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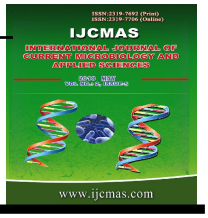

Original Research Article

Distribution of microbial population associated with crabs from Ennore seacoast Bay of Bengal north east coast of India

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A B S T R A C T

Ennore coastal area was highly contaminated with fecal coliform and other pathogenic microorganisms due to sewage effluents, anthropogenic sources, domestic wastes, urban runoff and rapid industrialization. The present study aims to evaluate the seasonal occurrence and distribution of microbial diversity of bacterial population present in crab samples collected from Ennore coast, Bay of Bengal, India. Samplings were carried out during postmonsoon, summer, premonsoon and monsoon seasons and analyses microbiologically. The bacteria were isolated from muscles, shells, gills and hepatopancreas of crab samples using Zobell's marine agar medium, selective, non selective media and identified using biochemical test and the results were compared with Bergy's Manual. The isolated bacterial species were predominately fecal coliforms associated with the tissues of crabs indicate possible fecal contamination that may have occurred through sewage effluents and others like, *Moraxella lacunata, Aeromonas salmonicida, Pseudomonas aeruguinosa* etc. are also found. During monsoon season maximum bacterial density (CFU) was found and minimum bacterial colonies were found during summer seasons. Shells and gills of crab samples showed higher number of bacterial species than muscles and hepatopancreas tissues. The results of this study clearly indicate that edible crabs are contaminated with coliforms and other pathogenic microorganisms. It is evident that environmental quality management and water quality should be considered where seafood plays an important role in marine habitat.

Keywords

Microbes; Pathogenic bacteria; Ennore; Crabs; Seasonal variation.

Introduction

Inorganic and organic contaminants entering coastal waters may be concentrated by edible marine organisms to varying degrees from either water, their food or sediments (Fowler, 1982). Understanding the transfer of contaminants through the food web is critical to predict the exposure of humans to contaminants either through subsistence or commercial consumption of seafood and the possible health consequences of such exposure. In addition, such information is crucial in making accurate risk assessment for seafood safety

purposes, a topic which is attracting much National and International attention.

Many bacterial species of enteric origin can be isolated from harbours which are located around sites of human habitation, including *Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus, Salmonella spp., Escherichia coli, Shigella* spp., *Listeria monocytogenes*, and *Klebsiella* spp. These bacterial species are commonly isolated from waters which contain fecal materials (Badley *et al.*, 1990; Jones and Summer-Barason, 1998; Martinez-Manzanarez *et al.*, 1992). Pathogenic bacteria in seawater are most abundant in sediments (Martinez-Manzanarez *et al.*, 1992) but are also seen in increased concentrations in the surface film, as compared with the water column (Plusquellec *et al.*, 1991). As a result, shellfish and other benthic fish, such as *Flounder*, show elevated levels of these bacteria, which can also cause disease in fish, as well as human hosts (Martinez-Manzanarez *et al.*, 1992; McVicar *et al.*, 1988). Untreated sewage can cause disease in humans as a result of eating contaminated shellfish and bottom dwelling species.

The global importance of food safety is not fully appreciated by many public health authorities despite the constant increase in the prevalence of food borne illnesses. The surveillance for food borne illness has been stressed because of centralization of food production and increased International trade and tourist, the responsibility for food safety has expanded from individuals to industries and government, and thus these changes have created potentials for epidemiological outbreaks of food borne diseases. The external surfaces of aquatic organisms are always in contact with microorganisms present in the water. Horsley (1977) has extensively reviewed the bacteria flora of fish. The bacterial flora, both internal and external of incubating rainbow (*oncorhynchus mykiss*) and brown trout (*Salmo trutta*) eggs was also studied by Barker *et al*. ,(1989). Vanderzant *et al.*, (1971) studied the microbial flora of pond-reared brown shrimp (*Penaeus aztecus*) in the digestive tract of Penaeid prawns such as *P.setiferus* and *P.japonicus* was studied by Hood *et al.*, (1971) and Yasuda and Kitao (1980) respectively.

Aquatic ecosystem although harbors a sizable population of microbes (Moriarty, 1976; Austin, 1982) are often considered as an index of water quality. These ubiquitous microorganisms do find various surfaces or organs of aquatic organisms for colonization. The present study provides an account of microbial communities isolated from shells, gills, muscles and hepatopancreas of crab samples and also the density of bacterial population from different tissues of crabs collected from Ennore area.

Materials and Methods

The most commonly occurring edible species such as *Portunus pelagicus* and *Portunus sanguinolentus* were used in these studies. Crabs were collected in Ennore coastal area during postmonsoon, summer, premonsoon and monsoon seasons of the period from July 2007- June 2009. The primary criteria for the selection of crabs for use in this study were that they are edible and availability during different seasons. Collected samples were aseptically transferred in iceboxes and transported to the laboratory where they were frozen until analysis. Twenty samples were analysed for each season.

Methods of Isolation of bacteria

The crab tissues such as gills, shells (carapace), muscles and hepatopancreas (1g) was inoculated into 9ml Zobell marine broth and nutrient broth with different NaCl concentration and incubated at 37°C for 24 h. From broth the sample was serially diluted and plated on nutrient agar and Zobell marine agar (ZoBell, 1941). This media contain all the nutrients necessary for the growth of marine bacteria. The media contain minerals that nearly duplicate the major mineral composition of sea water (Lyman and Fleming, 1940). Confirmation test were also performed using MacConkey agar, EMB (Eosin-Methylene blue agar) and Pseudomonas agar (APHA, 1998).

Quantitative analysis of bacteria

To estimate bacterial numbers, the inoculated plates were incubated at 25°C - 32°C for two days and duplicates were prepared for each dilution. Following incubation, the total number of colony forming unit (CFU) was determined and representative colonies were subcultured for identification. Bacterial numbers were calculated as the average of each set of duplicates and expressed as CFU/ml of the homogenate. Bacteria were isolated by a random collection of colonies from the agar plates. The colonies were purified by repeatedly sub culturing them on agar.

Bacteria identification

Morphological identification of the bacteria present in all samples was carried out with the Gram stain, acid fast stain and spore staining (cappuccino,1999) followed by biochemical tests as: TSI – triple sugar iron, methyl red, Voges Proskauer, motility, indole, citrate utilization, Hydrogen sulfide production, lactose and sucrose fermentation urease test, nitrate reduction, catalase, motility, fermentation of carbohydrates (Acid and gas production) oxidase and coagulase test (Norris and Ribbons, 1972).

The establishment of the genera and species present in the samples were identified according to their characteristics as outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994; Macfaddin, 2000).

Result and Discussion

The microorganisms enumerated from crab tissues are shown in Table 1. The greatest concentrations of bacteria isolated from shells and gills of the crab tissues as most of the bacterial communities isolated in the previous study from flora of the surrounding medium. Microbial communities associated with the external surfaces depend significantly on the environment of the host.

The micro-flora associated with gills is likely to have significant effect on crab as constant movement of water over gills might provide opportunity for contamination and colonization. Muscle and hepatopancreas tissues had relatively few bacteria. The minimum bacterial species were found in *Portunus pelagicus* when compared with *Portunus sanguinolentus*. In this study a total of 9 bacterial species was isolated and were predominant by gram-negative bacteria. Of these, *Alicaligenes faecalis, Pseudomonas aeruginosa, Moraxella lacunata,* and *Aeromonas salmonicida* were found common in all the tissues of crabs. The crab tissues such as shell, gills, muscles and hepatopancreas showed that mean value of CFU were found maximum

Table.1 Physiological characteristics of bacteria isolates from crab collected from Ennore, Tamilnadu, India

S.No	Organism names	G'stain	In	MR	VP	Cit	TSI	Cat	Oxi	Mot	H_2S	NO_3 Red	Ur	AF	Coagulase	Spore
1	*Aeromonas salmonicida*	G^{-ve} bacilli	-	-	-	-	A/A	+	+	+	-	+	-			
2	*Alicaligenes faecalis*	G^{-ve} cocci	-	-	-	-	K/K	+	+	+	-	-	-			
3	*Alicaligenes paradoxus*	G^{-ve} bacilli	-	-	-	+	K/K	+	+	+	+	+	-			
4	*Bacillus cereus*	G^{+ve} cocci						+	-	+		-	-	-	-	-
5	*E.coli*	G^{-ve} bacilli	+	+	-	-	A/A+G	+	-	+	-	+	-			
6	*Klebsiella pneumoniae*	G^{-ve} bacilli	-	-	+	+	A/A+G	+	-	+	-	+	-			
7	*Micrococcus luteus*	G^{+ve} cocci						+	+	+		-		-	-	-
8	*Pseudomonas aeruginosa*	G^{-ve} bacilli	-	-	-	+	K/K	+	+	+	-	+	-			
9	*Moraxella lacunata*	G^{-ve} cocci	-	-	-	-	K/K	+	-	-	-	+	-			

Note: - Negative, + Positive, A/A -Gulcose & lactose and /or sucrose fermentation, K/K -no fermentation, A/A+G- Gulcose & lactose and /or sucrose fermentation+ gas produced.

Table.2 Microorganisms identified from tissues of crabs collected from Ennore seacoast

Crabs	Shells(Carapace)	Gills	Muscles	Hepatopancreas
Portunus sanguinolentus	*Moraxella lacunata*	*Moraxella lacunata*	*Moraxella lacunata*	*E.coli*
	Bacillus cereus	*Bacillus cereus*	*Aeromonas salmonicida*	*Moraxella lacunata*
	Aeromonas salmonicida	*Aeromonas salmonicida*	*Alcaligenes faecalis*	*Aeromonas salmonicida*
	Alcaligenes faecalis	*Alcaligenes faecalis*	*Pseudomonas aeruginosa*	*Alcaligenes faecalis*
	Alcaligenes paradoxus	*Alcaligenes paradoxus*		*Pseudomonas aeruginosa*
	Klebsiella pneumonia	*Pseudomonas aeruginosa*		
	Pseudomonas aeruginosa			
	Micrococcus luteus			
Portunus pelagicus	*Moraxella lacunata*	*Bacillus cereus*	*Bacillus cereus*	*Alcaligenes faecalis*
	Bacillus cereus	*Alcaligenes faecalis*	*Alcaligenes faecalis*	*Alcaligenes paradoxus*
	Alcaligenes faecalis	*Alcaligenes paradoxus*	*Alcaligenes paradoxus*	*Klebsiella pneumonia*
	Alcaligenes paradoxus	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*E.coli*
	Klebsiella pneumonia	*E.coli*		
	Pseudomonas aeruginosa	*Pseudomonas aeruginosa*		

during monsoon season and were found minimum during premonsoon, summer and postmonsoon season. *Pseudomonas aeruginosa* found in higher density in shell tissues. *Bacillus cereus* was found in higher density in gill tissues. *Alcaligenes faecalis* in muscle tissue and *E.coli* in hepatopancreas were found in higher density. The seasonal counts of *pseudomonas aeruginosa* in crab shell samples ranged from 1.44± 0.77 to 3.66 ± 5.84 x10^5 CFU/g. *Bacillus cereus* counts ranged from 1.40 ± 049 to 3.72 ± 2.35 x10^5 CFU/g in gills, *Alcaligenes faecalis* counts ranged from 2.59± 2.98 to 4.75 ± 2.95 x10^5 CFU/g in muscles, *E.coli* counts ranged from 4.28 ± 2.13 to 7.64 ± 0.48 x10^5 CFU/g in hepatopancreas during seasons (Fig.1a-d).

In Fig.2 a-d the percentage composition of bacterial population during different seasons recorded that higher percentage was found during monsoon season (29% to 40%) followed by premonsoon, postmonsoon and summer seasons respectively in shells, gills, muscles and hepatopancreas of crab tissues.

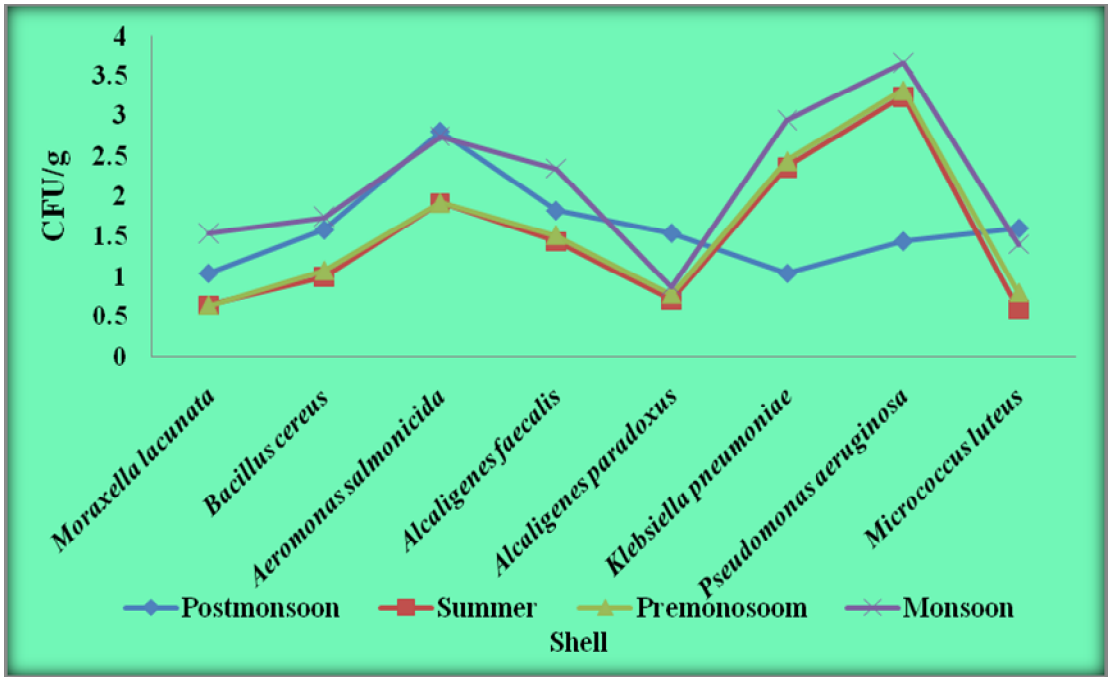
The percentage composition of each bacterial species was recorded in different tissues of crab samples. *Pseudomonas aeruginosa* (22%) *Aeromonas salmonicida* (17%) and *Klebsiella pneumonia* (16%) are dominant among other species in crab shells. In gills, *Moraxella lacunata* (21%), *Aeromonas salmonicida*, *Bacillus cereus* (18%) are dominant among other species. In crab muscles, *Alcaligenes faecalis* (36%) and *Alcaligenes paradoxus* (30%) are dominant among other species. In hepatopancreas, *E.coli* (39%) is dominant among other species (Fig.4a-d).

From the above results, bacteria such as *Bacillus cereus, Alcaligenes sp., Aeromonas salmonicida, Klebsiella pneumonia, Micrococus luteus* and *E.coli* are all indicators of sewage and sludge disposal into the coastal waters. *Aeromonas salmonicida* and *Pseudomonas aeruginosa* are facultative pathogens.

Studies on the microbial communities isolated from different organs (carapace, gills, muscles and hepatopancreas) of crabs collected from the study area showed a highly diverse and varied microbial population associated with different organs. Contamination of seafood with bacterial pathogens at source (i.e. in the sea) primarily arises from two different origins. The first with bacteria that occur naturally in the marine environment which, when consumed in seafood in large enough numbers, will cause illness in humans. Some species of the genus *Aeromonas sp.* are considered to some to possibly cause gastro-enteritis in humans and these may also be present naturally in the marine or, more especially, the estuarine environment.

Environmental loading of fecal by-products from humans and their associated animals is significant and can affect the quality of water and food resources in coastal ecosystems (Fayer, 2004; Kim *et al*., 2004). Bacterial infections in the heamolymph of blue crab contribute to severe crab mortalities, particularly within soft shell crab shedding operations (Krantz *et al*., 1969). In addition, infected crabs can represent a significant public health problem.

Figure.1 a. Seasonal variation of bacterial species from Ennore crab samples



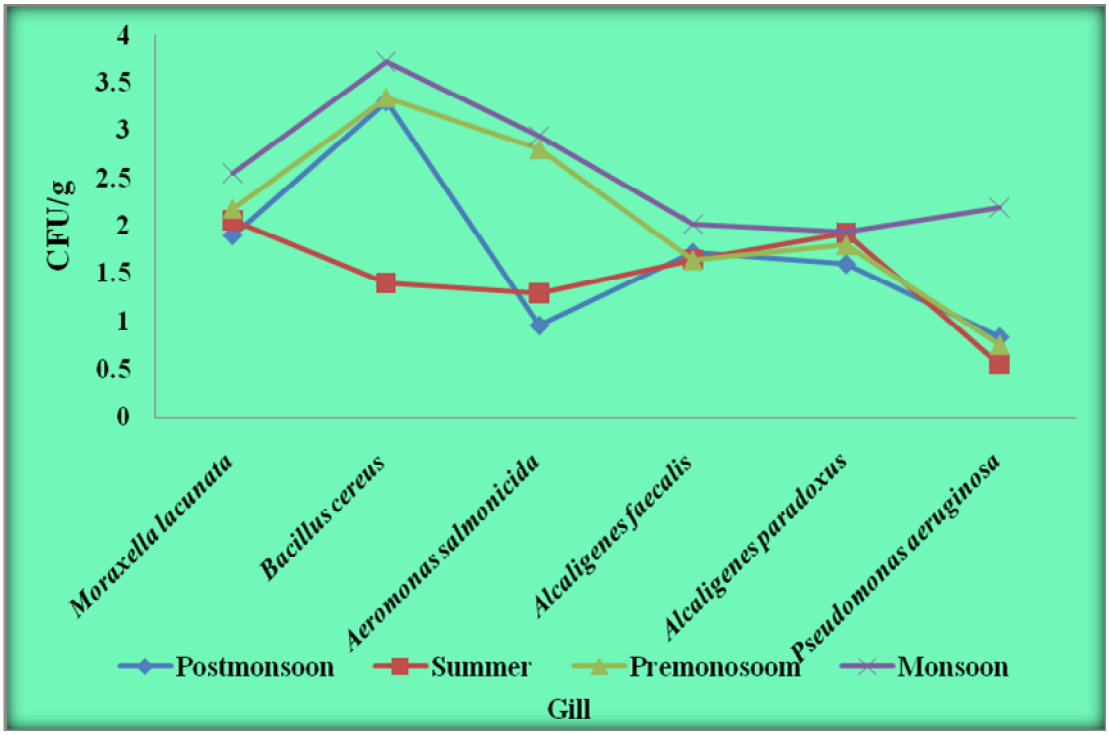
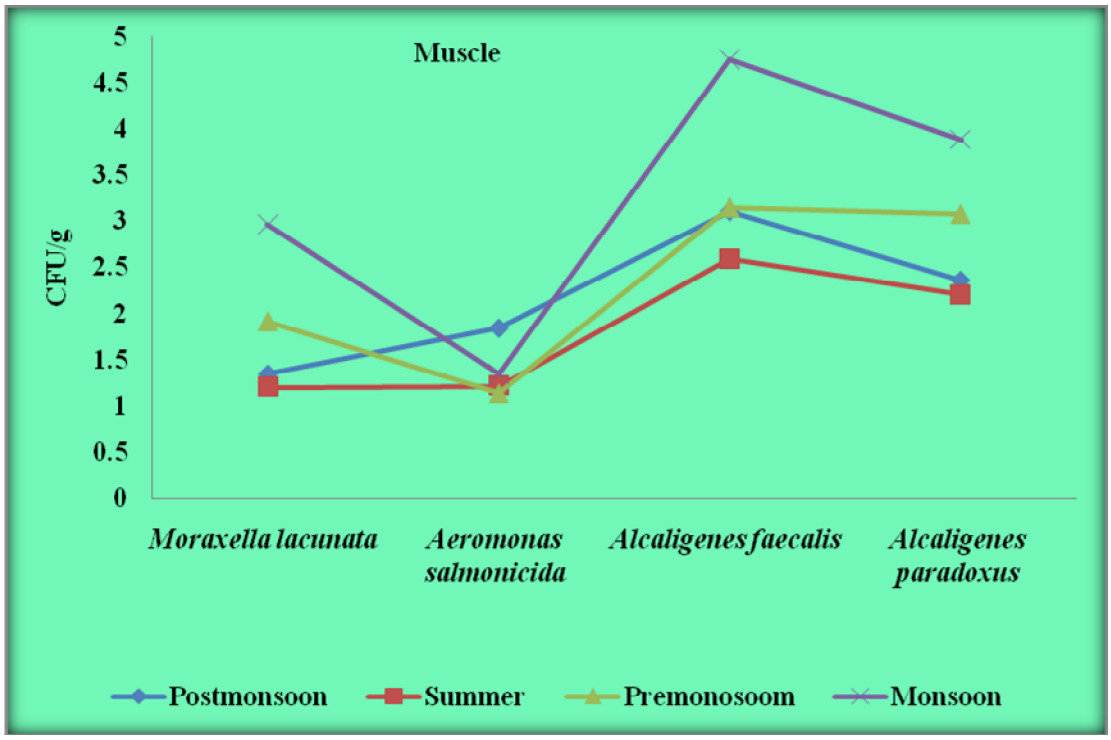


Figure.1 b. Seasonal variation of bacterial species from Ennore crab samples



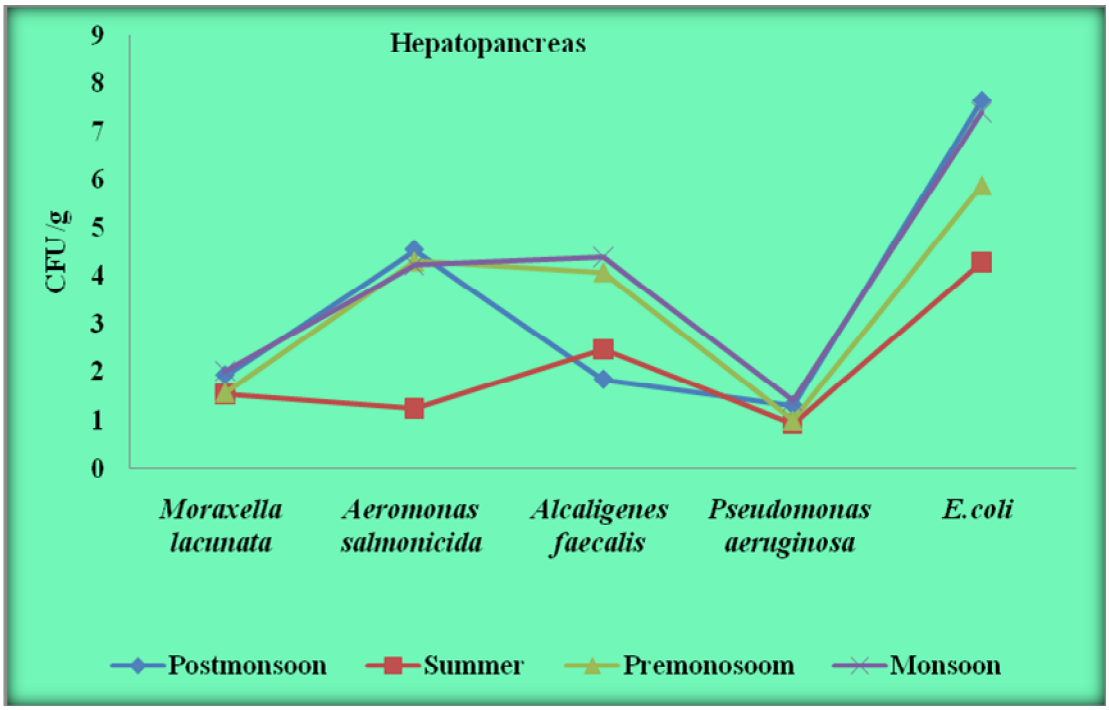
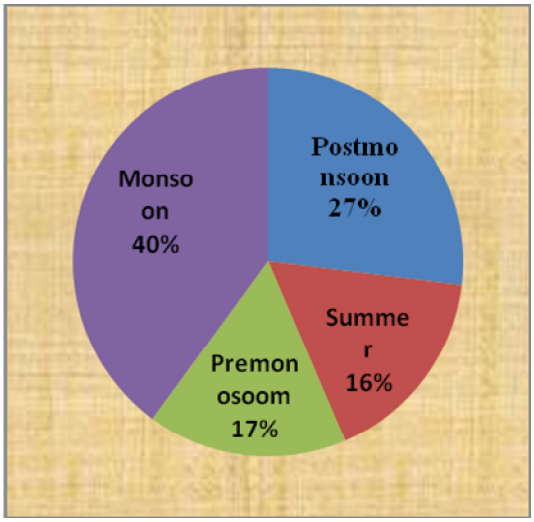
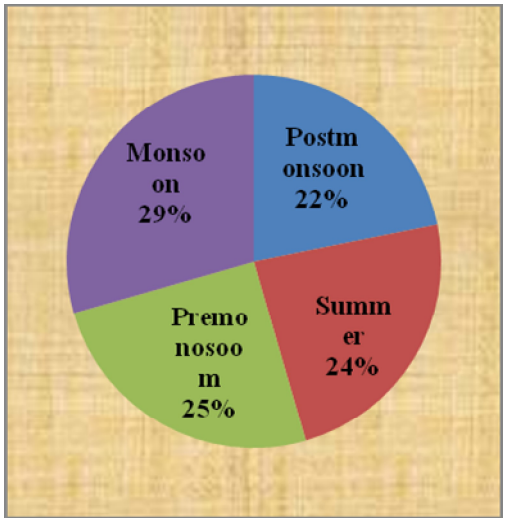


Figure.2 Percentage composition of bacterial population during different season in crab (g^{-1}) samples collected from Ennore.

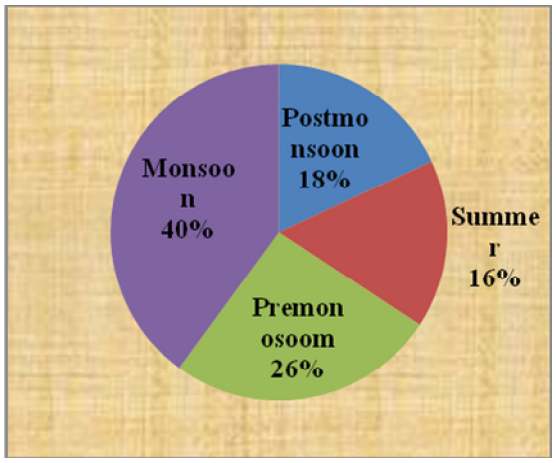
a. Shell



b. Gills



c. Muscles



d. Hepatopancreas

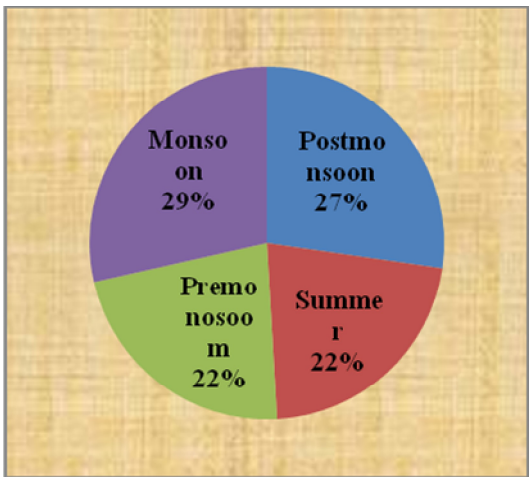
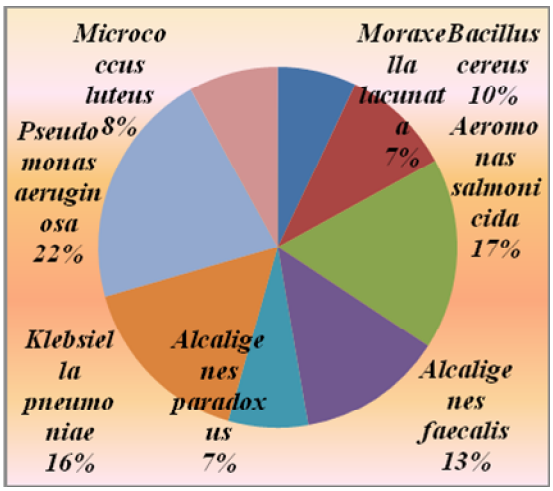
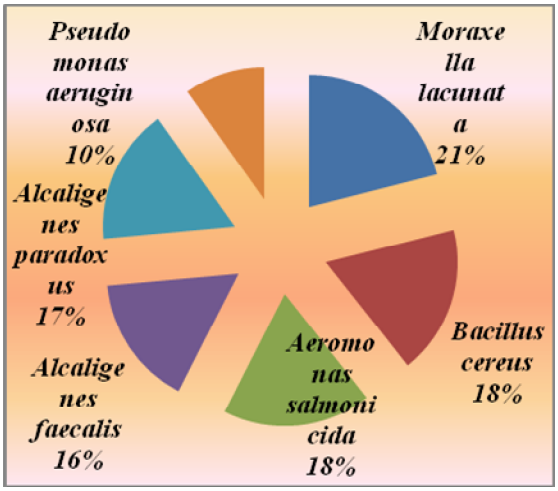


Figure.3 Percentage composition of bacterial species recorded from Ennore crab (g^{-1}) samples

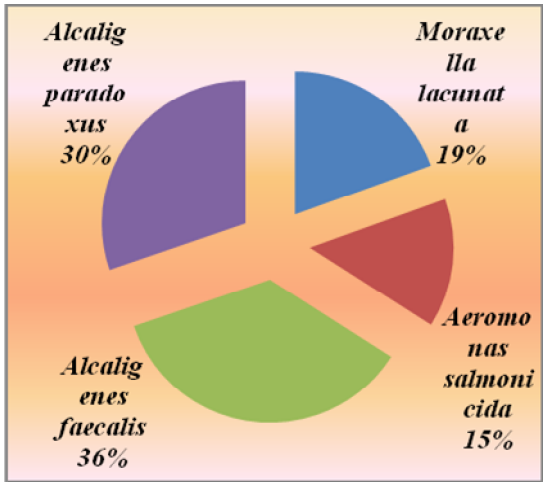
Shell



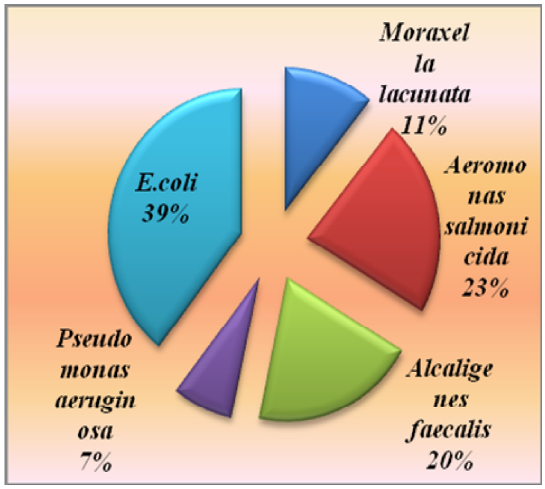
Gills



Muscles



Hepatopancreas



Several studies were subsequently published which considered the total heterotrophic bacterial flora of blue crab heamolymph (Colwell, 1975; Davies and Sizemore, 1982; Tubiash *et al*., 1975). Results of these indicate that greater than 80% of apparently healthy, and therefore marketable, blue crabs have bacterial infections was reported as a public health problem in the commercial preparation of crab meat (Fishbein *et al.*, 1970; Phillips and Peeler, 1972). Moribund blue crabs also contain bacteria (Krantz *et al*., 1969). Several out breaks of gastroenteritis due to

consumption of crabs have also been reported (Molenda *et al*., 1972; Peffers *et al.,* 1973; Sumner *et al*., 1971).

The present study is concurrent with Mohamad *et al.,* (1984) as greatest concentrations of bacteria occurred in the gills, and muscle tissues had relatively few bacteria from edible crabs collected from marine ecosystems. The presence of bacterial population in gills was comparatively similar with the work in Mohamad *et al*., 1984. It is a general profile of bacterial species, where *Klebsiella* sp. is suggestive of sewage contamination.

E.coli was found in gills and hepatopancreas of crabs collected from Ennore marine ecosystem. Fecal indicator organisms such as *E.coli* was found in water and sediment sample of Ennore seacoast (Mahalaxmi *et al.,* 2013). The bacterial populations during different seasons were also fluctuated widely depending on physico-chemical parameters of the environment (Mahalakshmi *et al*., 2012). Thampuran *et al*.,(2005) also reported that the microbial quality of the Tilapia indicated that all tissue samples except muscle were contaminated with fecal coliform where *Escherichia coli* is the most common contaminant and is often encountered in high numbers. The isolation of these groups of organisms indicted faecal and environmental pollution and these supported the findings of Yagoub *et al*., (2004). This also confirms the findings of Koutsoumanis and Nychas (2000), Gonzalez (2004) and Herrera *et al*., (2006), who isolated similar organisms from fish and fish products. It has been shown that *Escherichia coli* can survive for very long periods in tropical waters and once introduced may almost become indigenous to the environment (Fujioka, 1988).

Characteristically, there is occasional rainfall in monsoon season and premonsoon season along the east coast of Tamilnadu, whose run-off carries some fairly high load of faecal matter into the seacoast, resulting in the peaks exhibited by *Escherichia coli* counts during monsoon season and also higher densities of bacterial population in crab tissues. It is concluded that seasonal variability and prevalence of infection are of epidemiological significance (Blake, 1983; Holmberg, 1988). Seasonal difference was also found by Tubiash *et al.,* (1975) for Viriginia crabs. In contrast, Davis and Sizemore, 1982 did not find significant seasonal differences in bacterial counts in Texas crabs. They attributed this to the consistent warm winter temperatures along the Texas coast. Earlier studies suggests that rain may influence the bacteria-invertebrate interactions, possibly by altering the bacterial concentration in surface waters and/or the invertebrate feeding and depuration dynamics (Mourin˜o-Pe´rez *et al*., 2003). Other factors that could promote bacterial contamination of water located adjacent to urbanized coastlines include fertilizer runoff (through nutrient enrichment), discharges of heated water from industries, or algal blooms that can trigger explosive bacterial proliferation through enhanced bioavailability of dissolved organic material (Lipp *et al.,*2002; Mourin˜o-Pe´rez *et al*., 2003).

The isolation of *Pseudomonas* sp. from the collected crab samples is of highly importance because this bacterium plays a considerable role as potential pathogenic bacteria for human and as an indicator of food quality as spoilage organism. This is

in accordance with previously mentioned by Jeyasekaran *et al*., (2005) and Koutsoumanis and Nychas (2000) who identified pseudomonas as a good spoilage index. Although *Pseudomonas sp.* is not referred to as the cause of food borne illnesses they are closely associated to food deterioration (Tryfinopoulo, 2002). According to Tripathy *et al*., (2007) *Pseudomonas sp*. are frequently associated to fish and have been isolated from skin, gills and intestine. Their load is explained by the population density in water. In an aquaculture, especially *P. aeruginosa* and *P. fluorescens* have been considered opportunistic pathogenic species (Altinok *et al.,* 2006).

Aeromonas sp. has been recognized as potential food borne pathogens for more than 20 years. *Aeromonas salmonicida* can be causative agents not only of human enteritis (Sukroongreung *et al*., 1983), but also of a fatal septicaemia. *A. salmonicida* is the causative agent of the fish disease called furunculosis but human disease has not been described (Isonhood and Drake, 2002). *Aeromonas* is one of the major causes of bacterial infections affecting tilapia (Li and Cai, 2011).

Alcaligenes is commonly found in the environment (Papen and Von Berg, 1998; Wang *et al.,* 2011). Alcaligenes sp. had been isolated from water as well as in the mussel samples (Cavallo *et al.,* 2009). *Alcaligenes faecalis* which produce disease in crustaceans such as lobster, being isolated from the hemolymph and inducing a softening of the shells (Tindall, 2003; Buller, 2004). *Pseudomonas* is the most common genera in crustaceans, marine fish and bivalves (Alexopoulos *et al*., 2011). The group of bacteria related to the genus *Pseudomonas* is very broad and includes species pathogenic for humans and plants commonly found in fresh altered water. In the case of marine organisms species of the genus *Pseudomonas* have been isolated and identified from the microbiota of farmed fish such as rainbow trout (*Oncorhynchus mykiss*) (Salgado-Miranda *et al*., 2010), perch (*Perca fluviatilis*) (Goldschmidt-Clermont *et al.,* 2008) and rohu (*Labeo rohita*) (Ghosh *et al.,* 2010).

Some species of the genus *Aeromonas* are considered to some to possibly cause gastro-enteritis in humans and these may also be present naturally in the marine or, more especially, the estuarine environment. Although many such organisms pose significant health risks for immune-compromised individuals or other susceptible groups, several, such as *Pseudomonas* spp., *Aeromonas* spp. commonly form part of the natural flora of seafood. These observations and ensuing inferences of this study are useful for managing effluent out fall in to coastal ecosystem. However, we must rely on men to take social awareness and learn to care for the ocean, minimizing the contamination into the sea. Safeguarding the ecosystem from adding undesirable microbial population cells for evolving appropriate policies and regulation. Every effort leading to reduction in pollution indicator bacteria and microbes of human health concern has to be promoted and implemented.

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