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Occurrence of *phoA* and Shiga Toxin genes in Marketed Gandoffli, *Ruditapes decussates*

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Abstract

Generally, the majority of the food poisoning crisis from seafood comes out due to shellfish consumption, mainly gandofflibecause ofits filter feeders pattern. Many microbes are used as an indicator of the hygienic status of several foods, one of them is *Escherichia coli* which is used to detect fecal pollution in water and shellfish. Therefore, twenty gandoffli samples were randomly collected from local markets in Ismailia city, Egypt, for evaluation of *Enterobacteriaceae* counts, and identification of *Escherichia coli* and detection of *phoA* and Shiga toxin genes. The obtained results revealed that the total *Enterobacteriaceae* count of the gandoffli ranged from 7×10^2 to 7×10^5 cfu/g with an average of $5\times10^4\pm3.5\times10^4$cfu/g. The occurrence of *Enterobacteriaceae* members in gandoffli was represented by *E. coli* (99%), *Acinetobacter lwoffii* (99%), *Enterobacter hormaechei* (85%), *Klebsiella oxytoca* (95%), *Stentrophomonas maltophilia* (85%), *Moraxella lacunata* (85%), *Achromobacter xylosoxidans* (93%), and ESBL *E. coli* (100%). In addition, *E. coli* isolated from gandoffli were subsequently serologically typed into O103, O55, O 128, O 126, and O157 then confirmed using conventional polymerase chain reaction by the presence of alkaline phosphatase gene. Upon checking virulence genes in *E. coli*: *stx2* was absent in O157 and O103. Also, *stx1* was present in O157 and absent in O103. Its should be concluded that gandoffli were exposed to *Enterobacteriaceae* contamination from different sources during handling, storage and distribution. Gandoffli had *E. coli* and their toxin that can pose serious public health hazards to consumers. Strick hygienic measures must be applied through the chain of gandoffli production to ensure their safety for consumer consumptions

KEYWORDS
Enterobacteriaceae, *E. coli* , Gandoffli, RENDER MA120, Shiga-toxin.

INTRODUCTION

Seafood has assisted to overcome food shortages and gives an excellent complement to a healthy diet (FAO, 2010). Fresh seafood clams, mussels, and oysters are types of bivalve mollusks mainly called gandoffli, it has been known as a delicious human food worldwide.Gandofli has content which high-quality protein, microelements, and unsaturated fatty acids. Seafood-borne illnesses mainly occurred due to the consumption of raw seafood or inadequate cooking.

Bivalve mollusks are harvested as tender as from the sea, so they are very likable by the consumer. It resembles a simple also risky food to be consumed (Murchie *et al*., 2005). Bivalves are filter feeders, consequently, they may accumulate environmental biological and chemical hazards and act as a vector in delivering them to consumers (Lees, 2000). Bivalve shellfish infection takes placeas they are suspension feeders that selectively filter small particles of phytoplankton, zooplankton, viruses, andmicroorganismfrom the surrounding water and soil (Dunphy *et al*., 2006).

Serious security worries are associated with the consumption of uncooked shellfish due to the presence of organic dangers (Huss *et al*., 2000). In concern to food safety, many microbes are used as an indicator of the hygienic status of several foods, one of them is *Escherichia coli* which is used to detect fecal pollution in water and shellfish (Hodgson *et al*., 2017).

Nearly all of the food poisoning diseases from seafood show up to be due to shellfish consumption instead of finfish (Butt *et al*., 2004). The initial microbiota of seafood consists of microorganisms from the water of harvesting or growing areas (Parlapani, 2021). Shellfish-derived ailments can have herbal motives due to contaminants that are handy in the surroundings and for this reason a section of their regular biota (Shumway and Rodrick, 2009). Bivalves developed in unhygienic areas can act as sources for human pathogens particularly because of their filter-feeding conduct (Fusco *et al*., 2013; Dos Santos *et al*., 2022), and their growing usually close to wastewater or in polluted estuaries (Burkhardt and Calci, 2000).

Consumption of pathogen-infected mollusks affects human fitness at specific rates, consequently, it is quintessential to screen for microbial illness in seafood (Zgouridou *et al*., 2022). Therefore, this study was performed to evaluate the safety of gandoffli, *Ruditapes decussates* samples randomly collected from Ismailia markets though for evaluation of *Enterobacteriaceae* counts, identification of *E. coli*, and detection of *phoA* and Shiga

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toxin genes.

MATERIALS AND METHODS

Samples Collection

A total of 5 Kg fresh gandoffli, *Ruditapes decussates* samples (Balady and Arayes) were randomly collected from the Ismailia fish market during the period from October 2021 to January 2022. All samples were divided into groups, each single group has about 18-25 units of gandoffli and weights 250 g. All samples were immediately transferred without delay to Animal Health Research Institute, Ismailia branch laboratory under complete aseptic conditions in clean vessels inside the icebox for preparation for further microbiological evaluation.

Preparation of samples

After thorough washing of fingers with cleaning soap and water then alcohol decontamination, The shell of gandoffli used to be scrubbed with a sterile stiff bristle brush underneath bloodless potable water to put off the mud with extraordinary interest to crevices at the junction of the shells and then positioned on a smooth paper towel to drain. Closed clams used to be sterilized via alcohol and then flamed. The adductor muscular tissues have been reduced with sterile scissors and the covers have been eliminated and switch the gentle tissue to a sterile pattern container (ISO, 2013). Gandoffli samples represented by 25 g, were added to 225 mL of 0.1% (w/v) sterile buffered peptone water (Oxoid-CM509). The content was homogenized using a Lab Blender (Seward stomacher 400R/UK) for 2 min. resulting in forming the original homogenate with a concentration of 10-1. Further tenfold decimal serial dilution up to 10-7 was carried out twicefromthe original homogenate into tubes.

Determination of Enterobacteriaceae counts

Enterobacteriaceae counts have once decided the usage of violet crimson bile glucose agar medium (HI Media, M091), plates had been incubated at 37°C for 24±2 h (ISO, 2004). Suspected colonies, which confirmed purplish-red colonies surrounded with the aid of a pink region of precipitated bile acids have been enumerated to gain *Enterobacteriaceae* remember per gram.

Identification of Enterobacteriaceae

Iidentification of colonies grown in the previous step using morphological, biochemical methods and further biochemical identification using automated identification and antibiotic sensitivity testing ID&AST System, MA120 (Render, China) for identification of isolated bacteria using its specific *Enterobacteriaceae* kit according to manufacturer's instructions.

Isolation and identification of Escherichia coli

A loopful of pattern suspension was once aseptically transferred to sterile replica plates of MacConkey agar medium (Oxoid, CM015), and incubated at 35±2°C for 18±2 h. The colonies grew as red to brick-red colonies are picked up and streaked onto sterile reproduction plates of Eosin Methylene Blue agar medium (Lap, 61), and incubated at 35±2°C for 18-24 h. The traditional colony of *Escherichia coli* is chosen and has been tested through Gram's stain, motility test, indole, methyl red, Voges Proskauer, and citrate testes.

Serological identification of Escherichia coli isolates

All *E. coli* isolates in the preceding step had been subjected to serological typing through slide agglutination take a look at Lee *et al*., (2009) using general polyvalent and monovalent *E. coli* antisera (Seiken, Japan). Only sparkling bacterial way of life from 24-hour age colonies onto nutrient agar media have been used. Then two advantageous samples were dispatched to Reference Laboratory for Veterinary Quality Control - Dokki branch-A.R.C.-Egypt for PCR sequence.

Statistical analysis

Results were assessed for normality and data sets were set to follow a normal distribution (SPSS, 2016).

RESULTS AND DISCUSSION

Gandoffliisa nutritious food source consumed by the whole world (Fauconneau, 2002) and at the same time, they may be considered a good medium for bacterial flourishing. In addition, to their filter-feeding behavior, gandofflican accumulate a diversity of microorganisms (Croci *et al*., 2002).

Total Enterobacteriaceae count

Results recorded in Table 1 showed that *Enterobacteriaceae* count in gandoffli samples. The minimum, maximum, and mean values±standard error of *Enterobacteriaceae* count in gandoffli samples were $7x10^2$, 7×10^5 and $5\times10^4\pm3.5\times10^4$cfu/g, respectively. Although the examined gandoffli samples were fresh and were alive during evaluation, they did not meet the Egyptian standards (EOS, 2005) for *Enterobacteriaceae* count in shellfish (maximum 10^2cfu/g). Also, the obtained results are higher than European Union standards (EC, 2005, No 2073), where the limit for bivalves for direct human consumption is 230 *E. coli*/100 g of flesh and intra-valvular liquid of bivalves.

Table 1. Statistical analytical results for *Enterobacteriaceae* count (cfu/g) in Gandoffli (n=20)

Enterobacteriaceae count (cfu/g)	Results
Minimum	$7x10^2$
Maximum	$5x10^5$
Mean	$5x10^4$
±S.E	$3.5x10^4$

S.E. = Standard Error

Gandoffli had an average total *Enterobacteriaceae* count of $3.5x10^5$cfu/g (Hafez *et al*., 2022). Similar lower results were obtained by Amin (2021) who found that the mean values of the *Enterobacteriaceae* counts were $2\times10^2\pm1\times10^2$cfu/g for shrimp and $92\times10^1\pm2\times10^2$cfu/g for Gandoffli respectively.

The *Enterobacteriaceae* group has epidemiological importance as some of its members are pathogenic and may cause serious infections and food poisoning (Salem *et al*., 2018). *Enterobacteriaceae* were the most common organisms isolated from fish and shellfish, the occurrence of these organisms serves as an index of fecal pollution of water, besides many species of these microorganisms were found to be pathogenic. Shellfish acquire enterobacteria being in while in polluted water sources or subsequently while stored in vessels and as they are being processed. Improper storage of contaminated shellfish speeds multiplication to the quantity that can cause food poisoning (Bryan, 1980). *Enterobacteriaceae* count is known as an alternative index of seafood quality as it relates to ice storage, rinsing, and evisceration (Zambuchini, 2008). The total number of *Enterobacteriaceae* is regarded as an indication of possible enteric contamination

(Mercuri *et al*., 1978).

According to Table 2, the identified *Enterobacteriaceae* isolates using the further lab. Biochemistry (RENDER MA120) were *E. coli*, *Acinetobacter lwoffii*, *Enterobacter hormaechei*, *Klebsiella oxytoca*, *Stentrophomonas maltophilia*, Moraxellala cunata, and *Achromobacter xylosoxidans*. *E. coli* occurs in a percentage of 100, 25, 30, 40, 25, 25, 30, and 20% of gandoffli samples with the probability of 99, 99, 85, 95, 85, 85, 93, and 100%. The results suggest that gandoffli is contaminated with many genera of *Enterobacteriaceae*. The obtained results closely match with results obtained by Duran and Marshall (2005); Furushita *et al*. (2005); Ray and Kar (2006); Amoah *et al*. (2011); Rodriguez *et al*. (2011); Manikandan *et al*., (2015); Dadar *et al*., (2016); Zuo *et al*., (2018); Yang *et al*. (2020); Singh *et al*. (2020); Ni *et al*. (2021) and Khan *et al*. (2022).

Table 2. Occurrence of identified *Enterobacteriaceae* isolated from the raw Gandoffli using RENDER biochemistry (n=20).

Enterobacteria	Raw Gandoffli		Probability % by RENDER
	No.	%	
E. coli	20	100	99
Acinetobacter lwoffii	5	25	99
Enterobacter hormaechei	6	30	85
Klebsiella oxytoca	8	40	95
Stentrophomonas maltophilia	5	25	85
Moraxella lacunata	5	25	85
Achromobacter xylosoxidans subsp deni.	6	30	93
ESBL E. coli	4	20	100

Escherichia coli

According to the Table 3 *E. coli* was positive in all (100%) examined gandoffli samples. *E. coli* has been used to detect fecal pollution in shellfish (Hodgson *et al*., 2017). *E. coli* is the main disease-causing illness and food poisoning for seafood consumers (Hatha *et al*., 2005). The obtained results are nearly similar to those reported by Rodriguez *et al*. (2011), and Amr *et al*. (2012). Lower results were recorded by Ham (2008); Amoah *et al*. (2011) and Moreno Roldan (2013).

Table 3. Occurrence of *E. coli* in raw Gandoffli (n=20).

Raw Gandoffli samples		
	Number	%
Positive	20	100
Negative	0	0

Gandoflli grown in sewage-polluted areas can act as sources for human ailments given their filter-feeding behavior. Moreover, sewage canaffectmussels' micro-biota and fitness, thereby making them extrainclined to infections and diseases, mainto mortalities (Dos Santos *et al*., 2022). *E. coli* causes illnesses ranging from gastrointestinal tract-related complications such as diarrhea dysentery, urinary tract infection, pneumonia, and even meningitis (Johnson *et al*., 2006).

Table 4 shows the incidence of *E. coli* serotypes from raw gandoffli. the incidence of *E. coli* Serotypes isolated from raw gandoffli samples from Ismailia markets were O157, O103, O126, O128, O55, and O118 with the occurrence of 30, 25, 20, 20, 20, and 15% respectively. One sample may have one or more serotypes, so results here show that the O157 strain was present in 6 samples out of the 20 samples and so on. Similar results were obtained by Schimmer *et al*. (2008); Kambire *et al*. (2017); Miotto *et al*. (2019) and Ally *et al*. (2021).

Results obtained in Table 5 and Fig.1 showed the conventional polymerase chain reaction (cPCR) done for two *E. coli* isolates, O103 and O157. It revealed that both strains possess an alkaline phosphatase gene (*PhoA*) at 720 bp. While Shiga-toxin genes: *Stx1* at 614 bp (in O157 only) and *Stx2* at 779 bp were absent (in both O103 and O157).

Table 4. Occurrence of *E. coli* Serotypes isolated from raw Gandoffli Samples (n=20).

Sample	Raw Gandoffli	
E. coli strain (serotype)	No.	%
O_{157} K_{-}	6	30
O_{103} K_{-}	5	25
O_{126} K_{71}	4	20
O_{128} K_{67}	4	20
O_{55} K_{59}	4	20
O_{118} K_{-}	3	15

Table 5. Conventional Polymerase chain reaction (cPCR) for *E. coli* to detect alkaline phosphatase gene (*phoA*) and Shiga toxin genes (sxt1 and sxt2) from raw Gandoffli samples.

Serotype	*phoA* gene	*stx1* gene	*stx2* gene
O_{157}	positive	positive	negative
O_{103}	positive	negative	negative

PhoA: alkaline phosphatase gene; Stx: Shiga toxins genes.

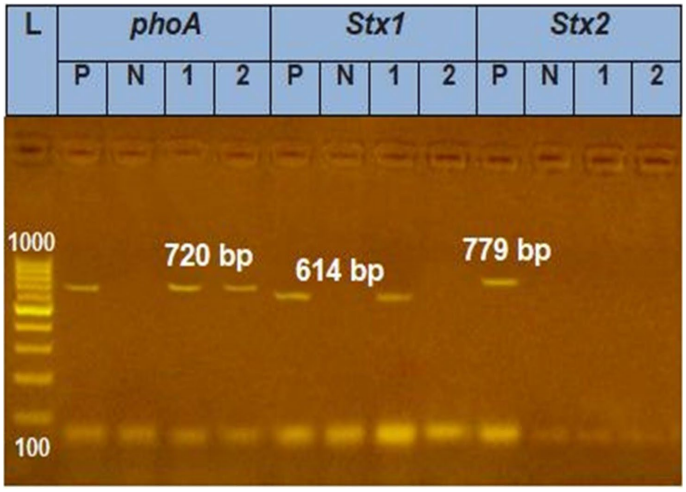

Fig. 1.Agarose gel electrophoresis of amplified *phoA* gene and Shiga toxin genes PCR product: Lanes 1- 2: positive amplification of 720 bp of *phoA* gene in the two tested samples; Lanes 1- 2: negative *stx1* and *stx2* genes in the two tested samples; L: Marker lane 100-1000 bp DNA ladder; Neg.: Negative contro; Pos. for *phoA*: Positive control, 1 and 2) at 720bp); Pos. for *Stx1*: Positive control and (1) at 614 bp); Bp: base pair.

The alkaline phosphatase gene is encoded by the *PhoA* gene, which is considered a house keeping gene found in all *E. coli* strains (Yu and Thong, 2009). The presence of this gene confirmed the identification of *E. coli* isolated from the gandoffli samples. The obtained results on *Stx1*and *Stx2* agreed with those obtained by Balière *et al*., (2015). Meanwhile, different results were obtained by Marceddu *et al*. (2017) and Ally et. al. (2021).

CONCLUSION

From the results, it could be concluded that raw gandoffli samples traded in Ismailia city were exposed to *Enterobacteriaceae* contamination from different sources during handling, storage and distribution. Gandoffli had *E. coli* and their toxin that can pose serious public health hazards to consumers. Strick hygienic measures must be applied through the chain of gandoffli production to ensure their safety for consumer consumptions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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