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Attempts for controlling of histamine by using bacillus polymyxa in salted fishes

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ABSTRACT

Histamine is the causative agent of scombroid fish poisoning which is considered as a foodborne chemical hazard. Histamine is produced in foods through decarboxylation of histidine by the activity of various species of bacteria. Fish and its fermented products are rich in free amino acids, making them susceptible to bacterial decarboxylase activity, and thus might contain a high level of biogenic amines including histamine. In this study, seventy-five random samples of salted fishes represented by Sardine, Fesiekh and Molouha (25 0f each) were collected from fish markets located in Menoufia governorate, Egypt. The collected samples were kept individually in an insulated ice box and transferred directly to the laboratory for determination of their contents of histamine. Further, the application of *Bacillus polymyxa* as a biological trial to control the histamine in such salted fishes was also studied. The mean values of histamine levels in the examined samples of sardine, Fesiekh and Molouha were 10.82±0.64, 16.05±0.71 and 19.78±0.95(mg/100g), respectively. The number of accepted samples of salted fish according to their histamine content were 20, 18 and 15 by 80%, 72% and 60% and unaccepted samples were 5, 7 and 10 by 20%, 24% and 40%, respectively. The effect of *B. polymyxa* culture ($1x10^7$ cfu/g) on the levels of histamine experimentally inoculated to salted sardine fillets (50 mg/Kg) was excellent where its level was decreased to 35.2 mg/kg after 1 day, 21.8 mg/kg after 2 days and 14.5 mg/kg after 3 days with reduction percentages of 29.6%, 56.4% and 71.0 %, respectively.

INTRODUCTION

Fish has a high-quality protein, excellent source of phosphorus, calcium and the provision of ß-complex vitamins are considered to be the preferred source of high nutritional value and highly desired food **(Hassan et al. 2007).**

Salted fish products are traditional salted and fermented fish products, prepared by adding 10-20% of clean table salt to raw fish, and then allowed to ferment for 3-6 months depending on the processing store until the fish tissue has solubilized **(Lee et al. 2016)**.

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High concentrations of histamine are found in the muscles of some species, such as tuna, bonito and sardines. Moreover, the biogenic amines content in fish varies depending on the season, climate, food, sex, genetics, physiological state, storage and sample tissue **(Lee et al. 2012).**

Biogenic amines have typically been produced by decarboxylation of free amino acids by certain bacteria. Histamine is produced in food through decarboxylation of histidine by the activity of various species of bacteria. In addition, the quantification of biogenic amines in foods can be used as indicator for the quality and freshness of foods **(Awan and Thomas, 2008).**

Histamine is the causative agent of scombroid poisoning and a foodborne chemical hazard. Although, scombroid poisoning is usually of mild symptoms including rash, urticaria, vomiting, diarrhea and itching of the skin, the severity of the illness varies depending on the amounts of histamine ingested and individual's susceptibility to histamine **(Taylor SL, 1986).**

At present, the application of starter cultures with high proteinase activity is common practice to accelerate fermentation time and the end of fermentation is determined by color, aroma and flavor. Salted fish products are reported to contain considerable amounts of biogenic amines such as histamine, in addition to many nutritious compounds **(Fu et al. 2008)**.

Starter cultures play a key role in reducing the content of biogenic amines and polycyclic aromatic hydrocarbons which results in protection of fermented foods from chemical hazards **(García-Díez and Saraiva, 2021).** Therefore, the current study was planned to determine histamine content in salted fish (sardine, fesiekh and molouha) and use Bacillus *polymyxa* as a biological trial to control the histamine in such salted fishes.

MATERIAL and METHODS

1. Collection of samples:

Seventy-five random samples of salted fishes represented by Sardine, Fesiekh and Molouha (25 0f each) were collected from fish markets located in Shibin- Elkom, Menoufia governorate, Egypt. The collected samples were kept individually in an insulated ice box and transferred directly to the laboratory for determination their contents of histamine. Further, the application of *Bacillus polymyxa* as a biological trial to control the histamine in such salted fishes was also studied.

2. Determination of histamine by ELISA (Leszczynskai et al. 2004):

2.1. Intended use and principle of the test:

This enzyme immunoassay is for the quantitative determination of histamine in different tissues of the body using supplementary kit (available for purchase separately, cat. no. (BAE -1100).

First, histamine is quantitively acylated. The subsequent competitive ELISA kit uses the microtiter plate format. The antigen is bound to the solid phase of the microtiter plate. The acylated standards controls, samples, and the solid phase bound analyte compete for a fixed number of antiserum binding sites. After the system is in equilibrium, free antigen and free antigen-antiserum complexes are removed by washing. The antibody bound to the solid phase is detected by an anti-rabbit IgG- peroxidase conjugate using TMB as a substrate. The reaction is monitored at 450 nm. Quantification of unknown samples is achieved by comparing their absorbance with a reference curve prepared with known standard concentrations.

2.2. Test procedure:

All reagents and samples are allowed to reach room temperature prior to use. Measurement in duplicates is recommended.

2.3. Preparation of reagents:

Wash Buffer

Dilute the 20 mL Wash Buffer Concentrate with distilled water to a final volume of 1,000 ml.

Storage: up to 6 months at 4 – 8°C.

Acylation Diluent

The Acylation Diluent has a freezing point of –18.5°C. To ensure that the Acylation Diluent is liquid when being used, it must be ensured that the Acylation Diluent has reached room temperature and forms a homogeneous, crystal-free solution before being used. Alternative the Acylation Diluent can be stored at room temperature (20–25°C), separate from the other kit components.

Acylation Reagent

Reconstitute each vial with 1.25 mL Acylation Diluent. The Acylation Reagent has to be newly prepared prior to the assay (not longer than 1 hour in advance). If more than 1.25 mL is needed, pool the contents of 2 or 3 vials and mix thoroughly.

2.4. Sample preparation and acylation:

2.4.1. Pipette 25 µL of standards, 25 µL of controls, 25 µL of plasma samples, and 10 µL of the tested samples into the respective wells of the Reaction Plate.

2.4.2. Add 25 µL of Acylation Buffer to all wells.

2.4. 3. Add 25 µL of Acylation Reagent to all wells.

2.4.4. Incubate for 45 min at RT (20-25°C) on a shaker (approx. 600 rpm).

2.4.5. Add 200 µL of distilled water to all wells.

2.4.6. Incubate for 15 min. at RT (20-25°C) on a shaker (approx. 600 rpm). Take 25 µL of the prepared standards, controls, and samples for determination of histamine levels.

2.5. Histamine by ELISA:

2.5.1. Pipette 25 µL of the acylated standards, controls, and samples into the appropriate wells of the Histamine Microtiter Strips.

2.5.2. Pipette 100 µL of the Histamine Antiserum into all wells and cover plate with Adhesive Foil.

2.5.3. Incubate for 3 hours at RT (20-25°C) on a shaker (approx. 600 rpm). Alternatively, shake the Histamine Microtiter Strips briefly by hand and incubate for 15 – 20 hours at 2 – 8°C.

2.5.4. Remove the foil, discard or aspirate the contents of the wells and wash each well 4 times thoroughly with 300 µL Wash Buffer. Blot dry by tapping the inverted plate on absorbent material.

2.5.5. Pipette 100 µL of the Enzyme Conjugate into all wells.

2.5.6. Incubate for 30 min at RT (20-25°C) on a shaker (approx. 600 rpm).

2.5.7. Discard or aspirate the contents of the wells and wash each well 4 times thoroughly with 300 µL Wash Buffer. Blot dry by tapping the inverted plate on absorbent material.

2.5.8. Pipette 100 µL of the Substrate into all wells and incubate for 20-30 min at RT (20 -25°C) on a shaker (600 rpm). Avoid exposure to direct sunlight.

2.5.9. Add 100 µL of the Stop Solution to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.

2.5.10. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to 450 nm with a reference wavelength between 620 nm and 650 nm.

2.6. Calculation of results:

Standard	Concentration of the standards					
	A	B	C	D	E	F
Histamine (ng/mL = µg/L)	0	0.5	1.5	5	15	50
Histamine (nmol/L)	0	4.5	13.5	45	135	450
Conversion:	Histamine (ng/mL) x 9 = Histamine (nmol/L)					

2.6.1. The calibration curve is obtained by plotting the absorbance readings (calculate the mean absorbance) of the standards (linear, y-axis) against the corresponding standard concentrations (logarithmic, x- axis). **2.6.2.** Use a non-linear regression for curve fitting (e.g., spline, 4-parameter, akima).

2.6.3. The concentrations of the plasma samples and the controls can be read directly from the standard curve.

2.7. Quality control:

It is recommended to use control samples at both normal and pathological levels. The kit controls or other commercially available controls should be within established confidence limits. The confidence limits of the kit controls are printed on the quality control (QC) Report.

2.7.1. Calibration:

The binding of the antisera and the enzyme conjugates and the activity of the enzyme used are temperature dependent, and the extinction values may vary if a thermostat is not used. The higher the temperature, the higher the extinction values will be. The extinction values also depend on the incubation times. The optimal temperature during the Enzyme Immunoassay is between 20-25°C. In cases of overflow, reading of the solution absorbance in the wells within 10 minutes, using a microplate reader set to 450 nm.

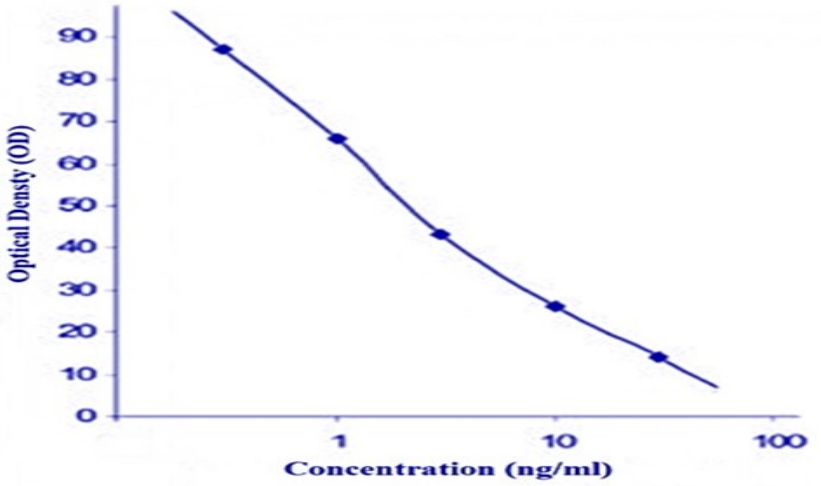

Figure (1): Calibration standard curve of histamine stipulated by ELISA

3. Experimental part:

The effect of *Bacillus polymyxa* for reducing the histamine concentrations of experimentally inoculated sardine fillets was studied as follow:

3.1. Preparation of bacterial suspension (Eom et al. 2015):

Bacillus polymyxa strain was cultivated in Brain Heart Infusion (BHI) Broth (Fluka, Sigma-Aldrich Chemie GmbH) for 24 hours at 37ºC to prepare an overnight culture. One ml of the cultivated bacterial suspension was decimally diluted in sterile peptone water (0.1%, w/v) (Merck, Darmstadt, Germany). Accordingly, the viable count of *Bacillus polymyxa* strain was carried out according to the plate count

method **[**A volume of the culture broth corresponding to approximately 5×10^7 bacteria was centrifuged (500 rpm for 15 minutes at 5ºC)], the bacterial pellets were washed twice with deionized water.

3.3.2. Binding assay:

The bacterial culture was adjusted finally to reach a final concentration of 1×10^7cfu/g as well as the histamine level adjusted to be 50 mg/Kg according to **Halttunen et al. (2008)** with some modifications. Accordingly, the tested fish fillet (without *Bacillus polymyxa*) was served as a control assay. However, the test group represented the samples contaminated with histamine as well as treated with *Bacillus polymyxa* were served as treated group. The samples were acidified with ultrapure HNO3 and examined at zero, 1, 2 and 3 days for measuring their histamine levels using ELISA technique and repeat this trail 3 times. The obtained results were statistically analyzed by application of student t-test according to **Feldman et al. (2003)**.

RESULTS

Table 1. Analytical results of histamine levels (mg/100g) in the examined samples of salted fish (n=25).

Salted fishes	+ve samples		Min	Max	Mean ± S.E
	No	%			
Sardine	17	68	1.6	24.5	10.82 ± 0.64^{C}
Fesiekh	19	76	3.1	29.8	16.05 ± 0.71^{B}
Molouha	23	92	3.7	41.9	19.78 ± 0.95^{A}

*Means with different superscript letters in the same row are significantly different (P<0.05).

Table 2. Acceptability of the examined salted fish samples based on their histamine content (n=25).

Salted fishes	Maximum Permissible Limit (mg/100g)*	Accepted samples		Unaccepted samples	
		No.	%	No.	%
Sardine	20	20	80	5	20
Fesiekh		18	72	7	24
Molouha		15	60	10	40
Total (75)		53	70.7	22	29.3

*Egyptian Organization for Standardization (2005)
No. 1725-2/2005 for salted sardine (2005)
No. 1725-1/2005 for Fesiekh (2005)
No. 1725-3/2005 for Molouha (2005)

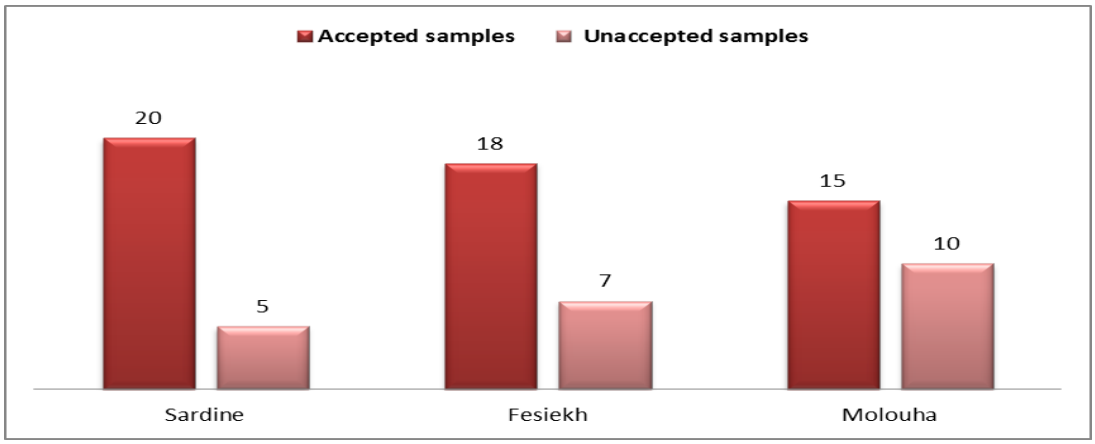

Figure (2): Acceptability of the examined salted fish samples based on their histamine content.

Table 3. Effect of *B. polymyxa* culture ($1x10^7$ cfu/g) on the levels of histamine experimentally inoculated to salted sardine (50 mg/Kg).

Group / Storage time	Control (mg/Kg)	*B. polymyxa* Treated group (mg/Kg)	Reduction %
Zero time	50	50	-------
1 day	50	35.2	29.6
2 days	50	21.8	56.4
3 days	50	14.5	71.0

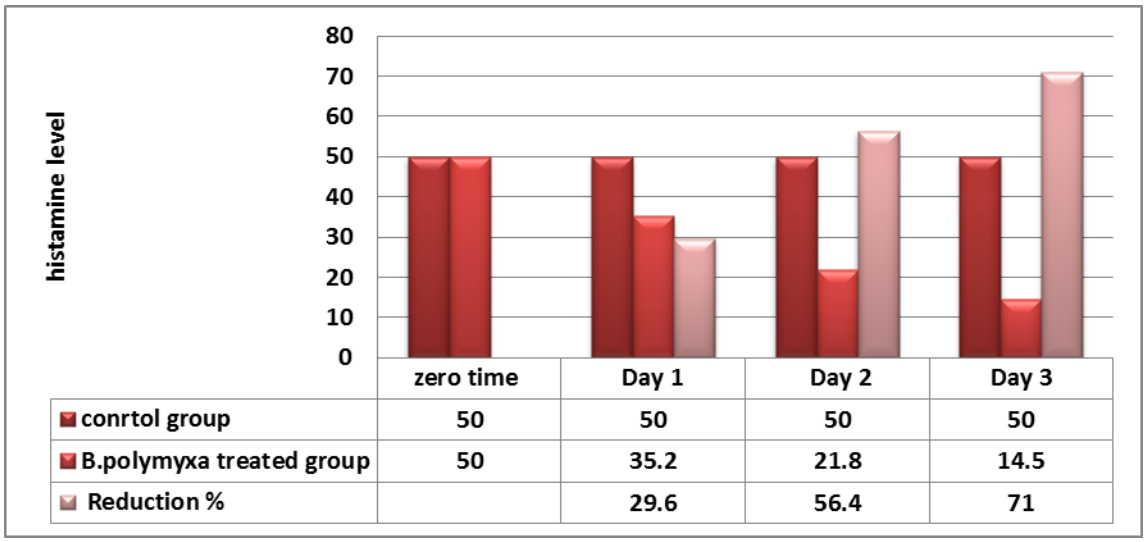

Figure (3): Histamine residues (mg/Kg) in the control and *B. polymyxa* treated salted sardine samples

DISCUSSION

It is evident from the results recorded in table (1) that histamine concentrations in Sardine, Fesiekh and Molouha were ranged from 1.6 to 24.5, 3.1 to 29.8 and 3.7 to 41.9 with an average of 10.82±0.64, 16.05± 0.71 and 19.78±0.95 mg %, respectively. There were significant differences between the examined samples **(P<0.05).**

The concentrations of histamine in sardine samples were higher than the results obtained by **El- Sayed (2010)** who found that mean histamine concentration of sardine was 6.91±0.46mg/100g.

The obtained results were lower than those of **Huda KE, 2014 (126.65**± 14.77) in Fesiekh samples. While, **Dalia A, 2016** recorded (33.12± 1.15 and 28.14± 1.02 mg %), **Azza EA and Weam B, 2011** (21.5 and 17.2 mg/100gm) and **Saad et al. 2021** (26.48±0.52 and 21.93± 0.40) in Fesiekh and Sardine, respectively.

The factors affecting histamine output is the favorable conditions for microorganism to produce decarboxylase enzyme which result in decarboxylation of free amino acids **(El-Mossalami and El- Agizy, 2005).**

In addition, during suitable temperature conditions, bacteria that decarboxylating histidine are naturally present in the skin, gills and gut of a freshly caught fish can multiply rapidly and form histamine. Once bacterial multiplication has occurred, the histidine decarboxylases enzyme activity can continue slowly even after bacterial growth has ceased at refrigeration temperatures **(Lehane and Olley, 2000)**.

Results achieved in table (2) and fig (2) indicated that 80%, 72% and 60% of the examined samples of sardine, fesiekh and molouha, respectively were accepted based on their content of histamine and this come in accordance with those reported by **Egyptian Organization for Standardization (2005)** which stipulated that the maximum permissible limit of histamine in sardine, fesiekh and molouha should not exceed 20mg%.

Lower results obtained by **Saad et al. (2021)** the accepted samples were 44% and 48% in fesiekh and sardine.

Histamine is one of the amines implicated in the toxicity of food, however at low levels histamine is not toxic; the presence of cadaverine and putrescine which have five times higher levels than histamine will contribute to histamine toxicity **(Emborg and Dalgaard, 2006).**

The consumption of histamine in the range of 8–40 mg,40– 100 mg or more in one meal may cause slight, intermediate and severe poisoning, respectively (**Parente et al. 2001**). Low level of biogenic amines does not cause a serious risk to human health, since the amine oxidase (mono amine oxidase and diamine oxidase) in human intestine can detoxify such amines (**Biji et al. 2016).**

However, scombroid fish poisoning will occur in a healthy individual only when a dose of at least 50 mg histamine is consumed. This generally occurs when the fish is having a histamine level of more than 200 mg/kg. Freshly caught scombrotoxin forming fish typically contain histamine level less than 2 mg/kg **(FAO/WHO 2013).**

Table (3) and fig (3) showed that the effect of *B. polymyxa* ($1x10^7$) on the levels of histamine experimentally inoculated to salted sardine (50 mg/Kg) was decreased to 35.2 mg/kg after 1 day, 21.8 mg/kg after 2 days and14.5 mg/kg after 3 days by a percentage of reduction reached 29.6%, 56.4% and 71.0%, respectively. Lower results were obtained by **Lee et al. (2016)** who recorded a reduction of histamine in inoculated samples was 34% at the end of fermentation, compared to control samples.

Higher results were obtained by **Saad et al. (2021)** who showed that the effect of *B. polymyxa* culture ($2x10^7$) on the levels of histamine experimentally inoculated to sardine fillets (40 mg/Kg) was decreased to 22.1 mg/kg after 8 hours, 14.2 mg/kg after12 hours and 8.9 mg/kg after 24 hours by a reduction percentage

of 44.7%, 64.5% and 77.8%, respectively.

Application of *B. polymyxa* as a starter culture in salted fish products fermentation is effective to inhibit biogenic amines accumulation and to enhance the safety of salted and fermented fish products **(saad et al. 2021).**

CONCLUSION

The present study declared that some samples of sardine, fesiekh and molouha obtained from various fish markets in Menoufia governorate, Egypt, exposed for human consumption were contaminated with histamine. Molouha contained the highest level of histamine, while sardine contained the lowest level of histamine. Application of *B. polymyxa* as a starter culture in salted fish products fermentation is effective to inhibit histamine accumulation and to enhance the safety of salted fish products.

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