Seafood a potential source of some zoonotic bacteria in Zagazig, Egypt, with the molecular detection of Listeria monocytogenes *virulence genes*

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Seafood,
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Summary
This article describes the results of a study conducted on 71 fresh seafood samples (fish and shellfish) marketed in Zagazig city, Sharkia province, Egypt, as well as on 50 human stool samples collected at the Zagazig University Hospital. The samples were examined for the presence of *Listeria monocytogenes* and *Escherichia coli*. The investigation of *L. monocytogenes* virulence genes was performed using Polymerase Chain Reaction (PCR), while the microbiological quality of the seafood samples was evaluated using the coliform count and aerobic plate count (APC) as indicators. Out of the examined 71 seafood samples, 20 (28.2%) were identified as *L. monocytogenes*, 15 (75%) of which were confirmed as virulent strains. Also, out of 50 human stool samples, only 1 (2%) was identified as virulent *L. monocytogenes*. *E. coli* serotypes were isolated from only 11.3% of seafood and 30% of human stool samples. In shellfish, the APC and most probable number of coliforms (MPC) were higher than those obtained from other fish samples. Multiplex PCR targeting internalin genes allowed simultaneous identification of *L. monocytogenes* and differentiation of virulent strains, thus enabling more timely detection of cases and sources of food borne listeriosis. The article concludes by stressing that the isolation of potentially virulent *L. monocytogenes* and *E. coli* from both seafood samples and humans emphasises the potential public health hazard caused by eating raw or undercooked shellfish.

Rilevazione molecolare dei geni di virulenza per Listeria monocytogenes *e di batteri responsabili di zoonosi in prodotti ittici commercializzati nei mercati di Zagazig in Egitto*

Parole chiave
Egitto,
Escherichia coli,
Internalina,
Listeria monocytogenes,
Listeriolisina O,
Molluschi,
Pesce,
Virulenza.

Riassunto
L'articolo descrive i risultati di uno studio condotto su 71 campioni di prodotti ittici freschi (pesci e molluschi), commercializzati nella città di Zagazig (provincia di Sharkia) in Egitto, e su 50 campioni di feci umane, prelevati da pazienti dell'Ospedale Universitario della stessa città, con l'obiettivo di valutare la presenza di *Listeria monocytogenes* ed *Escherichia coli*. I geni di virulenza per *L. monocytogenes* sono stati analizzati impiegando la polymerase chain reaction (PCR). L'analisi microbiologica dei campioni ha previsto la conta dei Coliformi e la conta aerobica su piastra. In 20 (28,2%) dei 71 campioni ittici esaminati è stata rilevata la presenza di *L. monocytogenes*. In 15 (75%) di questi 20 campioni e in 1 (2%) dei 50 campioni di feci umane sono stati riscontrati ceppi virulenti di *L. monocytogenes*. Sierotipi di *E. coli* sono stati isolati solo nell'11,3% dei campioni ittici e nel 30% dei campioni di feci umane. L'analisi microbiologica ha evidenziato nei molluschi valori più elevati rispetto ai campioni di pesce. L'utilizzo di Multiplex PCR sui geni dell'internalina ha permesso di identificare simultaneamente *L. monocytogenes* e altri ceppi virulenti rilevando tempestivamente i casi e le fonti di listeriosi di origine alimentare. Lo studio evidenzia il potenziale rischio per la salute pubblica derivante dal consumo di prodotti ittici crudi o non adeguatamente cotti.

Introduction

Consumers of seafood worldwide are becoming increasingly concerned about the safety and nutritional quality of their food. Nonetheless, seafood still plays a significant role in causing food borne diseases (55). Contamination of seafood with zoonotic bacteria could occur during slaughter, refrigeration and processing, resulting in the transmission of such bacteria to consumers (23). *Listeria monocytogenes* has been recognized as an important opportunistic human pathogen since 1929 and as a food borne pathogen since 1981 (35). The involvement of seafood in the transmission of listeriosis was suggested by Lennon *et al.* (39), who proposed that consumption of shellfish and raw fish was responsible for an epidemic of prenatal listeriosis in New Zealand in 1980. *L. monocytogenes* primarily affects children, elderly and immune-compromised individuals causing severe diseases such as septicemia, encephalitis and meningitis (62). It also causes abortion and stillbirth in pregnant women, in addition, *Listeria* infects healthy people causing fever, vomiting and diarrhoea (47). Multiple key virulence factors are important in *L. monocytogenes* pathogenesis (56), therefore it is necessary to identify virulent from avirulent strains by molecular techniques such as Polymerase Chain Reaction (PCR) (40) in order to implement effective control and preventive measures against *L. monocytogenes* infections.

Pathogenic strains of *Escherichia coli* are transferred to seafood through sewage pollution or by contamination after harvest (69). *E. coli* strains can cause a variety of diseases, including diarrhoea, dysentery, and haemolytic uremic syndrome (31, 67). Faecal coliforms and the aerobic plate count (APC) have been adopted as indicator to assess the quality of seafood flesh and, consequently, to predict the risk of seafood consumption (44, 71).

The aim of this study is to generate information on the prevalence of *L. monocytogenes* and *E. coli* biotypes in some seafood marketed in Zagazig city in Egypt, as well as in stool samples collected from patients attending the Zagazig University Hospital, Egypt. In addition, the detection and differentiation of virulent and avirulent *L. monocytogenes* isolates by PCR was performed. The coliform count and APC were used as indicators to assess the microbiological quality of the examined seafood.

Materials and methods

Sample collection and preparation

A total of 71 seafood samples, including 13 Nile-tilapias (*Oreochromis niloticus*), 10 mullets (*Mugil cephalus*), 10 bivalve mollusks (*Caelatura laronia*), 12 blue crabs (*Calinectes sapidus*), 12 tuna (*Thunnus thynnus*), and 14 shrimps (*Penaeus semisulcatus*) were collected from fish markets in Zagazig city. Also, 50 stool swabs were collected from diarrheic patients, with a history of chronic diseases, attending the University Hospital in Zagazig city.

Ten grams of the interior flesh content from the seafood sample were homogenized in 90 ml of sterile 0.1% peptone water using a blender for 2 min (55). The homogenate was firstly used for APC and most probable number of coliforms (MPN) determination and then incubated at 37°C for 24 hours for pre-enrichment of the samples. Stool samples were collected in a clean sterile container and then a swab from each sample was inserted in 0.1% peptone water for pre-enrichment and incubated at 37°C for 24 hours (61).

Isolation and molecular identification of *L. monocytogenes*

Isolation of L. monocytogenes

L. monocytogenes was isolated according to the US Food and Drug Administration (FDA) methods (27). For enrichment, 25 ml of the pre-enriched sample was added into 225 ml of *Listeria* Enrichment Broth (LEB) (Himedia, Catalogue # 569-500G, Mumbai, India) and incubated at 30°C for 2-7 days. For isolation, a loopful from the LEB culture was streaked onto OXFORD media (Himedia, Catalogue # MV1145-500G with *Listeria* Oxford supplement Himedia, Catalogue # FD071, Mumbai, India) and incubated for 24-48 hrs at 35°C.

The suspected colonies obtained by cultural methods were re-suspended in LEB and incubated at 30°C for 2 days. Bacterial cells were harvested in a microcentrifuge tube by centrifugation at 10,000 rpm for 30 sec. The supernatant was discarded and the bacterial pellet was suspended and washed in 200 µl physiological saline 0.9% and the suspension was then centrifuged again at 10,000 rpm for 30 sec. DNA extraction was performed according to the manufacturer guidelines using Bacterial DNA Extraction Kit (Spin-column) (BioTeke Corporation, Shanghai, China).

Multiplex PCR for detection of internalin genes

Purified DNA of the suspected colonies was subjected to a Multiplex PCR for the identification of *L. monocytogenes* and also for the detection of internalin virulence factors according to Liu *et al.* (40). Oligonucleotide primers (AlphaDNA, Montreal, Quebec, Canada) were used for the amplification of

Table I. *Primer sequences and expected product sizes of the Multiplex PCR.* The *inlA* primers were intended for species-specific recognition, and the *inlC* and *inlJ* primers were designed for virulence determination of *Listeria monocytogenes*.

	Gene	Primer sequence (5'→3')	Expected product size (bp)
inlA	*inlA-Forward*	ACGAGTAACGGGACAAATGC	800 bp
	inlA-Reverse	CCCGACAGTGGTGCTAGATT	
inlC	*inlC-Forward*	AATTCCCACAGGACACAACC	517 bp
	inlC-Reverse	CGGGAATGCAATTTTTCACTA	
inlJ	*inlJ-Forward*	TGTAACCCCGCTTACACAGTT	238 bp
	inlJ-Reverse	AGCGGCTTGGCAGTCTAATA	

L. monocytogenes internalin genes *inlA*, *inlC* and *inlJ*. Table I shows the sequence of the primers and the expected product sizes. The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2x power *Taq* PCR mastermix (BioTeke Corporation) and 40 pmol each *inlA*, 30 pmol each *inlC* and 20 pmol each *inlJ* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA was run in the PCR as a negative control. A positive control of serologically confirmed *L. monocytogenes* isolate was kindly obtained from the department of Food Control, Faculty of Veterinary Medicine, Zagazig University, Egypt.

The reaction conditions consisted of one cycle of 95°C for 2 min followed by 30 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 50 sec, a final cycle of 72°C for 2 min. The reaction was carried out in Primus (MWG-Biotech Thermal Cycler, Ebersberg, Germany). Amplification products were resolved in 1.2% (w/v) agarose gels along with 100 bp molecular weight ladder (BioTeke Corporation). The agarose gel was prepared in 1 x TBE (89 mM Tris-Borate; 2 mM EDTA; and pH 8.3) stained with 5 µM ethidium bromide. The gels were run in 1 x TBE, 5 µM ethidium bromide for at least 45 min at 100 V and then visualized under Ultra Violet light of ultraviolet transilluminator (Spectroline, Westbury, NY, USA).

PCR for detection of Listeriolysin O virulence genes

A second PCR was used for the detection of Listeriolysin O virulence gene (LLO) in the molecularly identified *L. monocytogenes* (34). The primers (AlphaDNA, Montreal, Quebec, Canada) had the following sequences: *hlyA-Forward*: 5'- CGG AGG TTC CGC AAA AGA TG-3' and *hlyA-Reverse*: 5'- CCT CCA GAG TGA TCG ATG TT-3' (45). The reaction was performed in 25 µl reaction volume containing 12.5 µl of readymade 2x power *Taq* PCR master mix and 100 nM of each *hlyA* primers and 2 µl of the purified DNA. A reaction mixture with no added DNA as a negative control and a positive control of serologically identified strain were run in the PCR reaction. The reaction conditions consisted of one cycle 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 1 min and a final extension at 72°C for 2 min. The visualization of the expected 234 bp amplified products was performed as previously described in the Multiplex PCR.

Isolation and identification of *E. coli*

One ml of the pre-enriched samples was directly inoculated into 9 ml MacConkey broth (Oxoid, CM 5a, Adelaide, Australia) and incubated at 37°C overnight for enrichment (9). After enrichment, a loopful from the incubated enrichment broth was streaked directly onto Eosin-Methylene-Blue agar EMB (Oxoid, CM69, Adelaide, Australia) and incubated at 37°C for 18-24 hrs. The suspected *E. coli* colonies were subjected to biochemical identification (6) and then the biochemically confirmed isolates were serotyped (37) using rapid diagnostic E.coli antisera sets (DIFCO Laboratories, Detroit Michigan, USA). Serotyping was performed at the Food Analysis Centre, Faculty of Veterinary Medicine, Benha University, Egypt.

Aerobic plate count and most probable number of coliforms

For the examined samples, the APC was determined according to Stevenson and Segner (63). The total APC per gram sample was calculated according to the following equation: total APC = number of colonies x dilution factor. The total APC was presented as colony forming units (CFU/g). The APC was considered acceptable, marginally acceptable or not acceptable according to the Food and Drug Administration, Centre for Food Safety and Applied Nutrition of United States (FDA CFSAN) (16).

The coliform counts were determined according to Thatcher and Clark (66). Positive tubes with acid and gas production were recorded and for each dilution, the results were presented as a fraction as follows: number of positive tubes/number of inoculated tubes. The MPN was then estimated using MPN index (67) and the concentration of coliform bacteria was presented as MPN/g of the sample.

A test of significance of observed differences in bacterial counts in the different seafood species examined was conducted using a one-way analysis of variance (ANOVA) computed by SPSS (version XI). $P < 0.05$ was regarded as statistically significant.

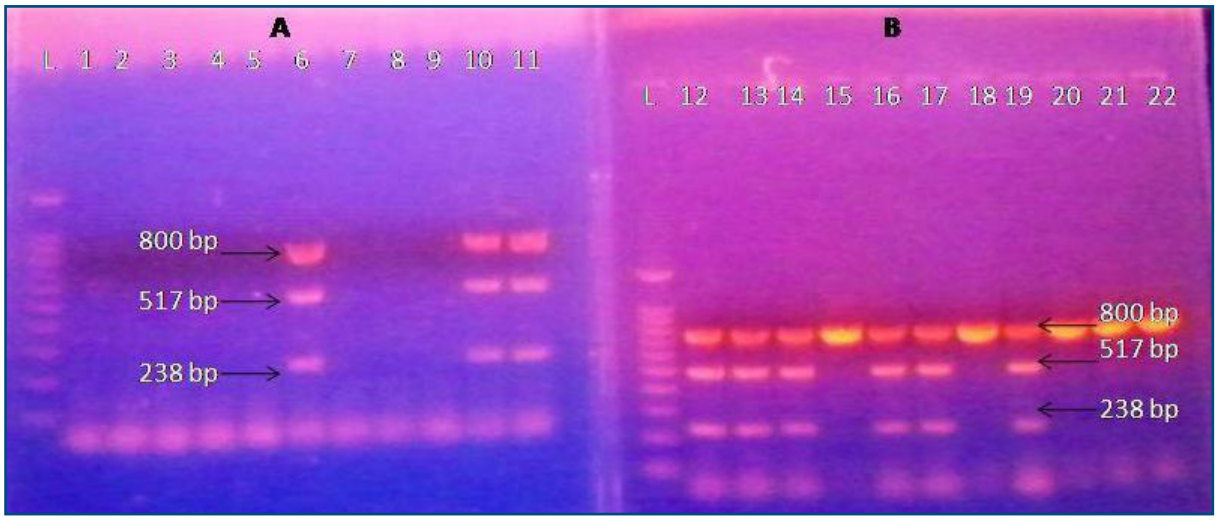

Figure 1. *Multiplex PCR of* Listeria monocytogenes *suspected isolates for the detection of* inlA, inlC *and* inlJ *genes.*
L: 100 bp ladder; 1: negative control; 12: positive control; 2-5 and 7-9: negative samples; 6, 10-11, 13-14, 16, 17, 19: virulent *L. monocytogenes*; 15, 18, 20-22: avirulent *L. monocytogenes*.

Table II. *Confirmed and virulent* Listeria monocytogenes *detected by Multiplex PCR.* Confirmed *L. monocytogenes* strains were detected by amplification of *inlA* gene, while virulent strains were distinguished by the presence of *inlC* and *inlJ* genes.

Sample type		Number of samples	Confirmed *Listeria monocytogenes*	Virulent *Listeria monoctogenes*
Shellfish	Blue crab	12	4 (33.3%)	3 (75%)
	Shrimp	14	6 (42.9%)	5 (83.3%)
	Bivalve mollusks	10	6 (60%)	5 (83.3%)
	Total	**36**	**16 (44.4%)**	**13 (81.3%)**
Fish	Nile tilapia	13	0	0
	Mullet	10	2 (20%)	1 (50%)
	Tuna	12	2 (16.7%)	1 (50%)
	Total	**35**	**4 (11.4%)**	**2 (50%)**
Total seafood		**71**	**20 (28.2%)**	**15 (75%)**
Human		**50**	**1 (2%)**	**100%**

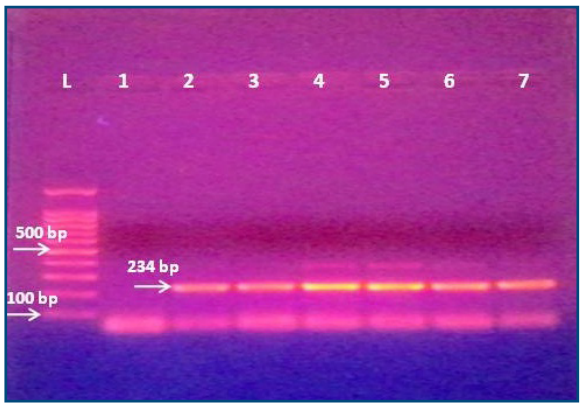

Figure 2. *Listeriolysin O PCR of* Listeria monocytogenes *suspected isolates.* L: 100 bp ladder; 1: negative control; 2: positive control; 3-7 positive *L. monocytogenes* for Listriolysin O.

Results

Prevalence of *L. monocytogenes* in seafood and in human samples

Seventy-one seafood samples were examined for the prevalence of *L. monocytogenes*, 20 (28.2%) were identified by the amplification of *inlA* gene which is a species-specific protein used for the identification of *L. monocytogenes* that produced 800 bp amplicon (Figure 1). Comparing the prevalence of *L. monocytogenes* in shellfish and other fish species, *L. monocytogenes* was isolated from shellfish with an overall percentage of 44.4% and 11.4% from the other fish samples (Table II). *L. monocytogenes* was isolated from diarrheic patients, with a history of chronic diseases, attending the University Hospital, Zagazig city (Table II).

Detection of virulent *L. monocytogenes* strains

L. monocytogenes strains harbouring internalin virulence genes were determined by the amplification of *inlC* and *inlJ* (Table II and Figure 1). The presence of LLO was investigated and detected in all virulent strains harbouring *inlC* and *inlJ* genes so to fully investigate the pathogenicity of *L. monocytogenes* to humans. Some *L. monocytogenes* isolates positive for LLO are shown in Figure 2.

Table III. *Serotypes and biotypes of* Escherichia coli *in the examined samples.* Serotypes of *E. coli* were identified by slide agglutination method using polyvalent and monovalent antisera.

Sample type		Number of samples	Number of *Escherichia coli* isolates (%)	*Escherichia coli* serotypes	*Escherichia coli* biotypes
Shellfish	Blue crab	12	1 (8.3%)	0124:K72(B17)	EIEC
			1 (8.3%)	0111:K58(B9)	EHEC
	Shrimp	14	0	-	-
	Bivalve mollusks	10	1 (10%)	0128:K67(B12)	ETEC
			1 (10%)	0127:K63(B8)	ETEC
Fish	Nile tilapia	13	1 (7.7%)	0111:K58(B9)	EHEC
	Mullet	10	1 (10%)	0128:K67(B12)	ETEC
	Tuna	12	1 (8.3%)	086:K61(B7)	EPEC
			1 (8.3%)	026:K60(B6)	EHEC
	Total	**71**	**8 (11.3%)**		
Human		**50**	3 (6%)	0111:K58(B9)	EHEC
			5 (10%)	0124:K72(B17)	EIEC
			2 (4%)	086:K61(B7)	EPEC
			5 (10%)	026:K60(B6)	EHEC
Total		**15 (30%)**			

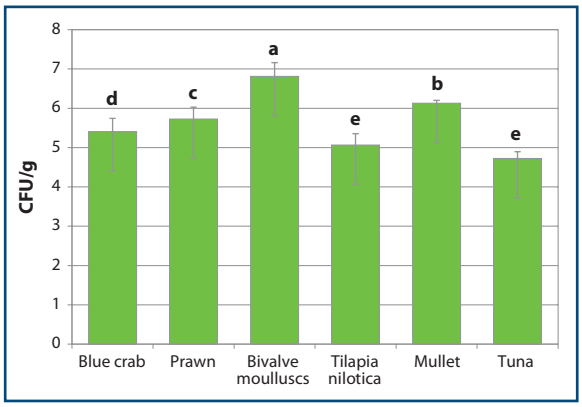

Figure 3. *Mean log-aerobic plate count per gram of examined fish and shell fish samples, error bars contain SD.* Means carrying different letter are significantly different ($p < 0.05$) based on one-way ANOVA.

Prevalence of *E. coli* in seafood and human samples

E. coli was isolated from seafood and diarrheic patients, the serotypes of the isolated *E. coli* isolates were determined (Table III). The results of the APC revealed that APC of examined blue crab ranged from 5.04 to 6.21 with a mean ± SD value of 5.405 ± 0.338 log CFU/g (Figure 3). The aerobic plate count of examined bivalve mollusks showed that 20% had bacterial load in extremely high numbers >7 log CFU/g, also no samples had acceptable level of total bacterial count (<5.69 log CFU/gm) and 80% of samples are marginally accepted (5.69 and 7 log CFU/g), the mean APC was 6.81 ± 0.349 log CFU/g. In shrimps, about 50% of the examined samples was accepted, while the other 50% was only marginally accepted according to FDA CFSAN. In Tilapia, APC was 5.06 ± 0.292 log CFU/g, all the examined Tilapia samples in the current study were within the acceptable level of APC (<5.69 log CFU/g). The results showed also that the APC in the examined Mullet fish ranged from 6.04 to 6.32, with a mean value of 6.18 ± 0.08 log CFU/g and 100% of samples marginally accepted (5.69 and 7 log CFU/g). The APC in the examined Tuna samples ranged from 4.59 to 4.84 with a mean value of 4.72 ± 0.174 log CFU/g and 100% of the samples were accepted (< 5.69 log CFU/g).

Most probable number of coliforms

The results showed that MPN of coliforms in examined crab and shrimp samples were respectively 1.42 ± 0.345 log MPN/g and 2.24 ± 0.362 log MPN/g (Figure 4). However, bivalve mollusks contain MPN of coliforms within average of 2.79 ± 276 log MPN/g. The MPN of coliforms in examined Tilapia, Mullet and Tuna were respectively 1.6 ± 0.256, 1.35 ± 0.241and 1.54 ± 0.306 log MPN/g.

Discussion

Seafood is able to carry potentially pathogenic bacteria for human beings to non-polluted water causing infection when fish are consumed or handled (11). Therefore, it is important to gather information about the prevalence of *L. monocytogenes* and *E. coli* in order to estimate the public health hazard

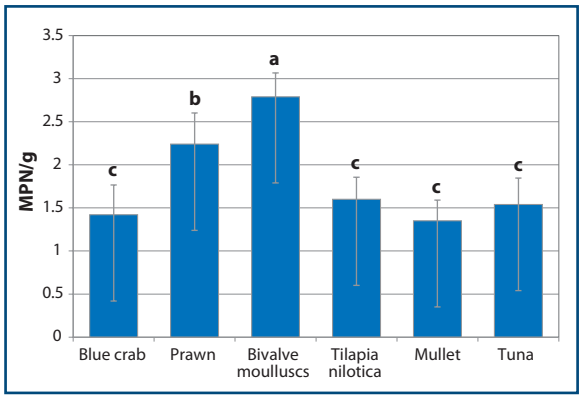

Figure 4. *Mean log-most probable number of coliform per gram of examined fish and shell fish samples error bars contain SD.*
Means carrying different letter are significantly different ($p < 0.05$) based on one-way ANOVA.

of shellfish and fish as a potential source for these pathogens. In this study, a total of 71 seafood samples (fish and shellfish) were examined for their contamination with *L. monocytogenes* and *E. coli*. Moreover, 50 stool samples from patients attending Zagazig University Hospital were examined for the presence of *L. monocytogenes* and *E. coli* serotypes.

The yearly medical costs and productivity losses from the acute illness from food borne listeriosis in the USA are estimated to be equal to the costs caused by *Salmonella* spp., twice the costs of *Campylobacter* spp. and 3 times the costs related to *E. coli* O157:H, despite the prevalence of these latter diseases being over 500, 700 and 25 times the number of listeriosis cases, respectively (13). Biochemical identification of *L. monocytogenes* is not always accurate and is not always accurate and depends on phenotypic characteristics of the bacteria (5). Of the 11 common *L. monocytogenes* serotypes, over 98% of clinical isolates from human listeriosis belong to only 4 serotypes (33, 41). Therefore, it is pivotal to distinguish between potentially virulent and avirulent strains by the amplification of different virulence genes such as *inlJ* (34). Some potentially virulent *L. monocytogenes* strains lack *inlJ* gene, an additional virulence-associated gene *InlC*, has been used in the Multiplex PCR in association with *inlJ* and *inlA* for rapid and simultaneous confirmation of *L. monocytogenes* species identity and its potential virulence (34, 36).

In this study, the isolation of *L. monocytogenes* by amplification of *inlA* gene indicates the usefulness of the Multiplex PCR as a rapid and accurate method compared to the time consuming and less accurate biochemical tests for the identification of *L. monocytogenes*. The obtained percentage of *L. monocytogenes* from seafood (28.8%) was nearly similar to previously reported studies in France (29%) (29) and in USA (30%) (28), while a relatively higher isolation rate of 39% was recorded in the Nordic countries (24). Other studies have found that the prevalence of *L. monocytogenes* in raw seafood is quite low, for instance: 0.8% in European fish (10), 2.3% in Ethiopia (48), and 12% in Portugal (45). In India, *L. monocytogenes* was isolated from seafood with the percentages of 1.8% (48) and 8% (64). The relatively low prevalence rate of *L. monocytogenes* reported in the aforementioned studies compared to the current study could be attributed to the use of molecular techniques for *L. monocytogenes* detection endorsed for this study and the difference in the water quality of the study areas.

The isolation of *L. monocytogenes* from shrimp (Table II) was similar to 44% percentage reported in Malaysia (4). In Gao region (India) 4.5% of seafood samples were positive for *L. monocytogenes*, of which, bivalves were found to have a maximum percentage of 12.5%, followed by prawns (3.84%) and finfishes (2.9%). These results are consistent with those obtained in this study, in which bivalves had higher prevalence rate. The higher isolation rate of *L. monocytogenes* in bivalves could be attributed to the fact that bivalves are filter feeders and they can accumulate more microorganisms than fish, from water impacted by sewage pollution (19). The consumption of bivalve mollusks is relatively high in Egypt due to the cheapest price compared to other seafood, therefore, the habit of eating these kinds of mollusks raw or undercooked constitute an important source of infection with *L. monocytogenes*. Lower prevalence of *L. monocytogenes* (12.1%) was reported in shellfish samples from India (35). The obtained results showed also that Tilapia was free from *L. monocytogenes*, which in turn has an important impact on public health considering the fact that this is one of the most popular fish types consumed in Egypt.

Since *L. monocytogenes* is commonly found in coastal waters and in surface waters of lakes (15), water should be regarded as the source of seafood contamination with *L. monocytogenes*. Other possible sources for contamination of seafood are soiled surfaces and boxes, as well as contamination from human and avian sources (54). Although *L. monocytogenes* has no hazard for consumers when seafood undergoes processing, they pose some risk to susceptible populations when consumed raw or lightly cooked. In addition, the possibility of cross contamination in the processing plant, kitchen or food service establishment is also of concern (68).

L. monocytogenes was previously isolated from human stool samples with low percentages of 0.7% (50) and 5% (58). The isolation of *L. monocytogenes* from diarrhoeic patients suffering from chronic diseases, although with low percentage, is of great importance because *L. monocytogenes* fatality rate

may be up to 30% (8, 59). The risks for human beings following the consumption of seafood contaminated with *L. monocytogenes* was previously documented in a Finnish retrospective study (42), which reported that a *L. monocytogenes* type recovered from several sporadic listeriosis cases turned out to be identical to an epidemic strain that originated from fish.

Similarly, a retrospective subtyping analysis of 42 human isolates identified in Italy through Multiplex PCR. In this case, the results showed that the human isolates tested positive for *inlA*, *inlC* and *inlJ* genes (43). Such results concur with those presented in this article, in which the obtained human isolate tested also positive for the aforementioned genes. Previously conducted studies reported that *L. monocytogenes* strains harbouring internalin genes *inlC* and *inlJ* could be unable to produce human disease via oral ingestion due to inability to cross the host's intestinal barriers during infection (40). Meanwhile, for a *L. monocytogenes* strain to cause infection in humans via ingestion, it requires involvement of other known virulence genes such as listeriolysin O. In this regard, a PCR which amplifies LLO has been used in the current study to fully investigate the potential of the isolated strains to cause human disease. The results indicated the detection of LLO in all virulent strains (Figure 2). These results highlight the usefulness of LLO as virulence indicator for *L. monocytogenes* causing human infection.

Testing of seafood for the presence of *E. coli* is still a gold standard used to assess the faecal contamination of seafood (20). *E. coli* was isolated from the examined seafood in the current study (Table III), lower percentage (6.7%) was reported in Korea (60), while higher percentage (48.95%) was documented in India (25). In Egypt, *E. coli* was also isolated from different seafood samples such as raw shellfish (48%, Suez Canal) (3) and bivalve mollusks, (30%, Ismailia) (32). In Domiatta, *E. coli* was isolated from shrimps, crabs and bivalve mollusks with the percentages of 8%, 12% and 80%, respectively (12). The differences in the prevalence of *E. coli* in seafood samples reported in the current studies and those previously reported could be due to environmental conditions, microbial quality of fish farms and sanitary conditions of markets. Similarly to the obtained results, serotypes O111 and O86 were previously identified in stool samples from diarrheic children in South Africa with nearly similar percentages of 6.5% and 4.8%, respectively (18). *E. coli* serotypes identified from human stool were similar to those identified from seafood in the present study suggesting the potential of *E. coli* transmission from seafood to human either by consumption or handling. However, the transmission of such serotypes could be further investigated using molecular typing methods to determine the genetic relationship of the serotypes identified from human and seafood sources.

The APC indicates the level of microorganisms in a product, quality, shelf life and post heat-processing contamination (44). It is useful in order to measure the conditions of the raw material, effectiveness of processing and hygienic conditions during processing, sanitary conditions of equipment and utensils and time temperature profile during storage and distribution. According to FDA CFSAN (16), the permissible limit of APC in seafood was reported to be less than 5.69 log CFU/g, while if the bacterial count is between 5.69 and 7 log CFU/g, it would be marginally accepted. Whenever the count is more than 7 log CFU/g, the quality of seafood would be unacceptable (16). Compared to APC in crab samples (Figure 3), lower APC ranged from 0.78 to 3.26 log CFU/g was obtained by Giuffrida (22), while higher rang of 6.53 to 9.23 log CFU/g was obtained by Gillman and Skonberg (21). The obtained results shrimp samples (Figure 3) coincide with Nayem (52) who reported APC in fresh water prawn in Bangladesh, with range of 4.83 to 6.2 log CFU/g. Nearly similar APC range of 6.39 to 6.43 log CFU/g in bivalve mollusks was obtained by Hatha (26) in India. Higher means for APC in Tilapia were reported in Nigeria by Adetunji (1) than those obtained in the current study (Figure 3). In Mullet samples, lower APC of 4.79 log CFU/g was detected in Nigerian Mullet fish (53), while higher APC of 5.90 to 8.95 log CFU/g was reported in Tuna samples in Taiwan (7). In the current study, APC arranged in descending manner as following bivalve mollusks > Mullet > shrimp > blue crab > Tilapia > Tuna (Figure 3). There is a significant difference between all examined species ($p < 0.05$), Tilapia and Tuna are not significantly different compared to each other, as they both have a low APC. The time of fish exposure to temperature during marketing is considered a critical factor, where the increase in temperature leads to multiplication of mesophillic microorganisms. This in turn explains the low APC reported in Tuna and Tilapia samples because these kinds of fish are of the same price (lower than other types of seafood) and are also popular to consumers in Zagazig, therefore the time of marketing is low due to high demand.

Counts of commensal coliform bacteria have traditionally been used to indicate the potential presence of pathogenic microbes of intestinal origin (2). Fish of good quality should have coliform count of less than 10/g according to the USA food and drug administration report (16). Higher MPN of coliforms in crabs was obtained by Hatha (26) in India, where counts ranged from 2.04 to 3.04 log MPN/g. Lower MPN of 1.32 log MPN/g were reported by Wentz (70). In shrimps, higher MPN of coliforms than the one obtained in this study were detected in fresh water prawn of Bangladesh where count ranged from

2.17 to 5.25 log MPN/g (52); while in Giza, Egypt, MPN of coliform count in raw shrimp samples was 3.72 log MPN/g (51). Once again bivalve mollusks were the most contaminated samples compared to other types of samples ($p < 0.05$). Nearly similar MPN of coliforms in 48 bivalve mollusks ranged from 2 to 2.69 log MPN/g were reported by Qadri (57), while higher MPN of coliforms (4.86 log MPN/g) was obtained in Alexandria, Egypt (14). The MPN of coliforms in examined Tilapia, Mullet and Tuna were 1.6, 1.35 and 1.54 log MPN/g, respectively (Figure 4). Nearly similar results were obtained by Landeiro (38) in Brazilian fish, where the detected value was 1.7 log MPN/g. There was no significant difference among these examined fish species ($p > 0.05$), which show the same level of sewage contamination in the site from which fish were harvested.

Conclusions

The results obtained from this study demonstrated the presence of *L. monocytogenes* and *E. coli* in seafood marketed in Zagazig city, Egypt. The presence of these pathogens and the high concentration of bacterial contaminants is an indication that the hygiene and safety of such seafood is compromised. Therefore, suitable processing parameters and post processing handling should be treated as important control measures to minimize or eliminate the hazard associated with these organisms. The detection of LLO in all potentially virulent *L. monocytogenes* strains is of great concern because this gene is an indicator of virulence and has a role in the pathogenicity of the organism in humans via the oral route. The current study recommends the use of the Multiplex PCR targeting internalin genes for the identification of *L. monocytogenes* and for differentiation virulent and avirulent strains instead of the conventional biochemical testing. Evaluation of the genetic similarity between *L. monocytogenes* and *E. coli* isolates from seafood and humans is recommended for further investigation of the public health potential of such isolates.

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