



Biogenic Amines Content and The Prevalence of *Staphylococcus aureus* in I Crab and Shrimp



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Abstract

SHRIMP AND CRAB are crustaceans that are recognized as novel sources of high biological value protein. They are also high in moisture, minerals like calcium and phosphorus, vitamins, including vitamin D, and omega-3 polyunsaturated fatty acids. Around the world, people are widely dependent on fish and shellfish, especially in Egypt, to make up for the lack of red meat. Naturally occurring toxins called biogenic amines (BAs) are produced when a wide range of microbes, including both pathogenic and nonpathogenic bacteria metabolize particular amino acids. One of the main foodborne pathogens that cause numerous cases of intoxications worldwide is *Staphylococcus aureus*. Nevertheless, little is known about the prevalence of *Staphylococcus aureus* and the numerous enterotoxins that are produced in the examined crab and shrimp. This study's main goal was to determine the amount of BAs generated in the studied shrimp and crab. Furthermore, the estimated daily intakes of BAs that the Egyptian population derived from eating these crustaceans were calculated. The other aim of the current study was to find out how common *Staphylococcus aureus* is in the edible tissues of shrimp and crab. In addition, the public health significance of BAs and *S. aureus* was also discussed. The obtained results revealed detection of histamine, cadaverine, putrescine, and tyramine from the examined crab and shrimp at variable rates. In addition, *S. aureus* was isolated from the examined crab and shrimp at 50%, and 70%, respectively. Therefore, strict hygienic measures should be followed during handling and processing of such shellfish.

Keywords: Crustaceans, *Staphylococcus aureus*, Biogenic amines, Egypt.

Introduction

Crustaceans, such as crab and shrimp, are considered to be excellent sources of protein with a high biological value, including approximately 19.2%. They also have a moisture content of about 75.3% and a carbohydrate content of around 3.6%. Additionally, they are rich in omega-3 polyunsaturated fatty acids, minerals such as calcium and phosphorus, and several vitamins, including vitamin D. Crustaceans contain elevated quantities of the amino acids alanine, arginine, aspartic acid, glutamic acid, glycine, and lysine, which make up 8.1%, 7.1%, 7.7%, 10.7%, 4.5%, and 5.5% of their

total protein, respectively [1-3]. Globally, particularly in Egypt, there is an increasing trend in the eating of fish and shellfish as a substitute for the insufficient availability of red meat. Furthermore, throughout their lifespan, crustaceans are subjected to a diverse range of microorganisms and xenobiotics.

Biogenic amines are frequently formed through the decarboxylation of free amino acids. They occur naturally in small amounts in food, but can also be synthesized in higher quantities under specific conditions. The main determinants influencing the development of biogenic amines include the

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freshness of the food, the storage temperature, the presence of microorganisms possessing the decarboxylase enzyme, and the presence of conditions that facilitate the growth of these microorganisms and the synthesis of their decarboxylase enzyme [4-7].

Histamine (HIS), tyramine (TYR), tryptamine (TRP), and putrescine (PUT) are the primary biogenic amines present in fish, shellfish, and other food types. These amines are formed through the enzymatic decarboxylation of histidine, tyrosine, tryptophan, and ornithine, respectively. They are of great importance in terms of their presence in these food sources [8, 9].

Histamine poisoning is a widely recognized form of chemical intoxication that has a short incubation period, often ranging from half an hour to one hour. A variety of symptoms such as urticaria, edema, localized inflammation, and rash are commonly observed [10]. In contrast, there is a scarcity of reports regarding cadaverine and putrescine poisoning. Putrescine and cadaverine have been associated with immediate negative effects, such as heightened cardiac output, lockjaw and paralysis of the limbs, expansion of the vascular system, low blood pressure, and slow heart rate (which could lead to heart failure and bleeding in the brain) [11, 12]. Furthermore, these substances have the ability to enhance the toxicity of other biogenic amines, such as histamine, through indirect means. There is less knowledge about the production of biogenic amines in shrimp and crab. The detection of particular microbes, such as *Staphylococcus aureus* (*S. aureus*), in food indicates that the food handlers have neglected to follow proper hygiene standards. The primary factors contributing to the contamination of cooked foods are inadequate personal hygiene, insufficient sanitation of storage and preparation environments, and filthy utensils. Furthermore, the improper handling of food is regarded as the primary origin of *S. aureus*, which is commonly seen in the respiratory tract, skin, and superficial wounds.

S. aureus is a Gram-positive bacterium that is pathogenic and can cause various zoonotic diseases and food poisoning [13]. Global occurrences of foodborne illnesses resulting from *S. aureus* and its enterotoxins have been documented internationally [14, 15]. According to a study conducted by the Center for Disease Control and Prevention, there were 241,188 reported instances of *Staphylococcus aureus* food poisoning (SFP) in the United States from 2006 to 2008. This resulted in 1064 hospitalizations and 6 deaths [16, 17]. SFP is characterized by a sudden and swift occurrence of symptoms such as nausea, vomiting, and abdominal cramps [18]. *S. aureus* is responsible for several disorders such as SFP, toxic shock syndrome, bacteremia, pneumonia, and soft tissue infections [19]. The pathogenicity of *S. aureus* is enhanced

through the action of many virulence factors, such as the genes that code for staphylococcal enterotoxins (SEs) [15].

Staphylococcal enterotoxins (SEs) are the primary causative agents of SFP and are produced by staphylococci that test positive for coagulase. Staphylococcal enterotoxins refer to gastrointestinal exotoxins, as stated by Argudín *et al.* [20]. The Staphylococcal enterotoxins remain viable in the digestive tract following ingestion by humans due to their ability to withstand high temperatures, proteolytic enzymes, and other environmental factors [21]. The identification of SEs is a reliable technique for confirming outbreaks because of their consistent characteristics and the ability of strains to produce enterotoxins. More than 20 enterotoxins of *S. aureus* have been discovered. Staphylococcal enterotoxins are categorized into classical genes and non-classical genes (referred to as new SEs) based on serological classification. The classical genes, namely sea, seb, sec, sed, and see, are the most prevalent enterotoxins in outbreaks of SFP, accounting for over 90% of cases. On the other hand, non-classical genes refer to newly discovered enterotoxins that have been found in only 5% of cases [21]. Consuming food that is contaminated with enterotoxigenic *S. aureus* can readily result in foodborne outbreaks, as the stable qualities of SEs and the low dosage needed might cause symptoms. The enterotoxins produced by *S. aureus* are widely recognized as the primary cause of SFP [22]. There has been less focus on the potential of shrimp and crab as sources of *S. aureus* and SEs. Considering the aforementioned facts, this study attempted to estimate the levels of produced BAs in the edible tissue of both shrimp and crab. In addition, the calculation of the estimated daily intakes of various biogenic amines generated in the shrimp and crab was performed. Furthermore, the occurrence of *S. aureus* in the edible tissue of the shrimp and crab was investigated. Furthermore, the presence of SEs in the analyzed samples was assessed.

Material and Methods

Collection of samples

Forty samples were taken from local fish markets at Suez City, with 20 samples each from crab species, *Portunus pelagicus*, and shrimp species, *Penaeus semisulcatus*. The samples were promptly kept in refrigerated containers, labeled, and promptly transported to the laboratory. Upon arrival, the examined shellfish had their inedible portions removed.

Sample preparation

Each sample was homogenized by combining ten grams with 100 ml of a 10% solution of trichloroacetic acid. The homogenization process lasted for three minutes at a centrifugal force of 17,608 g. Next, the homogenates were extracted in

darkness for one hour with agitation at a temperature of 4°C, and then subjected to centrifugation for 20 minutes at a speed of 1,956 g and a temperature of 4°C. The liquid portion of the samples was passed through a Whatman filter No. 1, and the resulting liquid was stored at a temperature of 4°C until it could be analyzed [23].

Analysis of biogenic amines (BAs) in crustacean samples using high-performance liquid chromatography (HPLC)

The levels of HIS, cadaverine (CAD), PUT, TYR, and TRP were measured in all the samples using the method described by Pinho et al. [24] for the subsequent step of dansyl-amine synthesis. The dansyl-amine was dissolved in 1ml of methanol and 10µl of the solution were injected into the HPLC system. The quantification of dansylamines was performed using high performance liquid chromatography (HPLC) with an Agilent 1100 HPLC system (Agilent Technologies, Germany) equipped with a UV detector (Model G 1314A) set to a wavelength of 254 nm. The HPLC gradient solvent program for the separation of biogenic amines is described in Table 1.

Calculation of the estimated daily intakes of BA

The formula used to calculate Egypt's estimated human daily intake (EDI) of total biogenic amines from consuming crustaceans is as follows:

$$EDI = C_i \times FIR$$

In Egypt, the rate at which crustaceans are consumed is referred to as the Food Ingestion Rate (FIR), whereas the amount of BAs in the tested samples is designated as C_i (mg/g). According to the Food and Agriculture Organization [25], the daily ingestion rate (FIR) of crustaceans in Egypt is 48.57 grams.

Bacteriological examination

Sample preparation is according to ISO 4833-1 [26].

Precisely, 225 ml of 0.1% sterile peptone water were added to 25 g of the sample and blended well for 1.5 minutes using a sterile blender. Subsequently, ten-fold serial dilutions were made.

Isolation and identification of Staphylococcus aureus

Using a sterile bent glass spreader, one milliliter from each of the created serial dilutions was evenly distributed on a Baird Parker agar plate. The plates were placed in an incubator set at a temperature of 37°C for duration of 48 hours. The colonies, which were glossy and black in color, were counted. The colonies presumed to be *S. aureus* were observed as black, shiny, circular, smooth, and convex with a narrow white border. They were surrounded by a clear zone that extended into the opaque medium [27]. *S. aureus* colonies were counted, and the number of *S. aureus* per gram was computed.

Staphylococcus aureus was identified through a series of examinations. First, a morphological examination was conducted [28], followed by biochemical identification [29]. The bacterium was then subjected to various tests including catalase activity, oxidase activity, mannitol fermentation, growth in the presence of 10% NaCl, bile esculent test, detection of hemolysis, coagulase test, thermostable nuclease test (D-Nase activity), detection of Arginine decarboxylase (ADH), and fermentation of sugars [30].

Examination of S. aureus isolates for enterotoxin production [31]

S. aureus strains were cultivated in tryptone soya broth supplemented with 5% sodium chloride using an orbital shaker (Lab-Line Instruments, Melrose Park, Calif.) set to 200 rpm. The cultures were then incubated at 37 °C for 24 hours. Following the growth phase, the culture underwent centrifugation at 900 rpm for 20 minutes. The resulting supernatant was examined to determine the presence of SEs. The presence of Enterotoxins was identified using the commercially available ELISA kits, following the directions provided by the manufacturer. Concisely, latex reagents that have been sensitized with antisera are combined with diluted supernatant and left to incubate overnight to detect SEA, SEB, SEC, and SED.

PCR Assay for the Detection of mecA and nuc Genes

DNA extraction was performed using the QIAamp kit. Genomic DNA was isolated from 24-hour cultures of *S. aureus* isolates in BHI broth. Commercial kits from Qiagen, GmbH, Germany were used, following the instructions provided by the manufacturer [32]. The quantity and purity of DNA were evaluated using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The presence of the methicillin resistance gene (*mecA*) and the thermonuclease coding gene (*nuc*) were identified using the polymerase chain reaction (PCR) method, with primers from Pharmacia Biotech, as previously described [33].

Multiplex polymerase chain reaction (PCR)

The multiplex PCR was conducted following the protocol described [34]. The reaction mixture consisted of a total volume of 25 µL, containing 80 mM MgCl₂, PCR buffer, 3.5 mM dNTP mix (Fermentas), 10 picomole µL⁻¹ of each primer, and 1 unit of Taq polymerase (BioSyntech Technologies). The bacterial suspension obtained from the rapid DNA extraction method was added at a volume of 1 µL. The amplifications were conducted using a Biometra-Trio Thermoblock thermal cycler. The thermal cycling profile consisted of an initial denaturation step at 94°C for 5 minutes, followed by 10 cycles of amplification (denaturation at 94°C for

30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 45 seconds), and 25 cycles of amplification (denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute). The process was concluded with a final extension step at 72°C for 10 minutes. Following amplification, a volume of 10 µL from the reaction mixture was placed onto a 2% agarose gel and subjected to electrophoresis. This was done to determine the sizes of the amplification products, using a 100-bp molecular size standard ladder provided by MBI Fermentas. The gel was subsequently stained with ethidium bromide and then photographed under UV light. The PCR results were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) together with a gel pilot 100 bp ladder (QIAGEN, USA).

Statistical analysis

The collected results were evaluated statistically using the Analysis of Variance (ANOVA) test, as described [35].

Results and Discussion

Biogenic amines are formed in the final products of high protein foods due to either autolysis or bacterial decomposition. In the present study, BAs were formed in the edible tissue of the examined crab and shrimp. Histamine, CAD, PUT, and TYR were formed at 70%, 40%, 50%, and 20% of the examined crab samples, respectively. While such rates were 80%, 50%, 70%, and 30%, respectively in the examined shrimp samples. Tryptamine was not detected in any of the examined samples of the both species (Fig. 1). The recorded mean residual concentrations of the detected BAs in the crab edible tissue were 9.01 ± 2.85 , 5.30 ± 1.67 , 8.64 ± 2.73 , and 1.80 ± 0.56 for HIS, CAD, PUT, and TYR, respectively. Such values were 13.25 ± 4.19 , 8.28 ± 2.61 , 9.18 ± 2.90 , and 3.30 ± 1.04 , respectively in the examined shrimp samples (Fig. 2). Calculation of the EDI values (mg/day) for the average consumption of crab and shrimp revealed potential daily exposure of the Egyptian population to the following concentrations of the tested BAs: 437.82 (HIS), 257.42 (CAD), 419.64 (PUT), and 87.42 (TYR), respectively when crab tissues were consumed. While such EDI values were 643.55 (HIS), 402.15 (CAD), 446.15 (PUT), and 160.28 (TYR), respectively when shrimp tissues were consumed. It notes worthy to confirm that shrimp consumption might increase the human exposure to histamine higher than the recommended histamine level of 500 mg/day (Fig. 3). It was clear from the obtained results that shrimp had higher occurrence rates and concentrations of different BAs compared to the crab. In addition, HIS was the most prevalent BAs compared to other detected BAs. In agreement with the reported concentrations of the present study, Kim *et al.* [36] recorded histamine levels of 36.6–

2123.9 mg/kg in fish and shellfish retailed in the Republic of Korea. Cadaverine and putrescine were the most dominant biogenic amines formed in the spotted crab retailed in India [37].

Histamine is the main cause of a serious condition known as scombroid poisoning, or HIS-intoxication. This condition occurs when a person consumes high levels of histamine. Common symptoms of this condition encompass chest pain, and manifestations impacting the neurological and cardiovascular systems, gastrointestinal discomfort, and anaphylaxis [38]. The eating of raw or processed fish has been identified as the cause of several outbreaks of HIS poisoning in Taiwan [39]. Similar cases of HIS-induced intoxication were documented in Australia from 2001 to 2013, primarily caused by the ingestion of tuna [40]. In addition, a reported occurrence of HIS-intoxication was seen in children who had consumed canned sardines in a kindergarten in the Vojvodina province of northern Serbia [41]. This illustrates the diversity among individuals in terms of their vulnerability to experiencing symptoms of HIS poisoning. Possible variables that may be contributing to this difference include variances in age, eating habits, and genetic susceptibility [42]. Paulsen *et al.* [43] proposed a maximum permissible concentration of TYR in Austria at 950 mg/Kg. These findings indicate that the people of Egypt would not suffer negative consequences as a result of the levels identified in the current study. It is important to mention that TYR, a recognized vasoactive biogenic amine, has been linked to problems such as hypertensive crises, cardiac failure, and elevated heart rate [44]. Hence, it is imperative to exercise extreme vigilance, especially for individuals who are highly vulnerable. Consuming larger quantities of CAD can lead to an increase in the toxicity of HIS. CAD is additionally linked to stomach and intestinal malignancies [44]. The limited availability of data on NOAEL and the risk assessment of PUT can be explained by the fact that PUT has the lowest level of harmful effects [45]. In contrast, the use of PUT may potentially amplify the harmful effects of TYR and HIS by hindering their oxidative inhibition mechanism [46]. Furthermore, there is a correlation between PUT and neurological illnesses and gastrointestinal cancers [44].

The second section of the present study was to investigate the prevalence of *S. aureus* in the examined crab and shrimp. The recorded results in Fig. 4, 5, and 6 showed that the prevalence rates of *S. aureus* in the examined crab and shrimp samples were 25% (5 out of 20 samples), and 35% (7 out of 20 samples), respectively. The recovered *S. aureus* isolates harbored *nuc* gene which is a species-specific marker for *S. aureus*. Methicillin Resistant *S. aureus* "MRSA", *mecA* gene was also detected in the examined *S. aureus* isolates that recovered from crab at 20% (1 out of 5 isolates) and from the examined

isolates that recovered from the shrimp at 42.9% (3 out of 7 isolates). Total *S. aureus* counts were 2.61 ± 0.11 , and $2.92 \pm 0.19 \log_{10}$ cfu/g in crab and shrimp, respectively. This result demonstrated that 28.57% (2 samples out of 7) of the examined shrimp samples had total *S. aureus* counts higher than the established Egyptian Organization for Specification [47] which recommends that *S. aureus* counts should not increase than $3 \log_{10}$ cfu/g, with all crab samples had acceptable *S. aureus* counts. Regarding enterotoxin production, 20% (1 out of 5 isolates) of the recovered *S. aureus* isolates from crab samples, with SEC the detected enterotoxin. In case of *S. aureus* isolates recovered from shrimp samples, 42.86% of the recovered isolates were found to have enterotoxin; with SEA detected at 28.57% (2 out of 7 isolates) and SED detected at 14.28% (1 out of 7 isolates). *S. aureus* is responsible for many cases of foodborne intoxication worldwide. *S. aureus* enterotoxins are the responsible for the onset of such intoxication cases leading to variable symptoms including abdominal cramps, vomiting, diarrhea with no fever and short incubation period [15]. In agreement with the obtained results of the present study, *S. aureus* was found in 5% of the uncooked samples of fish and shrimp, 17.5% of the frozen samples, and 12.3% of the ready-to-eat sea food samples collected from India [48]. Besides, MRSA was isolated from 35.2% of sea food samples collected from Kerala, India [49].

Conclusion

The obtained results of the current study revealed formation of biogenic amines in the retailed crab and shrimp. In addition, *mecA*-resistant *S. aureus* gene was isolated from the examined samples at variable rates. *Staphylococcus aureus* enterotoxins were detected in the examined crab samples at 10%, and in the examined shrimp samples at 30%. Therefore, strict hygienic measures should be followed during handling of crab and shrimp.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study did not use any living animal nor human subjects.

TABLE 1. HPLC parameters adopted during BAs analysis

HPLC parameters	Value
Time intervals	0, 10, 15, 20, and 25 minutes
The flow rate	1 ml/min for all intervals
The percentage of solvent A (0.02N acetic acid)	60%, 20%, 15%, 60%, and 60% for the respective time intervals
The percentage of solvent B (methanol)	20%, 40%, 35%, 20%, and 20%
The percentage of solvent C (acetonitrile)	20%, 40%, 50%, 20%, and 20% for the respective time intervals

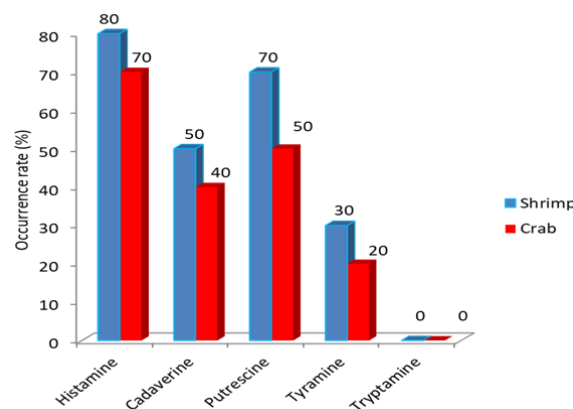


Fig. 1. Occurrence rates (%) of different biogenic amines in the examined crab and shrimp samples

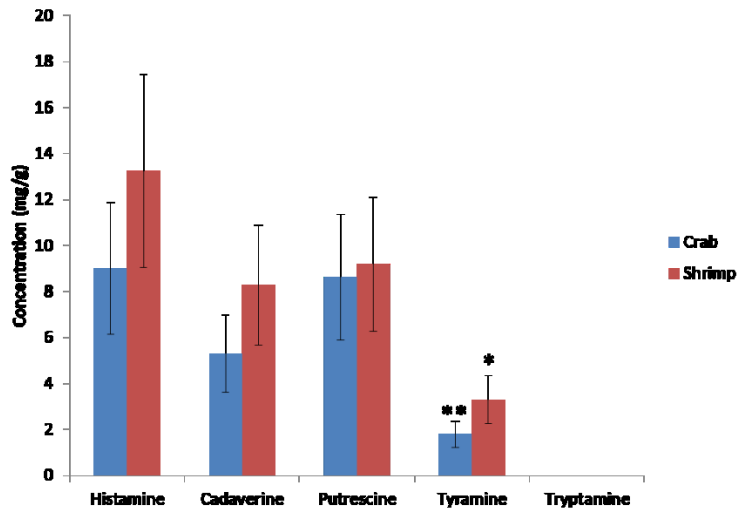


Fig. 2. Concentration of different biogenic amines (mg/g) in the examined crab and shrimp samples

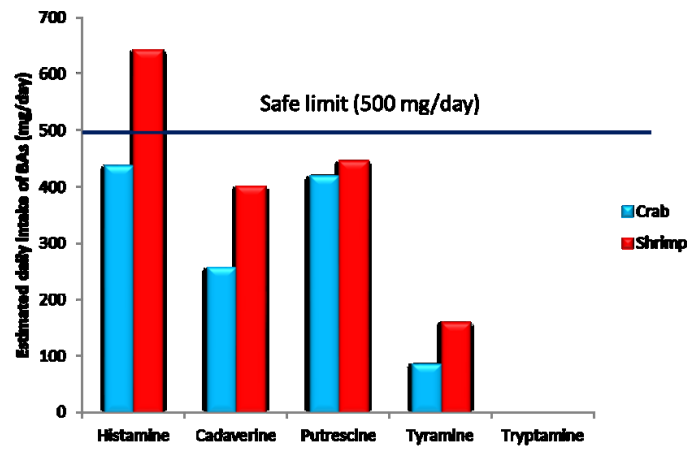


Fig. 3. Estimated daily intakes of Biogenic amines (mg/day) due to consumption of crab and shrimp in Egypt

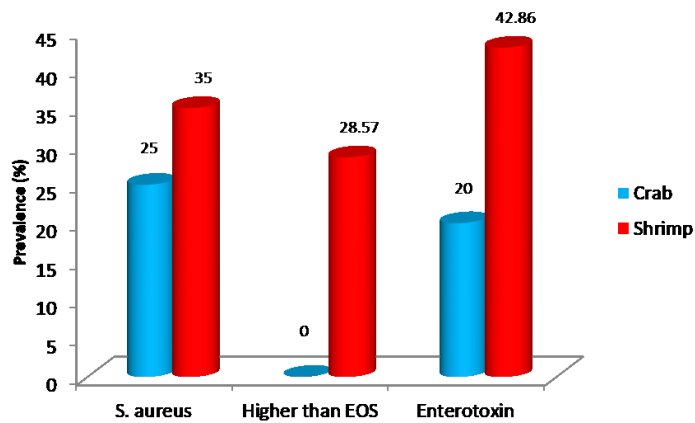


Fig. 4. Prevalence rate of *S. aureus*, enterotoxin and samples exceeding the permissible count of *S. aureus* in the examined crab and shrimp

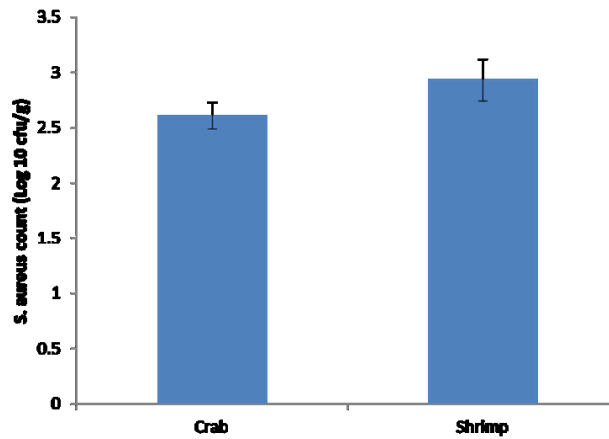


Fig. 5. *S. aureus* count (log₁₀ cfu/g) in the examined crab and shrimp. Values represent means ± SE of *S. aureus* counts in the positive samples of crab and shrimp.

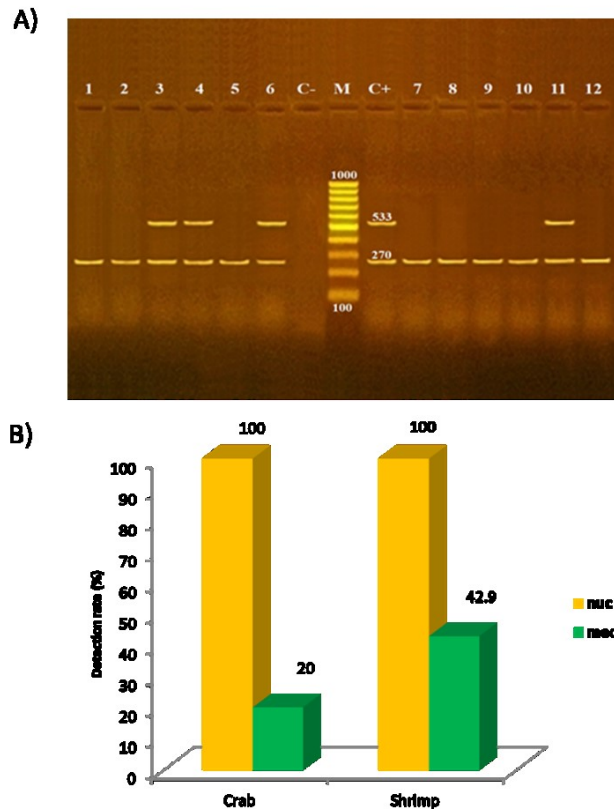


Fig. 6. A) Agarose gel electrophoresis of multiplex PCR of *nuc* (270bp) and *mecA* (533bp) genes of *S. aureus*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive for *nuc* and *mecA* genes. Lane C-: Control negative. Lanes from 1 to 12: Positive *S. aureus* strains for *nuc* gene. Lanes 3, 4, 6 & 11: Positive *S. aureus* strains for *nuc* and *mecA* genes. Lanes from 1 to 7: *S. aureus* isolated from the shrimp samples. Lanes from 8 to 12: *S. aureus* isolated from the crab samples. B) Detection rates (%) of *nuc* and *mecA* genes in the recovered isolates.

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تواجد الامينات الحيوية والمكورات العنقودية الذهبية في الكابوريا والجمبري

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الملخص

يعتبر الجمبري والكابوريا من القشريات التي تعتبر مصادر جديدة للبروتين عالي القيمة البيولوجية. كما أنها تحتوي على نسبة عالية من الرطوبة والمعادن مثل الكالسيوم والفوسفور والفيتامينات، بما في ذلك فيتامين د، وأحماض أوميغا 3 الدهنية المتعددة غير المشبعة. يعتمد الناس في جميع أنحاء العالم على الأسماك والمحاريات بشكل كبير، خاصة في مصر، لتعويض النقص في اللحوم الحمراء. يتم إنتاج الامينات الحيوية التي تحدث بشكل طبيعي عندما تقوم مجموعة واسعة من الميكروبات، باستقلاب أحماض أمينية معينة. واحدة من مسببات الأمراض الرئيسية المنقولة بالغذاء والتي تسبب العديد من حالات التسمم في جميع أنحاء العالم هي المكورات العنقودية الذهبية. ومع ذلك، لا يُعرف سوى القليل عن انتشار المكورات العنقودية الذهبية والعديد من السموم المعوية التي تنتج في الجمبري والكابوريا. كان الهدف الرئيسي لهذه الدراسة هو تحديد كمية الامينات الحيوية المتولدة في الجمبري والكابوريا. علاوة على ذلك، تم حساب المدخول اليومي المقدر من الـ الامينات الحيوية الذي يستمده السكان المصريون من تناول هذه القشريات. وكان الهدف الآخر من الدراسة الحالية هو معرفة مدى تواجد المكورات العنقودية الذهبية في الجمبري والكابوريا. وبالإضافة إلى ذلك، تمت مناقشة أهمية الامينات الحيوية والمكورات العنقودية الذهبية في الجمبري والكابوريا. وبالإضافة إلى وجود الهيستامين والكادافيرين والبيوتريسين والتيرامين في الجمبري والكابوريا المفحوص بنسب متفاوتة. بالإضافة إلى ذلك، تم عزل المكورات العنقودية الذهبية من الكابوريا و الجمبري المفحوصين بنسبة 50% و 70% على التوالي. ولذلك ينبغي اتباع تدابير صحية صارمة أثناء التعامل مع هذه القشريات.

الكلمات الدالة: الامينات الحيوية، المكورات العنقودية الذهبية، الجمبري والكابوريا.