg/100g in samples. On the other hand, higher histamine results were reported by El Hariri, *et al.* (34) estimated the level of histamine in the *S. pilchardus* muscle at the rejection time (12 days) preserved in ice was 16.2 mg/100g. Li *et al.* (35) reported that mean histamine content of samples was16.4±2.6 mg/100g. Aruna *et al.* (36) reported that histamine level of frozen sardines were ranged from 12 to 32 mg/100g. The strong evidence exists that the toxic effects of histamine affected and increased by the biogenic amines in fish tissue by inhibiting intestinal histamine-metabolizing enzymes such as diamine oxidase (13). Histamine-forming bacteria species which isolated in this study have been previously recorded by different investigators (37-39). However, the *Enterobacter* strains were isolated from many scombroid fish species (40). This increment in histamine concentration for sardine samples caused by consumptions of poor-quality raw food and bad handling techniques, improper storage and defective processing methods (17). Salting techniques can remove the bacterial contamination but unable to destroy histamine (scombroid) toxin of fish poisoning (41). If histamine levels in fish and its products are more than 50mg/100g, it is reported as the hazard action level of histamine (42). According to, U.S. Food and Drug Administration (U.S.FDA) histamine should be found at 50ppm of histamine to be enough to cause the scombroid poisoning signs, While, the Egyptian Organization for Standardization "EOS" (2005); permits histamine to be found at 20mg/100g (9, 43). The higher levels of fish histamine affected by certain factors such as natural microorganisms which depend on habitat, storage temperature after caught and improper handling on board the harvest vessel. Naturally, live fish contains different bacterial species on; external surfaces, gills, and in the intestinal content but after the death of fish, the defense mechanisms of fish can't inhibit the growth of bacteria which allowing the replications of microorganisms. Sabry et al.,(44) showed the occurrence of histamine producing bacteria associated with histamine production in muscles of retail sardine and mackerel in Egypt. The sardine samples showed a relatively high level of histamine than the legal limit, possibly

due to temperature abuse. The habitats in marine water differ and can be affected by the origin of fish contamination. The main approach after fish catching depend on the melting temperature in addition to the presence of some conditions which controlling the speed of reaching these temperatures such as fish size, fish initial temperature and the ice content of fish (45-47).

Conclusion

Results in this study indicated to that histamine and the bacteria forming histamine were recorded in all examined sardine samples. Temperature, salt concentration and water activity greatly influenced the histamine production in Sardine samples. The rate of histamine accumulation was rapid in salted fish. Increase of bacterial counts and histamine content slowed down with the decrease of storage temperature.it is referred that hygienic conditions and storage time/ temperature can significantly affect the frozen fish quality. bacteriological quality of anaerobic, *Enterobacteriacea spp. E. coli* and *Staphylococcus spp.* counts with no coliforms, was $8\times10^{3,}$ $5\times10^{4,}$ 5×10^{4} , 3×10^{4} log CFU/g, respectively. The histamine content in all samples was less than 10 mg/100 g USFDA guideline value. Molecular Identification of Sardines samples declared as following; 25/50 (50%), 10/50 (20%), 10/50 (20%), 5/50 (10%) for *Klebsiella pneumonia, Staphylococcus xylosus, Escherichia coli* and *Enterobacter cloacae* respectively. The isolated histamine-forming bacterial species which have been demonstrated in the examined sardine samples. There must be an attention to the fishing process which should be carried out under good hygiene to decrease the initial bacterial load.

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