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Bacteria forming histamine and shelf life of sardine (*Sardina pilchardus*) at different temperatures and storage times with an emphasis on histopathological changes in the skeletal musculature

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ABSTRACT

Sardines contain high levels of histidine within their muscles which is converted to histamine (HIS) by histidine-decarboxylase forming bacteria. Our investigation aimed to study the effect of storage time and temperature on the HIS levels of different muscular areas of sardine. Thirty fresh sardine samples were divided into two groups (n=15). Group (1) was kept at 0 ºC while group (2) was kept at 21 ºC. All fish were examined for freshness at time intervals of 0-, 6-, 12-, 18-, and 24-hr post fishing using Quality Index Method (QIM) and European grading scheme (EU) grading scheme. At the same time intervals, muscle samples were collected from the abdominal, dorsal, and tail regions for histamine measurement, bacterial isolation and identification as well as for histopathological examination. Our results revealed that the fish freshness was decreased by the storage time and temperature. Moreover, both bacterial count and histamine levels in different muscular areas were increased also throughout the storage time and temperature. Higher levels were observed in the abdominal muscle more than tail muscle and dorsal muscles. *Enterobacter aerogenes, Enterobacter cloacae complex, Lelliottia amnigena-1 (Enterobacter amnigena-1)* were identified with Vitek2 which gave 99 % confidence of identification, additionally, we localized gram-negative bacteria throughout the muscle tissue, primarily in the intermuscular region, around the blood vessels, and in the myofibrils. We concluded that during storage, there was a positive relationship between the HIS levels and the histamine-forming bacteria (HFB) numbers, sensory scores, and microscopic lesion scores. Also, the identified isolated bacteria differ from the previously recorded isolates in sardines and its related species at other localities; so the bacterial isolates might depend on the fishing locality.

INTRODUCTION

The most common biological risks found in seafood are biogenic amines (BAs), and the most important of which is histamine (HIS) **(Visciano *et al.*, 2012)**. Histamine (2-[3H-imidazol-4-yl] ethanamine) is a biogenic amine (BA) that has been linked to Scombrotoxin fish poisoning (SFP) outbreaks. SFP linked to fish eating and is characterized by symptoms similar to those seen in IgE-mediated food allergies **(EFSA, 2017; Peruzy *et al.*, 2017).** Diamine oxidase and histamine N-methyltransferase in the

intestine detoxify dietary HIS, however, some pretentious as other BAs and alcohol can block these detoxification pathways **(Murru *et al.*, 2017).** In fact, HIS is the only BA is having 50 mg/kg regulatory limitations in seafood **(FDA, 2011).** The term "scombroid" comes from the Scombridae family of fish, which includes tuna and mackerel **(Hungerford, 2010).** Sardine, carangids, herring, and anchovies, among other dark muscle fish, are prone to HIS formation because their muscles are histidine-rich **(Choudhury *et al.*, 2008; Feng et al., 2016).** In marketed fish species high in histidine, the allowable quantity of histamine should not exceed 200 mg/kg **(FAO/WHO, 2012, 2018).**

Histamine is formed in raw fish by the action of histidine decarboxylase, which produced by the histamine forming bacteria (HFB) following temperature and time abuse **(Visciano *et al.*, 2012)**. HFB is found naturally in the gills and gut of live sardines and makes up 1% of the typical microflora, while other sources include environmental contact surfaces **(Tao *et al.*, 2011)**. Fish with high HIS concentrations may not show indications of rotting **(Barbosa *et al.*, 2018)**. HFB produces HIS during the growth process, and once created, the enzyme can continue to make HIS in the fish even if the bacteria are not present **(Adams *et al.*, 2018; EFSA, 2015).** At high temperatures, histamine production is increased and therefore, the most crucial factor in preventing histamine generation is to quickly cool the fish after fishing **(FDA, 2011)**. Otherwise, longline capturing results in delayed removal of fish from the water, causing certain fish to overheat **(Köse, 2010)**. Mishandling of fish at a high temperature can also dramatically increase histamine production **(Visciano *et al.*, 2012)**.

High-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay are two techniques, mostly used for detection of histamine in fish muscles. HPLC is frequently used to quantify and validate histamine in fish and fishery products **(Jinadasa *et al.*, 2016)**. Due to its high sensitivity, superior linearity, and accurate quantitative analysis, HPLC is used for the quantitative analysis of biogenic amines. But it has some disadvantages, such being expensive and time-consuming **(Muscarella *et al.*, 2005)**.

There is currently insufficient information available in Egypt about HIS and HFB in sardine. As a result, the goal of this study to see how several parameters impacting histamine levels in different muscular parts of sardines, such as storage time, temperature, and bacterial count and effect of these factors on microscopical lesion scoring of different muscles. This study may aid in the understanding of sardine's shelf life, spoiling, and bacterial growth management. Additionally, it will help in the creation of better techniques for preserving the sardine's quality throughout storage and transportation

MATERIALS AND METHODS

All the procedures applied to the cached fish were approved by the institutional fisheries care and use committee (IACUC) of Cairo University (approval number: Vet CU 8032022402).

2.1. Fish collection and grouping

30 fresh sardines *(Sardina Pilchardus)* (20g±20) purchased from a fisherman in Bahary rings, Alexandria, Egypt. All fishes were separated into two groups (n=15) as follows: first group was kept in crushed ice (0ºC) while the second group was kept at room temperature (21ºC).

2.2. Sensory evaluation

The freshness degree of all fish in each group was assigned by an odd number of panellists via observing the appearance of fish surface, colour, odour, texture, slime, eyes, gills, and belly and finally, we measured the acceptance rate of fish according to the method that is determined by **Triqui and Bouchriti, (2003).** All the previously mentioned sensory parameters were inspected in 0-, 6-, 12-, 18-, and 24hr from fishing and assessed by using the QIM and the EU is grading of blue fishes shown in supplementary file (Tables S1& S2). Concerning the QIM, the degree of freshness was graded on a numerical scale from 0-4. Briefly, score (0) means very fresh fish, 1= slight deterioration, 2= mild deterioration, 3= moderate deterioration, and 4 indicates severe deterioration**.** All the individual scores were added to obtain the total score that known as a quality index (QI). By using the EU grading scheme, the QI score of 1-3 corresponding to E grade, a score of 4-9 corresponding to high A grade, score of 10-17 corresponding to low A grade, score 18-22 corresponding to B grade, and score of higher 22 corresponding to C grade.

2.3. Sampling

We collected muscle specimens from the dorsal, abdominal and tail areas from five fishes in each group at 0-, 6-, 12-, 18-, and 24hrs post-fishing and divided into two parts. Frist part were kept on -20°C for 15 days for further bacterial isolation and HIS analysis, while the second part was fixed at 10% neutral buffered formalin for histopathological examination.

2.4. Histopathological examination

The traditional procedure was used to prepare the formalin-fixed different muscle samples, which include xylene to eliminate extra alcohol and gradient concentrations of alcohol for tissue drying. Then embedded in paraffin wax, sliced at a thickness of 4.5 μm, and stained with hematoxylin and eosin (H&E) for histological examination. We used an Olympus DP27 camera connected to Olympus CellSens dimension software to take pictures of the H&E-treated samples before they were autolyzed using an Olympus BX43 light microscope (**Bancroft, 2013**).

The severity of muscular autolysis in both treatments was assessed using a semiquantitative scoring method in accordance with the protocol described by **Sayed *et al.* (2020)**. Muscular vacuolations, splitting of myofibrils, and widening of the intermuscular space (IMS) are the criteria used to score muscular lesions. In order to assess the severity of histological abnormalities in various muscular sections, seven microscopic regions per five sections in each group were blindly assessed. All the pathological lesions were graded using an ordinal scale from 0 to 4 as follow: (0) normal histology corresponding to 0-2% of muscular damage, (1) slight changes representing 2-10% of muscular damage, (2) mild changes representing 10–40% of muscular damage, (3) moderate changes representing 40–75% of muscle damage and (4) severe changes representing >75% of muscular damage.

2.5. Determination of histamine content in fish muscle

We homogenized 0.5 g muscle specimens from the collected parts by using a cold buffer (PBS). ELISA method used to determine HIS levels, according to the instruction of the manufacturer histamine kit (Sunlong Biotech Co., China).

2.6. Bacteriological examination

2.6.1. Enterobacteriaceae count

10 g muscle sample from different parts (abdomen, dorsal and tail) were homogenized using 0.1% peptone water in Stomacher 400 blender (Seward Lab., Worthing, United Kingdom). The surface spread plate technique applied for bacterial count, using violet red bile glucose agar (VRBGA). The plates inoculated in duplication with 0.1 ml of the original suspension and its prepared decimal 6 dilutions and incubated at 37ºC for 24 hours. All red colonies surrounded by a purple zone were counted and the average CFU / gram sample was recorded.

2.6.2. Isolation and identification of histamine forming bacteria

The Vitek2 compact system was used according to the manufacture’s instruction (Biomeriux VITEK-2 Compact ref Manual – Ref-414532) to identify the isolated strains after purifying and selecting suspected Enterobacteriaceae colonies.

2.7. Statistical analysis

All parametric values were represented as means ± standard error. Using the statistical package programme (SPSS version 20), the recorded findings were investigated using one-way analysis of variance (ANOVA) and post hoc Duncan's test; P values less than 0.05 represent statistical significance **(Kao and Green, 2008)**. The histopathological scoring and sensory evaluation scoring were represented as a median and analyzed using the Kruskal Wallis H test followed by the Mann-Whitney U test as nonparametric values **(Hazra and Gogtay, 2016)**.

RESULTS

3.1. Sensory evaluations

There was no change in the sensory evaluations of sardine at 0hr indicating their freshness. There was marked decrease in the quality of sardine by increasing the storage time and temperatures. The fish freshness was decreased faster at 21ºC than 0°C. The panellists accepted all fish stored at 0ºC till 24hr, and reject fish stored at 21ºC for 24hr **(Table 1)**.

Table (1): Quality Index Method (QIM) and EU grading score for Sardine

	At 0°C					At 21°C				
	0hr	6hrs	12hrs	18hrs	24hrs	0hr	6hrs	12hrs	18hrs	24hrs
General appearance	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	0 ^{a}	1 ^{b}	2 ^{c}	3 ^{d}	3 ^{d}
Surface	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	0 ^{a}	1 ^{b}	2 ^{c}	3 ^{d}	3 ^{d}
Stiffness	0 ^{a}	0 ^{a}	1 ^{b}	2 ^{c}	2 ^{c}	0 ^{a}	0 ^{a}	2 ^{c}	3 ^{d}	3 ^{d}
Flesh firmness										
Eyes										
clarity	0 ^{a}	0 ^{a}	0 ^{a}	1 ^{b}	2 ^{c}	0 ^{a}	1 ^{b}	1 ^{b}	2 ^{c}	2 ^{c}
pupil	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	1 ^{b}	0 ^{a}	0 ^{a}	1 ^{b}	0 ^{a}	2 ^{c}
shape	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	0 ^{a}	1 ^{b}	2 ^{c}	2 ^{c}	2 ^{c}
Cover										
Bloodiness	0 ^{a}	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	0 ^{a}	1 ^{b}	1 ^{b}	2 ^{c}	2 ^{c}
Gill										
Colour	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	2 ^{c}	0 ^{a}	1 ^{b}	1 ^{b}	2 ^{c}	2 ^{c}
Odour	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	2 ^{c}	3 ^{d}
Abdomen (belly-burst)	0 ^{a}	0 ^{a}	0 ^{a}	0 ^{a}	1 ^{b}	0 ^{a}	0 ^{a}	1 ^{b}	2 ^{c}	3 ^{d}
Flesh appearance	0 ^{a}	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	0 ^{a}	1 ^{b}	1 ^{b}	1 ^{b}	2 ^{c}
Quality index	0	1	6	9	11	0	8	15	22	27
EU grade	E	E	HA	HA	LA	E	HA	LA	B	C
Fish acceptance	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Accept**	**Borderline**	**Reject**

Values are represented as median (n=9). Different small letters at the same row mean significant difference between groups at $P \leq 0.05$.

Note: Quality index, the summation of Scores to produce an total sensory evaluation; E, Extra accepted; High A, Highly accepted; Low A, Low accepted; B, Borderline; C, Not admitted. Quality index

3.2. Histopathological examination

Muscle samples obtained from abdominal, dorsal, and tail area of sardine showed normal histological structures with normal minimum splitting of muscle fibers (MF) at 0hr. Additionally, there are no bacterial colonies found in a different muscular area **(Fig. 1)**.

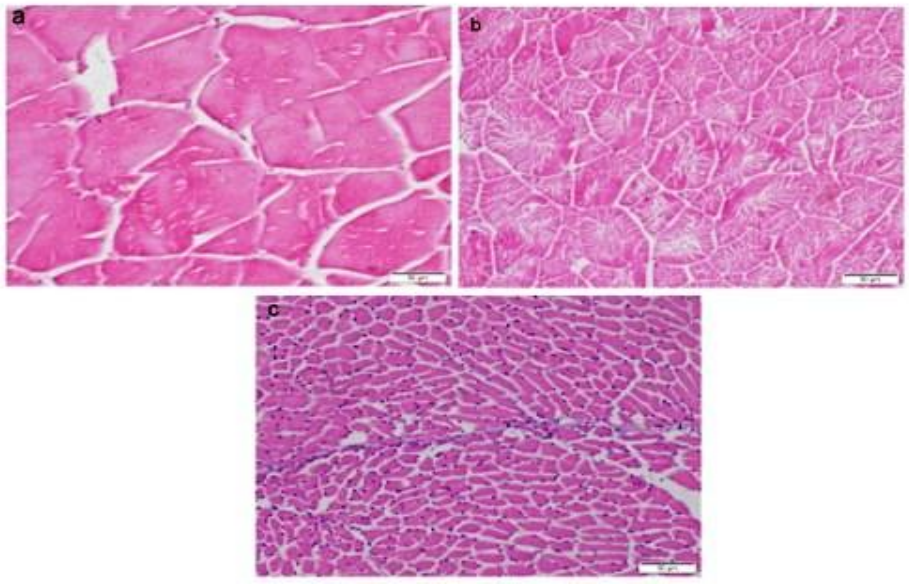

Fig. 1: Photomicrograph of sardine muscle sections at 0hr representing; (a) Abdominal, (b) Dorsal, (c) Tail muscle. All sections stained by H&E and showing normal histological structure.

At 0 hr, the abdominal muscle showed mild to moderate splitting of MF and widening of intermuscular spaces with mild vacuolations at 6hrs **(Fig. 2a)**. While at 12-, 18-, and 24hr showed moderate to severe splitting of MF, and widening of IMS **(Fig. 2b-d)**. At 24hrs, no bacterial colonies were seen in the abdominal muscle **(Fig. 2e)**. On the other side, the dorsal and tail muscles showed normal histological structure till 12hrs **(Fig. 2f-g, 2k-l)** while, at 18- and 24hrs the muscular sections becomes autolyzed **(Fig. 2h-i, 2m-n)**. At 24hrs, no bacterial colonies were seen in the dorsal and tail muscle when modified grams stain was used **(Fig. 2j, 2o)**.

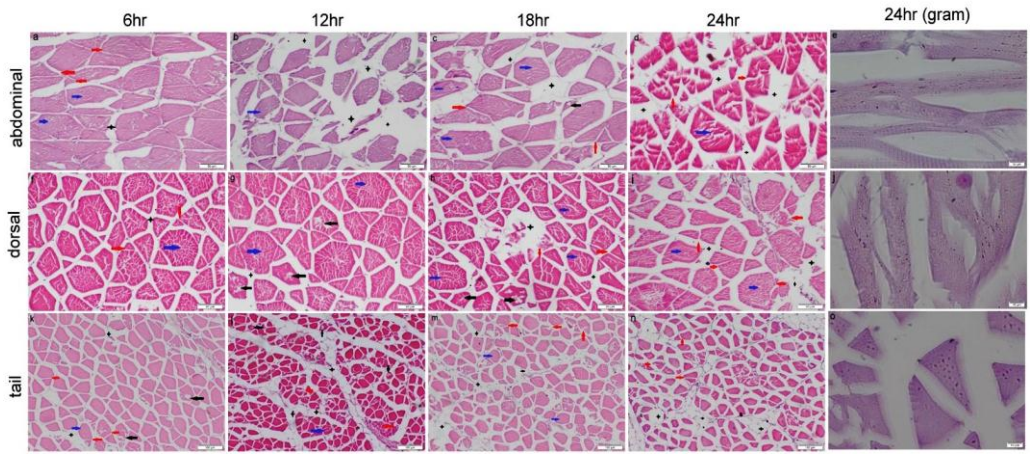

Fig. 2: Photomicrograph of sardine muscle sections kept at 0°C at different storage time representing; (a-e) Abdominal, (f-j) Dorsal, (k-o) Tail muscle. Note: vacuolation of muscle splitting of myofibrils (black arrows), splitting of myofibrils (blue arrows), broken myofibrils (red arrows), widening of intermuscular space (black stars). All sections stained by H&E but sections e, j & o that were stained by the modified grams stain and didn't show any bacteria within myofibers.

At 21 hrs, the beginning of muscular autolysis was at 6hr in the abdominal muscle **(Fig. 3a)**. Additionally, there were moderate to severe splitting of MF, and widening of IMS at 12hrs (**Fig. 3b)** and sever splitting MF, broken myofibrils, and widening of IMS at 18- and 24hrs (**Figs. 3c-d)**. At 24hrs, the abdominal area showed a large number of rod-shaped gram-negative bacteria within MF using modified gram stain **(Fig. 3e)**. Otherwise, both dorsal and tail muscle exhibit normal histological arrangement till 12hrs except some sections showed mild alteration **(Figs. 3f-g, 3k-l)**. Additionally, at 18- and 24hrs showed sever histopathological alteration **(Figs. 3h-i, 3m-n)**. The predominant microscopic lesions were splitting of MF, vacuolations, broken myofibrils, and widening of IMS. Moreover, Gram-negative bacilli were seen in multifocal aggregations in both dorsal and tail area within the MF at 24hrs **(Figs. 3j, 3o)**. By increasing the storage time, the severity of muscular autolysis has been increased. The application of modified grams stain displayed a significant increase in the bacterial count throughout the time in both fish and evidenced by rod-shaped gram-negative bacilli localized within the MF, IMS, and in the perivascular areas. The highest number of bacteria was recorded in the abdominal muscle followed by the tail and dorsal muscle.

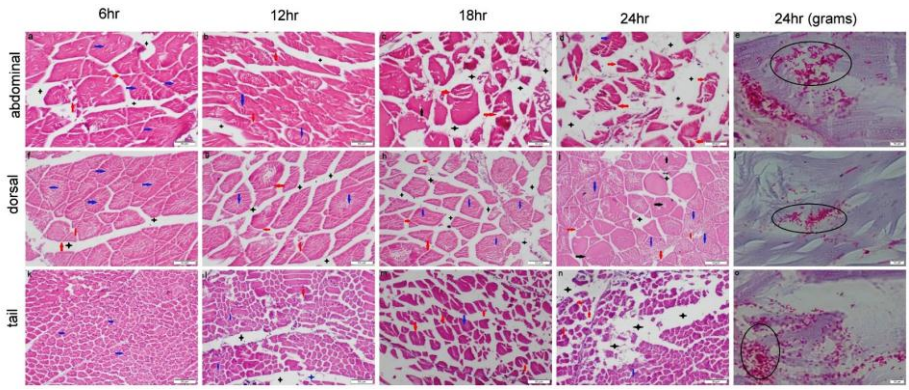

Fig. 3: Photomicrograph of sardine muscle sections kept at 21°C at different storage time representing; (a-e) Abdominal, (f-j) Dorsal, (k-o) Tail muscle. Note: vacuolation of muscles splitting of myofibrils (black arrows), splitting of myofibrils (blue arrows), broken myofibrils (red arrows), widening of intermuscular space (black stars). All sections stained by H&E but sections e, j & o that were stained by the modified grams stain and showing a huge number of gram negative red bacilli within myofibers (circle).

Table (2) provides a summary of the results of the muscular lesion score at different storage conditions. The fish kept at 21°C for either 18 or 24 hours showed the highest scores in all parameters, followed by the fish kept at 0°C for 24 hours. On the other hand, different muscle sections taken from fish that had been kept at 0°C for either 6 or 12 hours showed the lowest score.

3.3. Histamine contents in fish muscles

Throughout different storage temperature and time, the muscular content of HIS showed marked variation. At 0hr, HIS didn't exceed the permissible limits in different muscular areas of fish. Within the same storage time, the fish stored at 21°C have a higher level of HIS than those stored at 0°C. By increasing the storage time, HIS content showed a significant increase in all muscle areas. Additionally, their levels were higher in the abdominal muscle than the tail muscle and finally the dorsal muscle **(Table 3)**.

3.4. Isolation and identification of histamine forming bacteria

The total plate count (TPC) in different muscular parts of fish at various storage times and temperature is shown in **(Table 4).** There are significant differences in TPC relation at the storage time and temperature. Values of fishes stored at 21°C showed higher growth than those stored at 0°C even though, the initial count is the same. Furthermore, the TPC was markedly elevated in all muscle samples throughout the storage time, but, the growth rate was faster in abdominal muscle > tail muscle > dorsal muscle. There were positive correlations between the HIS levels and TPC during the storage. Vitek2 gave 99% to 96% confidence, identity of the following strains: *Enterobacter aerogenes, Enterobacter cloacae complex, Lelliottia amnigena-1(Enterobacter amnigena-1). Enterobacter aerogenes* and *Enterobacter cloacae complex*

were the predominant strain recorded at 6-, 12-, 18-, and 24hrs in both storage conditions while *Lelliottia amnigena-1 (Enterobacter amnigena-1)* was identified only in 18- and 24hrs at 21°C.

Table (2): The microscopic lesion scoring of muscular autolysis in different areas

	At 0hr	At 0°C				At 21°C			
		6hrs	12hrs	18hrs	24hrs	6hrs	12hrs	18hrs	24hrs
Abdominal muscle									
Vacuolization	0^{a}	0^{a}	1^{b}	1^{b}	1^{b}	0^{a}	1^{b}	0^{a}	0^{a}
Splitting	0^{a}	2^{c}	3^{d}	3^{d}	4^{e}	2^{c}	3^{d}	4^{e}	4^{e}
Broken myofibrils	0^{a}	1^{b}	2^{c}	2^{c}	3^{d}	1^{b}	2^{c}	3^{d}	4^{e}
Widening of intermuscular space	0^{a}	0^{a}	1^{b}	3^{d}	3^{d}	1^{b}	2^{c}	4^{c}	4^{e}
Dorsal muscle									
Vacuolization	0^{a}	0^{a}	1^{b}	1^{b}	0^{a}	0^{a}	0^{a}	0^{a}	1^{b}
Splitting	0^{a}	2^{b}	2^{b}	3^{c}	3^{c}	3^{c}	3^{c}	4^{d}	4^{d}
Broken myofibrils	0^{a}	0^{a}	1^{b}	2^{c}	3^{d}	2^{c}	3^{d}	4^{e}	4^{e}
Widening of intermuscular space	0^{a}	0^{a}	0^{a}	2^{b}	3^{c}	0^{a}	2^{b}	3^{c}	4^{d}
Tail muscle									
Vacuolization	0^{a}	1^{b}	2^{c}	3^{d}	3^{d}	0^{a}	0^{a}	0^{a}	2^{c}
Splitting	0^{a}	1^{b}	2^{c}	2^{c}	2^{c}	2^{c}	3^{d}	4^{e}	4^{e}
Broken myofibrils	0^{a}	1^{b}	1^{b}	2^{c}	3^{d}	0^{a}	1^{b}	3^{d}	4^{e}
Widening of intermuscular space	0^{a}	1^{b}	2^{c}	2^{c}	2^{c}	0^{a}	2^{c}	4^{d}	4^{d}

Values are represented as median (n= 21) (7 random microscopic fields in 3 sections representing 3 fish). Different small letters in the same row mean significant difference between groups (different time and temperatures) at $P \leq 0.05$.

Note: (0) normal histology 0-2% muscular damage, (1) slight changes 2-10% muscular damage, (2) mild changes 10–40% muscular damage, (3) moderate changes 40–75% muscular damage, (4) severe changes >75% muscular damage.

Table (3): Effect of storage time and temperature on the muscular histamine contents (mg/kg)

	0hr	0°C				21°C			
		6hr	12hr	18hr	24hr	6hr	12hr	18hr	24hr
Abdominal	0.4± 0.2 $^{a, A}$	10± 1.2 $^{b, B}$	45± 0.0 $^{c, B}$	70± 3.5 $^{d, B}$	146± 2.3 $^{e, B}$	45± 1.7 $^{c, A}$	145± 2.9 $^{e, B}$	244± 1.2 $^{f, C}$	450± 1.2 $^{g, C}$
Dorsal	0.3± 0.1 $^{a, A}$	3± 1.2 $^{a, A}$	39± 0.6 $^{b, A}$	55± 2.9 $^{c, A}$	137± 1.7 $^{d, A}$	40± 1.2 $^{b, A}$	135± 0.6 $^{d, A}$	200± 2.9 $^{e, A}$	412± 4.0 $^{f, A}$
Tail	0.4± 0.06 $^{a, A}$	6± 1.7 $^{a, AB}$	42± 1.2 $^{b, AB}$	64± 1.7 $^{c, AB}$	142± 1.2 $^{d, AB}$	42± 1.2 $^{b, A}$	144± 0.6 $^{d, B}$	230± 1.2 $^{e, B}$	426± 2.3 $^{f, B}$

Values are represented as mean ± SEM (n= 3 fish at different time). Different small letters in the same row mean a significant difference between groups (different time and temperatures) at $P \leq 0.05$. While, different capital letters at the same column mean significant difference between different muscular areas (in the same time) at $P \leq 0.05$.

Table (4): Effect of storage time and temperature on the bacterial growth (cfu/g)

	0hr	0°C				21°C			
		6hr	12hr	18hr	24hr	6hr	12hr	18hr	24hr
Abdominal	$<10^2$± 0.0 $^{a, A}$	1.2×10^3± 57.7 $^{ab, C}$	2×10^3± 57.7 $^{b, C}$	5×10^3± 115.5 $^{c, C}$	7×10^3± 57.7 $^{d, C}$	6×10^3± 57.7 $^{cd, C}$	1.1×10^4± 115.5 $^{e, C}$	2×10^5± 577.4 $^{f, C}$	4×10^6± 577.4 $^{g, C}$
Dorsal	$<10^2$± 0.0 $^{a, A}$	3×10^2± 57.7 $^{ab, A}$	5×10^2± 57.7$^{ab, A}$	1.2×10^3± 57.7 $^{ab, A}$	2.2×10^3± 57.7$^{b, A}$	1.9×10^3± 57.7 $^{ab, A}$	6×10^3± 115.4 $^{c, A}$	1.3×10^5± 577.4 $^{d, A}$	1.5×10^5± 1154.7 $^{e, A}$
Tail	$<10^2$± 0.0 $^{a, A}$	6×10^2± 57.7$^{a, B}$	1.4×10^3± 57.7 $^{ab, B}$	3×10^3± 57.7$^{bc, B}$	5×10^3± 115.5 $^{c, B}$	3.4×10^3± 57.7 $^{bc, B}$	9×10^3± 461.8 $^{d, B}$	1.4×10^5± 288.6 $^{e, B}$	3×10^5± 1154.7 $^{f, B}$

Values are represented as mean ± SEM (n= 3 fish at different time). Different small letters in the same row mean a significant difference between groups (different time and temperatures) at $P \leq 0.05$. While, different capital letters in the same column mean significant difference between different muscular areas (in the same time) at $P \leq 0.05$.

DISCUSSION

Sardine is the most popular and consumed pelagic fish species in Egypt and the Middle East **(El-Sherbiny and Sallam, 2021)**. It enters the Egyptian markets either through importation as frozen fish or through the fresh capture fisheries throughout the Red Sea and Mediterranean coasts of Egypt **(FAO, 2004)**. Scombroid fish poisoning is an allergy like reaction which occur due to consumption of fish containing high level of histamine **(EFSA, 2017)**. Histamine is playing an important role in many physiological and pathological processes within the body **(Kovacova-Hanuskova *et al.*, 2015).** It is the only biogenic amine with regulatory limits of 50 mg/kg in fish, seafood, and fishery product **(FDA, 2011).** Scombroid fish and other dark muscle fish such as sardine and anchovies are prone to form histamine, as their muscles are histidine-rich **(Choudhury *et al.*, 2008; Feng *et al*., 2016).** Histidine is converted to HIS by the action of bacterial enzyme histidine-decarboxylase (HDS) (**Hungerford, 2010).** The current study aims to investigate the effect of storage time and temperature on the degree of fish freshens, histamine levels, bacterial count, and histopathological alteration in different muscle areas of sardine.

In the current investigation, the EU grading method and QIM were used to determine the fish's quality. Our research showed that the quality of fish dropped over the course of storage time and temperature, but that it did so more quickly at 21°C than at 0°C. Meanwhile, the muscle histamine level increased over the course of storage time and temperature. The amount of histamine in fish kept at 0°C does not go beyond the legal limit. Our research showed that there is no correlation between histamine concentration and fish quality which is consistent with several previous studies **Shakila *et al.*, (2003); Mercogliano and Santonicola, (2019).**

Our results of histopathological examination showed that the muscular autolysis score was significantly increased throughout increased storage time, and was faster in 21 ºC than 0 ºC. Additionally, it's higher in abdominal muscle than the tail and dorsal muscle. The main muscular alteration were splitting of MF and breakdown of muscles with widening of IMS. Our results showed there were a positive correlation between histopathological changes and bacterial growth, which identified by modified gram stain. The higher temperature accelerates the muscle autolysis by increasing both bacterial growth **(Ghaly *et al.*, 2010)** and rate of glycolysis **(George *et al.*, 2016)**. According to reports, fish muscles autolyzed more quickly than mammalian muscles **(Roberts, 2012).** Additionally, fish cells use glucose and glycogen rapidly due to stress during fish handling and transportation, which result in raises lactic acid, lower PH levels and eventually accelerate the muscular autolysis **(Gatica *et al.*, 2008).** In the current investigation, we found a large number of rod-shaped gram-negative bacteria in the perivascular area, intermuscular space, and muscle fibers. The number of bacteria was higher in abdominal muscle > tail > dorsal muscle.

Histamine is not equally distributed throughout the entire rotten fish, and the difference can be ten times greater. This may help to explain why eating the same fish results in a dose–response relationship with consumers **(Lehane and Olley 2000).** The HIS content also revealed differences in certain muscle regions. HIS levels were substantially higher in abdominal muscle compared to the tail and dorsal muscles, which is consistent with **Mercogliano and Santonicola, (2019).** Both the bacterial count results and the HIS muscle content data follow the same pattern and showed a considerable rise over the course of storage time and temperature. Histamine formation is negligible in fish stored at 0°C or below, but it is formed after exposure of fish to a temperature above 4 °C during and/or after capture and may reach to the toxic levels **(Evangelista *et al.*, 2016)**.

Acinetobacter, *Enterobacter*, *Lelliottia* and *Shewanella* are the gut natural microflora which important for Fish health **(Gómez and Balcázar 2007; Nayak 2010; Yilmaz *et al.*, 2018)**. Many different bacterial species of the Enterobacteriaceae family are known to possess histidine decarboxylase activity and have the ability to produce histamine, including *Morganella morganii, Klebsiella pneumoniae, Hafnia alvei, Enterobacter aerogenes, Enterobacter cloacae*, **(Kim *et al.*, 2003).** Via utilizing the violet red bile glucose agar, we used the surface spread plate method to count the number of bacteria. It is created for the enumeration of *Enterobacteriaceae* based on the use of the indicator system glucose and neutral red as well as the selective inhibitory components crystal violet and bile salts. As a result, a large number of undesirable organisms are prevented from growing, while sought-after bacteria will assimilate glucose and create purple zones around the colonies. *Enterobacter aerogenes* and *Enterobacter cloacae complex* are the predominant strains recorded in 6-, 12-, 18-, and 24hrs in both storage conditions while *Lelliottia amnigena-1* was identified only in 18- and 24hrs at 21°C. In this concern, the identified isolated bacteria differ from the previously recorded isolates in sardines that including *Enterobacter cloacae, Raoultella planticola, Citrobacter freundii, and Enterobacter aerogenes* **(Sabry *et al.*, 2017)**, *Pseudomonas, Vibrio, Klebsiella, E. coli, Salmonella, Proteus* **(AE Refai *et al.*, 2020)**. The difference of isolated strains might depend on the handling procedures, holding times and temperatures. In addition, the character of the microflora can be influenced by the fish's feeding habits, geographical location, and the season, ocean temperature **(Lehane and Olley 2000)**. Additionally, the rate of bacterial growth was faster in the abdominal muscle than the tail muscle and finally than the dorsal muscle indicating the favorable association between the muscular content of HIS and bacterial count during the storage. Our investigation about higher HIS levels in the abdominal muscle than the tail or dorsal muscle, may be explained by this fact.

CONCLUSION

Our results revealed that the freshness of sardine was decreased by the storage time and temperature. Meanwhile, histopathological alterations, bacterial count, and histamine levels in different muscle areas were increased throughout the storage time and temperature. All the measuring parameters were recorded in the abdominal muscle higher than the tail and dorsal muscle. The main isolated bacterial strains are *Enterobacter aerogenes*, *Enterobacter cloacae complex*, and *Lelliottia amnigena-1* which identified with vitke2.

From our results, we recommend refrigerating fish at temperature below 0℃ as soon as possible from the time of catching to the time of consuming to avoid bacterial contamination. Furthermore, the fish with a bad odor, sunken eye, and brownish or greenish colored gill shouldn't be consumed. Also, the fish should be obtained from reputable retail outlets. On the other side, people who eat fish in a restaurant, there is no way to know if this fish is spoiled or not but, we can recommend them to avoid eating fish parts that containing high levels of histamine such as abdominal and red muscles. Additionally, if someone gets a scombroid poisoning from fish markets or restaurant you must tell the public health authorities to avoid further outbreaks.

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