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Dynamics of bacterial pathogens associated with the clam *Tapes decussatus* in three main Egyptian fisheries

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ABSTRACT

Production and consumption of bivalves are affected by microbial pathogens that could harm bivalves and human consumers. The present study quantifies the dynamics of seasonal and location variations of bacteriological contamination of the clam, *Tapes decussatus*, in Egyptian fisheries as potential candidate for aquaculture in Egypt. Clams were monitored for one year for contamination with the potential clam pathogens; *Vibrio* and *Aeromonas* spp. and the human pathogens; *Escherichia coli*, *Staphylococcus* and *Salmonella* spp. The result have shown significant seasonal variations in the spread of *Vibrio* spp., *Salmonella* spp. and *Aeromonas* spp. Similarly, *Staphylococcus* spp.. *Vibrio* spp. and *Salmonella* spp. were significantly affected by collection sites. *E. coli* did not show statistically significant variations among stations or different seasons for the same station. In conclusion, the examined sites are not currently suitable for bivalve aquaculture or safe consumption unless bacteriological examinations and treatments are applied to avoid shellfish associated disease transmission.

Indexing terms/Keywords

Shellfish; Bivalve; Clam; *Tapes decussates*; Biosafety; *Vibrio*; *Salmonella*; *Aeromonas*; *Staphylococcus*; *E. coli*; fishery, Aquaculture; Egypt,

Academic Discipline And Sub-Disciplines

Environmental Microbiology, Marine microbiology, Shellfish Biosafety, Fisheries, Aquaculture

SUBJECT CLASSIFICATION

Environmental Microbiology

TYPE (METHOD/APPROACH)

Survey, Monitoring

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INTRODUCTION

Bivalve shellfish mollusks, such as oysters, mussels and clams, are globally important sources of food. They are particularly important in developing countries, mostly because they are easily collected in shallow areas and have high nutritional value [1]. Shellfish also provide high quality proteins with all dietary amino acids essential for maintenance and growth of human body. Moreover, lipids of marine origin are rich in Omega-3(n-3) polyunsaturated fatty acids and they have hypocholesterolemic effect when supplemented in human diet [2].

Bivalves are filter-feeders that concentrate microorganisms from large volumes of the surrounding water. Since they are often consumed raw or slightly cocked, contaminated bivalves pose health risks to consumers [3,4]. The presence and concentration of microorganisms in bivalve vary temporarily within the same habitat [5], as well as among different habitats [6], due to environmental factors that come to play individually or synergistically [7,8]. Many bacterial pathogens found in shellfish including those associated with faecal contamination are involved in food borne diseases in humans (*Salmonella*, *Staphylococcus*, and *Escherichia coli*). Naturally occurring marine bacteria belonging to the genus *Vibrio* such as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* are among the most frequently detected species [9,10,11,12]. Furthermore, the prevalence of *Aeromonas* spp. in the aquatic environment has been recognized as a potential health risk.

Detailed understanding of the dynamics of bacterial contamination associated with shellfish products would allow the development of successful microbial monitoring and control strategies such as phage therapy [12,13]. European Shellfish Directive (854/2004/EC) classifies shellfish harvesting according to *E. coli* concentrations within shellfish flesh (CFU/100 g) to four distinct groups. These include class A (<230), class B (>230 and <4600), class C (4600> and <46000) and class D (>46000), with only class A that may go for direct consumption. Classes B and C require depuration and/or proper treatment method(s) to meet class A. Finally, the harvesting from class D is totally forbidden. *Salmonella* should be absent in all samples ready for direct consumption [12, 14, 15].

The present study aimed to evaluate the possible risks associated with shellfish consumption in six selected sites representative to three cities that are famous as main *Tapes* clam natural fisheries in Egypt. This was carried out by monitoring seasonal and regional dynamics of representative disease-causing bacterial species including *Salmonella, Staphylococcus, Vibrio* and *Aeromonas* in addition to *E. coli* in the clam species *Tapes decussates* for one year.

MATERIAL AND METHODS

Clams were collected from six active bivalve fishing sites in three different cities along the Egyptian coastal water (Figure 1): Ismailia (2 sites in Temsah Lake, I and II), Alexandria (2 sites in the Eastern Harbor, NIOF and EL-Kashafa and 1 site in El-Max) and Damietta (1 site in clam farm in Ezbet Elborg).

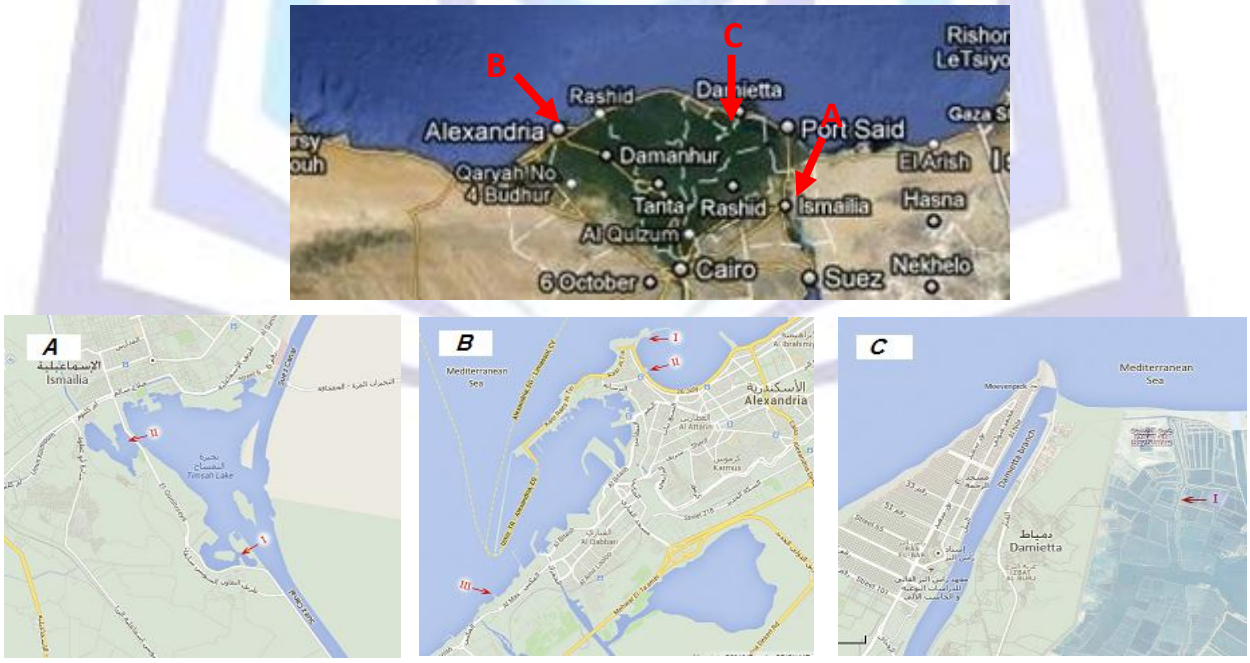

Figure 1: A map showing sampling sites in three Egyptian cities including Ismailia (A), Alexandria (B) and Damietta (C).

Clam sampling and transport

Samples of carpet shell clam *T. decussatus* were monthly collected from six different commercial Egyptian fisheries, in polythene bags placed in isothermal boxes that allowed keeping a refrigeration temperature. Samples were transferred for bacteriological analysis in the National Institute of Oceanography and Fisheries (NIOF), Alexandria branch. The time from

harvesting to the beginning of analyses did not exceed 18 h. Samples were then analyzed for contamination by *Salmonella, Staphylococcus, Vibrio, Aeromonas* and *E. coli.*

Quantification of targeted pathogenic bacterial groups

Methods described in Practical Food Microbiology [14] were used for processing of samples, in addition to isolation and identification of bacterial species of interest. *Salmonella Shigella* (SS) agar plates were used for isolation of *Salmonella* spp. Violet red bile agar with MUG (VRBA with MUG) was used for the isolation and differentiation of *E. coli* based on their ability to produce the enzyme β-glucuronidase visualized by UV induced florescent emission [16]. Agar plates of thiosulfate-citrate-bile salts-sucrose (TCBS) were used for the detection of *Vibrio* spp. [14, 17, 18]. Mannitol salt agar plates were used for isolation and enumeration of *Staphylococcus* spp., and *Aeromonas* agar plates [16] were used for the isolation of *Aeromonas* spp. The bacterial counting results are presented in CFU/g.

Statistical Analysis

All statistical analyses were conducted using Microsoft Excel 2013. Bacterial counting means were used to evaluate the significance of variations between sites as well as between seasons using the two-way ANOVA test followed by LSD post-hoc test with a significance level of $\alpha = 5\%$.

RESULTS AND DISCUSSION

Qualitative and quantitative monitoring of indicator bacterial pathogens in *Tapes decussatus*

In general, consumption of raw bivalve mollusks requires serious safety precautions to diminish the outbreaks of foodborne diseases. It is much easier to quantify bacterial indicators in harvesting waters than in bivalves flesh. Nevertheless, the guidelines to classify harvesting areas of bivalve based on the quantification of the levels of microbiological indicators in animal flesh and not in the harvesting waters [12]. Therefore, it was reasonable to analyze indicator bacterial species that constitute potential hazards to public health in clams collected from six different natural fisheries to evaluate these fisheries for suitability of their bivalves for human consumption as well for suitability as potential aquaculture grow out sites.

When clams from different sites were analyzed for contamination by *Salmonella, Staphylococcus, Vibrio, Aeromonas* and *E. coli*, qualitative analysis indicated that most of the examined clam samples carry all the bacterial contaminants under investigation. The percentages of positive results to total sample numbers are shown in Table 1 which demonstrates that a variety of disease-causing bacterial populations are harbored by clam samples. Based on these results, each of the examined sites has at least bacterial strains belonging to the genera *Vibrio, Aeromonas* and *E. coli*. This observation is possibly a result of sewage contamination in these areas resulting in the presence of a wide variety of pathogenic microbial populations which are easily concentrated in the bivalve flesh as a result of its feeding mechanism.

Table 1: Percentage of positive samples compared to total samples examined

Bacterial types	*Vibrio* spp.	*Salmonella* spp.	*Aeromonas* spp.	*Staphylococcus* spp.	*E. coli*
Positive samples (%)	100	93	100	72.2	100

Quantification of targeted indicator bacterial groups associated with *T. decussatus* was carried out on monthly basis at each of the experimental harvesting sites. The counting results of *Vibrio* spp. are graphically presented in Figure 2 as an example, while the detailed quantification results of the other bacterial species are not shown graphically. However, the dynamics of bacterial contamination in response to seasonal and site variations are fully described below based on the means of counting results for each bacterial type calculated at both season and sampling site levels.

Each of the examined bacterial species showed marked counting fluctuations in response to temporal as well as location variation. As shown in Figure 2, *Vibrio* spp. counts were generally very low in the clam samples collected in August through November and started to increase in December. This is followed by a drop in February and again rising in March to reach maximum during April, May, and June especially in the two harvesting sites of Ismailia. Detailed quantification of the other examined indicator bacterial species showed that *Salmonella* counts in *T. decussatus* were generally found to be maximum in El-Max , Alexandria with highest record during the months of April, May and June and minimum during the winter and autumn. The contamination levels of *Aeromonas* in *T. decussatus* showed a different pattern compared to *Vibrio* and *Salmonella*, as the highest counts were exhibited in October and November in all examined sites and moderate levels were observed in April and May.

The quantification results indicated also that the highest levels of *Aeromonas* content in *T. decussatus* were observed in the harvesting sites of Alexandria and Ismailia. *Staphylococcus* spp. counts were very low over the year except the period between April and June. On the other hand, *E. coli* was detected with various levels in almost all collection sites during the monitoring period with many sites that exceeded the limits (230 CFU/100 g) assigned for direct consumption (class A). In addition to our results, the findings reported by Pereira *et al.* (2015) demonstrate a higher complexity of the whole bacterial community and an increase of abundance of the main pathogenic bacteria in the summer, suggesting that this season is the critical time frame for the application of a treatment strategy such as phage therapy.

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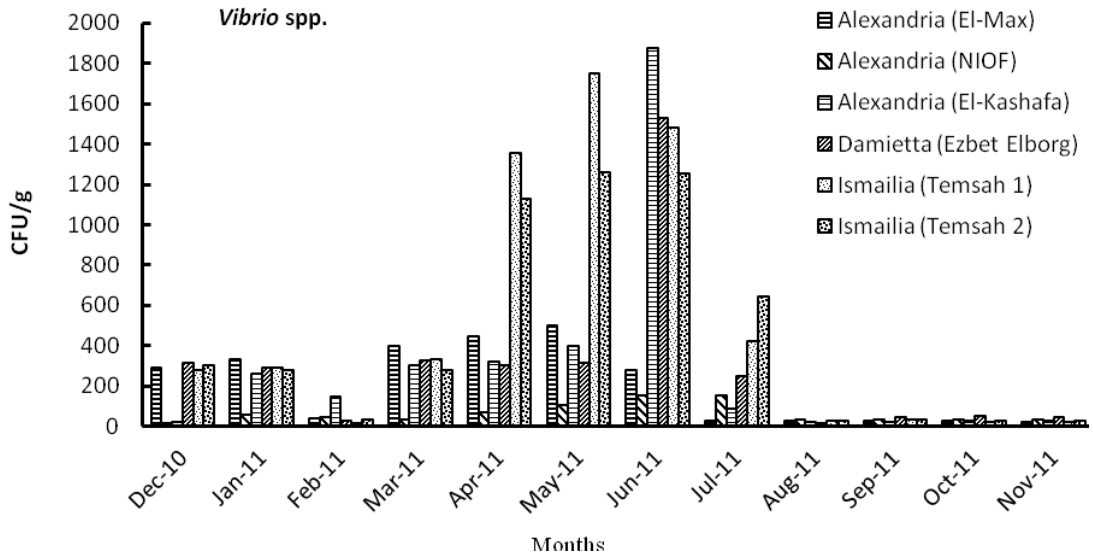

Figure 2: Monthly count of *Vibrio* spp. in *T. decussates* samples collected from different commercial clam fisheries**.**

Dynamics of bacterial contamination in response to seasonal variations at different sites

To evaluate the seasonal dynamics of the bacterial communities associated with clams at all sampling sites, the quantification results of each indicator bacterial type were subjected to analysis of variance (ANOVA). This was carried out by using the counting means calculated at both season and sampling site levels, as the raw data. As shown in Table 2, seasonal variation had significant effects on the densities of *Vibrio, Salmonella, Aeromonas* and *Staphylococcus* as clam contaminants. The results demonstrated also that the concentrations of *Vibrio* and *Salmonella* differed significantly from one sampling site to another. On the other hand, *Aeromonas* and *Staphylococcus* counting records showed insignificant variations between different sites. Furthermore, it appears that *E. coli* concentrations were not significantly affected by seasonal and regional variations.

Table 2: Two-Way ANOVA test of seasonal and site variations

Source of variation	*Vibrio* spp.	*Salmonella* spp.	*Aeromonas* spp.	*Staphylococcus* spp.	*E. coli*
Seasons	7.55×10^{-11} *	0.006628187 *	0.00625 *	0.000605 *	0.349977
Sites	0.00298 *	0.000107727 *	0.444373	0.586711	0.303488
Interaction	0.008103 *	0.001823529 *	0.051077	0.928344	0.783404

*Significant at α = 5%

The graphical representations (Figures 3 and 4) of the quantification averages indicate that the results were commonly heterogeneous in terms of the spread of the five examined indicators. As described below, each bacterial group showed particular prevalence patterns in response to seasonal fluctuations and sampling site variation with the highest counting results recorded by *E. coli* followed by *Staphylococcus* spp. and *Vibrio* spp.

Escherichia coli:

The presence of *E. coli* is traditionally understood as the main indicator that a water environment is contaminated by faecal material of human or other warm-blooded animals. Nevertheless, disease outbreaks associated with the consumption of shellfish contaminated by *E. coli* are reported [9]. Some strains of *E. coli* are able to cause diseases ranging from mild to cholera-like diarrhea and may lead to potentially fatal complications such as hemolytic uremic syndrome [19, 20]. As shown in Figures 3A and 4A, *E. coli* was present in all sites and all seasons with concentrations that are above the permissible limits of class A (230 *E. coli* 100 g^{-1}), which is the only category suitable for direct human consumption [14]. In agreement with the present results, several studies confirmed the contamination of many bivalve samples with *E. coli*. For instance, the results of a study conducted by Collin *et al.* [1] showed that 38% of the samples were contaminated with more than 60,000 MPN (most probable number) per 100-gram flesh, which lead to prohibition of these sites in Mozambique. Another study conducted in Southern Italy during the years 2011-2012 by Fusco *et al.* [21] in

which 59 bivalves were examined for bacterial and viral contamination showed that 27% of the collected samples showed positive *E. coli* results.

In contrast, the study carried out by Soegianto & Supriyanto [22] demonstrated that, *E. coli* enumeration values in bivalve samples collected from East Java coast, Indonesia ranged from undetectable level to 4800 MPN/100 g. Moreover, it has been reported by Clements *et al.*[23] that clear spots were detected within an area classified as class B (from 230 to 4600 *E coli* /100 g) in commercial intertidal mussel bed at Conwy Morfa in North Wales, UK. In these spots, *E coli* counts were below the lower limit of class B areas which makes these clear spots suitable for harvesting and direct consumptions. Accordingly, more efforts are needed to ensure the ranking of a given water body or commercial fishery before any regulation or closure decision can be taken. Therefore, there is need in large number of sampling points in any local areas for continuous monitoring of designated fisheries or potential aquaculture grow out site before it can be confirmed as contaminated. Recent study also in Egypt investigated the Mediterranean coast between Rasheed and Burullus (60 km) for exploitation of a variety of clams particularly *Chamelea gallina* through FAO EastMed project [24]. The microbiological examination revealed that clams from this area are less contaminated with pathogens when compared to the sites evaluated in the present study which could be possibly the result of being an open water area.

Staphylococcus spp.:

Staphylococcus spp. are everywhere and impossible to eradicate from the environment [20]. They present a potential hazard to consumer health as many of them are possibly found in foods due to environmental, human, and animal contamination [25]. *S. aureus*, as an example, is a versatile human pathogen capable of causing staphylococcal food poisoning, toxic shock syndrome, pneumonia, postoperative wound infection and nosocomial bacteremia [26].

It has been reported by Pomykała *et al.* [9] that 9% of bivalve samples collected in 2011 from commercially available raw shellfish in Poland were contaminated by coagulase-positive *Staphylococcus* spp., while all examined Japanese carpet shell mollusk samples showed negative results. On the other hand, previous local studies carried out by Ahmed & Eid [27] showed that *Staphylococcus* spp. could represent a health risk in association with bivalve consumption in Egypt. Consequently, raw shellfish should be microbiologically analyzed on regular basis to ensure their suitability for human consumption.

The results of the present investigation demonstrated that the highest prevalence of *Staphylococcus* spp. (872 CFU/g) was noted in spring which is followed by marked decreases during summer and autumn (Figure 3B). The lowest mean concentration (42 CFU/g) was observed in winter. These results suggest that the spring season is a critical period during which a microbial control treatment such as phage therapy should be applied [12].

However, with respect to regional variation, Figure 4B indicates that *Staphylococcus* spp. are present in all the experimental harvesting areas with obviously diverse contamination levels. Clam samples obtained from NIOF (A2) followed by the Eastern Harbor (A3) in Alexandria recorded the highest mean *Staphylococcus* concentration (535 CFU/g and 429 CFU/g, respectively). Comparatively, the lowest *Staphylococcus* contamination level (96 CFU/g) was recorded in El-Max (A1) which is also belonging to Alexandria governorate.

Vibrio spp.:

The obtained counting results of *Vibrio* spp. confirm that there is a strong correlation between the presence of *Vibrio* spp. and food poisoning associated with seafood consumption including bivalves [28,29]. The mean value of *Vibrio* spp. counting in clam (805 CFU/g) was significantly higher in spring than in the other three seasons (Figure 3C). Lower *Vibrio* spp. counts were recorded during summer followed by *winter* and *autumn* with mean values of 533, 193 and 87 CFU/g, respectively. The increase in *Vibrio* spp. count during spring might be due to more suitable temperatures in this period of the year (18.6 ±2.8 °C) [30]. More or less, all *Vibrio* species grow well at 18–22°C [17]. Another study was conducted by DePaola *et al.* [31] who studied the abundance of total *V. parahaemolyticus* in Alabama oysters at two sites (Cedar Point Reef and Dauphin Island Bay Reef). Their study showed that *V. parahaemolyticus* abundance started to increase by April through September and started to decrease in late September.

Although the present study did not identify the types *Vibrio* spp. present in clams, their observed high mean values indicate that consumption of raw or insufficiently cooked clams from the studied Egyptian fisheries can pose health risks on consumers especially in spring. With respect to spatial variations, the results shown in Figure 4C indicate that the highest count mean was in Ismailia Temsah 1 (504 CFU/g) followed by Ismailia Temsah 2 (440 CFU/g) and the lowest mean was in Alexandria NIOF (62 CFU/g). The mean value recorded in Temsah 1 showed more than six fold increases when compared to the NIOF sampling region in Alexandria.

Salmonella spp.:

The quantification results obtained in this study showed that 93% of the samples were contaminated with *Salmonella* with varying levels which are significantly affected by seasonal changes. As shown in Figure 3D, the mean values were relatively high in the spring and summer seasons (117 and 118 CFU/g, respectively) compared to autumn and winter (42 and 55 CFU/g, respectively). Parallel to these findings, the data published by Haley *et al.* [32] showed that concentrations of *Salmonella* were significantly higher in the summer months compared to other seasons ($P < 0.05$).

High counts of *Salmonella* were recorded in Alexandria, particularly in El-max station (Figure 4C) which might be attributed to the huge amounts of drainage water from El-Umoum drain as well as mixed wastes from Lake Maryout which reach seawater through El-Max pumping station [33]. Lake Maryout receives most of its water from a heavily polluted drain (El-

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Qalaa drain) which carries effluents from the East Treatment Plant, raw wastewater, irrigation drainage and agriculture runoff [34].

In humans, *Salmonella* causes non-typhoidal salmonellosis and typhoid fever [35]. The public health permissible limits for *Salmonella* in bivalves, is 0.0 CFU/ 25 gram of flesh to ensure safe products [14]. Therefore, the *Salmonella* counts recorded in the present study is alarming.

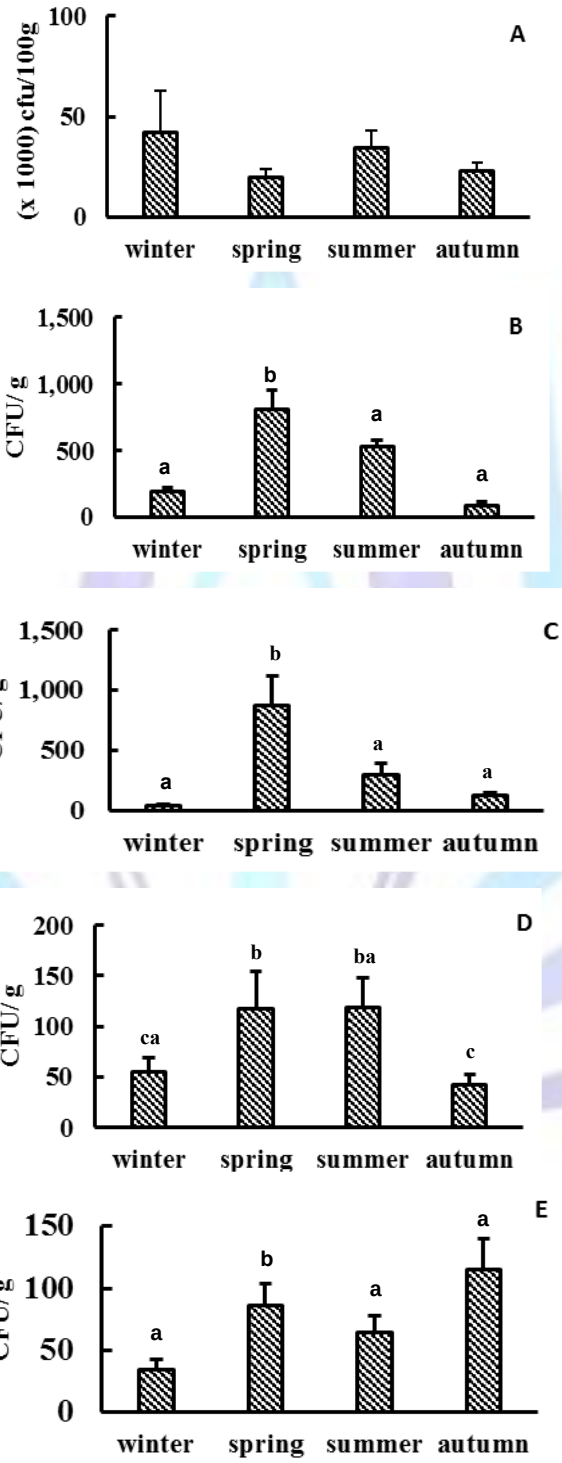

Figure 3: Effect of seasonal variations on bacterial contamination of clam samples presented as counting means. A: *E. coli*, B: *Staphylococcus*, C: *Vibrio*, D: *Salmonella*, E: *Areomonas* (seasons with different letters are significantly different at α = 5%).

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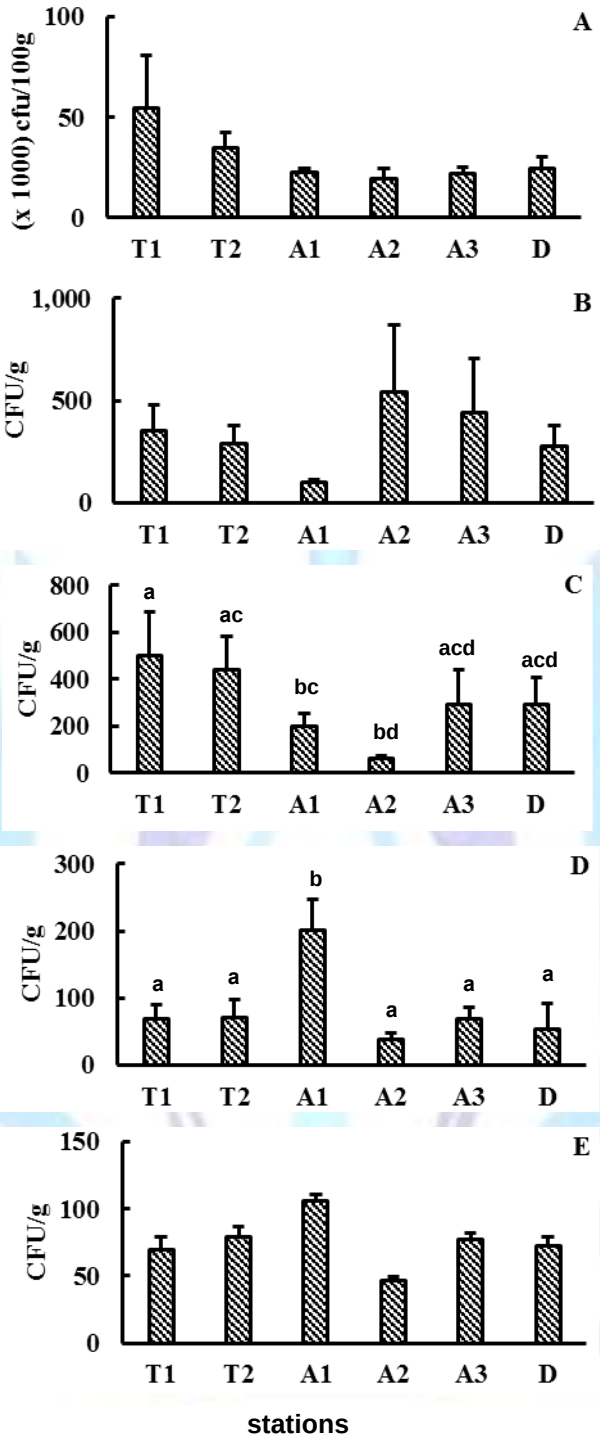

Figure 4: Counting means of bacterial communities associated with clam samples collected from different harvesting sites A: *E. coli*, B: *Staphylococcus*, C: *Vibrio*, D: *Salmonella*, E: *Areomonas* (sites with different letters are significantly different at α = 5% - T1: Temsah 1, T2: Temsah 2, A1: El-max, A2: NIOF, A3: El-Kashafa, D: Demietta)

Aeromonas spp.:

Aeromonas spp. have been described as probable cause of illness, including enteritis and traveler's diarrhea [35]. They may be considered as a contaminant of seafood, since they are ubiquitous in different water environments [36]. In the present study, *Aeromonas* spp. were reported in almost all sites throughout the year. As shown in figure 3E, the highest mean counts of *Aeromonas* (115 CFU/g) were recorded in autumn followed by spring (86 CFU/g), whereas the lowest counts were observed during summer and winter (64 and 34 CFU/g, respectively). In accordance with the present results, are those reported by Warren *et al.* [37] concerning spatial abundance of *Aeromonas* spp. in bivalves, from two urban playa lakes in Lubbock, Texas. They found that, the abundance was significantly affected by seasonal changes and the densities were maximum in the warmer months of the year, particularly from mid-summer to late autumn. These results support the speculation that there is a permanent risk of shellfish infections transmission throughout the year despite the fact that the most common pathogenic bacteria in shellfish reach the highest density during the summer season [12].

With respect to location, *Aeromonas* spp. were reported in almost all sites throughout the year confirming that they are associated with clams in Egyptian coastal water (Figure 4E). El-Max harvesting site in Alexandria recorded the highest mean value (105 CFU/g). In contrast, the lowest mean (46 CFU/g) was found to be in the sampling site NIOF which is also located in Alexandria. The rest of stations showed means that ranged between 69 CFU/g in Ismailia Temsah 1 and 79 CFU/g in Ismailia Temsah 2.

In conclusion, the emergence of infections outbreaks as a result of shellfish consumption represents an important human health problem. Therefore, the bacterial monitoring studies carried out in this work have public health as well as economic importance. The observed fluctuations of the selected indicator bacterial pathogens in response to seasonal and regional variations suggest that bivalve fisheries harvested from natural Egyptian fisheries are likely to be contaminated with a variety of considerable pathogens that cannot be overlooked. Moreover, the bacterial counting results obtained in this work do not accurately represent the actual total levels of microbial contamination within harvesting areas, but still provide general idea about the status of these fisheries. However, it is also clear that the most critical period for shellfish disease transmission is the summer season during which a variety of pathogenic bacteria reach their highest densities. Advanced treatments of wastewater disposed in marine environments, the application of more varieties of microbiological testing procedures and the development of successful microbial control strategies such as environmental phage therapy would allow minimizing such food prone pathogen transfer potentials.

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