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ORIGINAL ARTICLE

Culture-dependent bacteria in commercial fishes: Qualitative assessment and molecular identification using 16S rRNA gene sequencing

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Abstract Fish contamination has been extensively investigated along the Saudi coasts, but studies pertaining to bacterial pathogens are scarce. We conducted qualitative assessment and molecular identification of culture-dependent bacteria in 13 fish species from three coastal sites and a local fish market in Jeddah, Saudi Arabia. Bacterial counts of gills, skin, gut and muscle were examined on agar plates of Macconkey's (Mac), Eosin Methylene Blue (EMB) and Thiosulfate Citrate Bile Salts (TCBS) culture media. Bacterial counts significantly differed between species, sources and feeding habits of examined fishes. *Mugil cephalus* exhibited higher counts on TCBS (all body parts), Mac (gills, muscle and gut) and EMB (gills and muscle). Fishes from Area I had higher bacterial loads, coinciding with those in seawater and sediment from the same site, indicating direct association between habitat conditions and the levels of bacterial contamination. By feeding habit, detritivorous fish harbored higher counts than herbivorous and carnivorous species. Bacterial counts of skin were higher in fish from market than field sites, and positively correlated with other body parts indicating the relation of surface bacterial load on the overall quality of fish. *Rahnella aquatilis* (Enterobacteriaceae) and *Photobacterium damselae* (Vibrionaceae) were among the dominant species from fish muscle based on 16S rRNA sequencing. These species are known human pathogens capable of causing foodborne illness with severe antibiotic resistance. Opportunistic pathogens, e.g. *Hafnia* sp.

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(Enterobacteriaceae) and *Pseudomonas stutzeri* (Pseudomonadaceae) also occurred in fish muscle. The inclusion of bacterial contamination in future monitoring efforts is thus crucial.

1. Introduction

Fishes are known for their exceptional health benefits, particularly against cardiovascular diseases (He et al., 2004; Maehre et al., 2015) and for infant brain development (Boucher et al., 2011; McCann and Ames, 2005) due to their long-chain polyunsaturated fatty acids (PUFAs) content. Despite such nutritional benefits and culinary fondness, infection of fishes by pathogenic microorganisms and other contaminants is a major concern for seafood consumers. Fish contaminated with bacterial pathogens could cause severe foodborne illness and offset the health benefits of PUFAs (Huss, 1997). The primary sources of microbial pathogens in fish are anthropogenic activities that generate point and non-point pollution in coastal waters. There are also naturally occurring waterborne pathogens like vibrio that could cause human illness by way of food consumption. Hence, the monitoring of bacterial pathogens potentially provides early warning to safeguard seafood consumers from the threats of contamination.

Microorganisms in the marine environment have both beneficial and harmful functions. They execute biogeochemical cycles that are a critical process in marine environments (Hewson et al., 2007). Many microbial species are pathogenic to humans. They are known to produce toxins that cause lethal diseases such as paralytic shellfish poisoning, neurotoxic shellfish poisoning, diarrheic shellfish poisoning, amnesiac shellfish poisoning, and ciguatera fish poisoning (Bienfang et al., 2011; Garthwaite, 2000; Watkins et al., 2008). Microbial contaminants mainly consists of pathogenic groups of bacteria, viruses, and parasites. Bacteria represents a majority of pathogens in fish that are capable of causing foodborne diseases in humans. Bacterial abundance in fish species generally varies based on environmental and biological factors. Some fishes are inherently more prone to contamination depending on the species, feeding pattern, age, size, harvest season, habitat characteristics, and geographical location (Novotny et al., 2004). Hence, studies on bacterial abundance in fish should be made at a larger spatial scale, considering the potential differences in susceptibility to contamination.

Jeddah is the second largest city in Saudi Arabia and located on the Red Sea coast (western seaboard). It has the largest seaport along the Red Sea coast, with a total throughput of 4700 vessels (56% container ships) and 52 million metric tons of cargo (69% discharged, 31% loaded) in 2011 (Saudi Port Authority, August 2012). Rapid industrialization and urbanization in the Jeddah area have raised concerns over potential impacts on public health and the environment. Contaminated effluents through point and non-point sources may enter the coastal waters of Jeddah and eventually find their way into the body tissues of edible fish. This presents as a significant concern as fish is a major dietary protein source for both Saudis and expatriates in the area. In spite of significant fish consumption, scientific investigation of marine microbial contamination, particularly on bacterial pathogens in seafood, generally remains scarce in Saudi Arabia. Monitoring the microbial as well as chemical contaminants in commonly consumed fish will thus help in safeguarding the public from any potential adverse risks. Overall, our present study examined the levels and potential health risks of heavy metal, organic and microbial contaminants in commonly consumed fishes from the Jeddah area. This paper presents our findings on the bacterial contamination in fishes collected from three coastal sites and the local fish market in Jeddah. Bacterial abundance in different body parts (gills, skin, gut and muscle) of fishes and in environmental samples (seawater and sediment) from the fishing sites was determined by a culture-dependent method using three selective isolation media. In addition, the dominant bacterial species in muscles of the fish species were identified by gene sequencing technique.

2. Materials and methods

2.1. Sampling

Fish samples were collected from three sites (Areas I, II and III) near Jeddah City (Fig. 1). Area I is a semi-enclosed lagoon lined with several stormwater drainage outlets, a corniche, city road network, hotels, restaurants, a private harbor, and commercial buildings within a hundred meters from the shorefront. Area II is a reef complex fronting the international port, handling mainly cargo ships. Area III is an open-water fringing reef site near the largest discharge outfall of a wastewater treatment plant located south of Jeddah City. Bacterial analyses were made on 13 commonly consumed fish which were selected based on a consumption pattern survey in Jeddah region (Table 1). Fish were self-caught by baited hook and line or gill-net from the sampling sites. We also obtained fish samples from the central fish market in Jeddah City and a nearby landing site (the latter for *Hipposcarus harid* only). Collected fish samples were individually wrapped in aluminum foil and brought to the laboratory in refrigerated condition. Sediment and seawater samples were collected from all fishing sites (Areas I, II and III) and a control site about 70 km northwest of Jeddah City for comparison. Sediment samples were collected using a Van-Veen grab, while seawater samples were collected from 1 m below the surface using a horizontal acrylic water sampler (Wildco Alpha). The subsamples were then immediately transferred to falcon tubes and brought to the laboratory in ice coolers.

2.2. Sample processing

Samples of gills, skin, muscle and gut of fish were aseptically obtained by dissection and then prepared for plating following Andrews and Hammack (2001). All the dissecting apparatuses, such as scalpel, forceps, scissors, knives, mortar, pestle and glassware, were sterilized with 100% ethanol and kept in hot air oven at 180 °C for 6 h prior to dissection. Gills were

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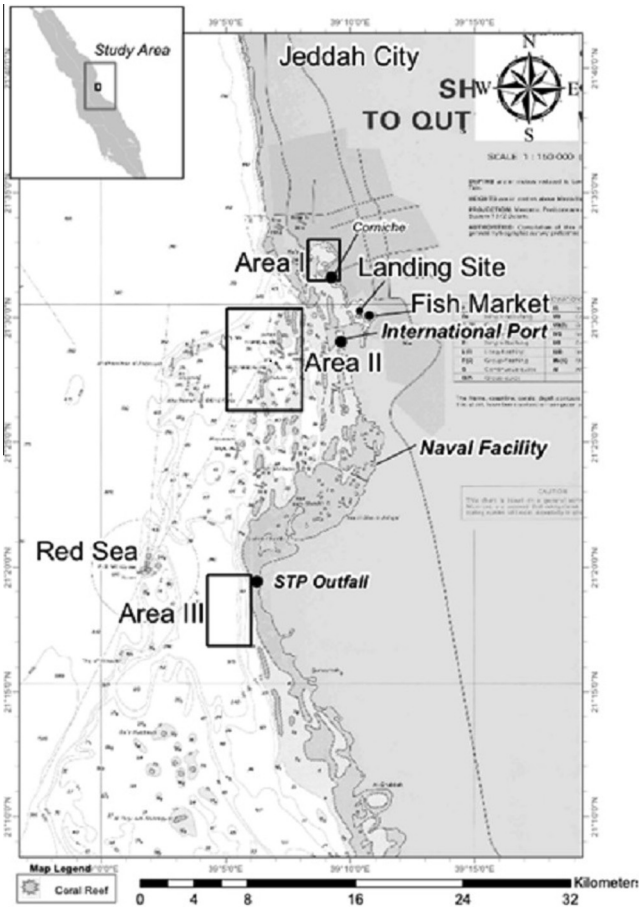

Figure 1 Map of study area showing the locations of sampling sites (Areas I–III, landing site and fish market). Control site (not shown) is about 70 km northwest of Jeddah City.

removed from both sides of the fish head and homogenized to a composite sample. Skin was removed from the right side of fish behind the dorsal fin after removing the scales with a sterilized knife. Muscle (flesh) samples were collected through incision on the same side and section of the fish body. An opening into the body cavity was carefully made with a sterile scalpel to remove a small portion of the gut. All samples were taken in the same order from the same body sections uniformly across fish species. After dissection, 1 g of each dissected organs were separately homogenized using a mortar and pestle.

The homogenized tissue, seawater and sediment samples were serially diluted before plating them for enumeration. Serially diluted samples of sediment and fish tissues were plated on different selective media for the estimation of bacterial counts. Bacterial counts in the seawater samples were estimated by Most Probable Number (MPN) method.

2.3. Enumeration

Three selective isolation media, Macconkey's agar (Mac), Eosin Methylene Blue agar (EMB) and Thiosulfate Citrate Bile Salts agar (TCBS), were used for isolation. Isolation media (Hi Media, Mumbai) were prepared by suspending the Mac (51.5 g/L), EMB (36 g/L) and TCBS (89 g/L) in sterile distilled water mixed with 50% of filtered seawater. Media were completely dissolved by heating on a hot plate and sterilized by autoclaving at 15 lbs pressure and 121 °C temperature for 15 min. TCBS media was not autoclaved but heated until boiling after suspending them in previously sterilized 50% seawater. Media were then cooled up to 50 °C and aseptically poured into Petri plates under sterile conditions. Enumeration of bacterial load in sediment and fish tissues were done by spread plate method. The plates were incubated in inverted position at 35 ± 2 °C for 18–24 h (Slaby et al., 1981). Bacterial colonies were counted and expressed in Colony Forming Units (CFU) per gram of given sample (Collins and Lyne, 1984). Broths of the same culture media were used for estimating the bacterial load in seawater samples through MPN technique.

2.4. Bacterial identification

Bacterial groups isolated from the muscle samples were morphologically categorized based on their colony shape, size and color. Dominant bacterial groups in the muscle samples were sub-cultured and a single colony was subjected to colony PCR using 16S rRNA forward and reverse primers. The primers 27b F – 5′ AGAGTTTGATCCTGGCTCAG 3′ and 1492u R – 5′ TACGGYTACCTTGTTACGACTT 3′ were used for the amplification. DNA polymerase Accuprime

Table 1 Fish species used for microbiological analysis, their local name(s), feeding habit and sampling site.

No.	Species	Local name	Feeding behavior	Areas			Market	Landing site
				I	II	III		
1	*Chanos chanos*	Salmani	Herbivorous	√			√	
2	*Mugil cephalus*	Arabi, Bori	Detritivorous	√			√	
3	*Carangoides bajad*	Bayad	Carnivorous		√		√	
4	*Lethrinus lentjan*	Shaoor, Sheiry	Carnivorous		√	√	√	
5	*Epinephelus tauvina*	Tauvina	Carnivorous		√		√	
6	*Plectropomus pessuliferus*	Najil	Carnivorous		√		√	
7	*Variola louti*	Louti, Sharef	Carnivorous		√	√	√	
8	*Cephalopholis argus*	Hamour	Carnivorous		√	√	√	
9	*Siganus rivulatus*	Sijan, Safi	Herbivorous				√	
10	*Lutjanus bohar*	Bohar	Carnivorous				√	
11	*Plectropomus truncatus*	Tarathi	Carnivorous				√	
12	*Aphareus rutilans*	Mosa, Faris	Carnivorous				√	
13	*Hipposcarus harid*	Harid	Herbivorous				√	√

(Invitrogen) was used to amplify 16S rRNA gene. The PCR mix was then subjected to Exosap-IT (GE Healthcare) PCR clean up protocol. The nucleotide sequence of 16S rRNA gene was determined by Sanger sequencing using Applied Biosystems 3730xl DNA Analyzer. The 27b F and 1492u R primers were used for setting up PCR reactions using the Big Dye Terminator from ABI. Sequences from forward and reverse primers were aligned using Vector NTI software (Invitrogen) and the contigs were subjected to BLAST (Zhang et al., 2000) for nucleotide similarity search.

Phylogenetic tree was constructed using BLAST pairwise alignment tool using Fast Minimum Evolution method (Desper and Gascuel, 2004). The genus and species were identified based on the lowest *E*-value in BLAST. Multiple sequence alignments of nucleotides were performed using Clustal Omega web tool (Sievers et al., 2011). Sequences of different genus and those with differences within same genus were submitted to GenBank/NCBI database for accession number (Table 2).

Phylogentic analyses of the isolates were carried out using Phylogeny (Dereeper et al., 2010; http://www.phylogeny.fr) using tools such as MUSCLE (Edgar, 2004) for multiple sequence alignment, Gblocks for curating sequences (Castresana, 2000), PhyML for constructing tree (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006) and TreeDyn for analyses of tree (Chevenet et al., 2006).

2.5. Statistical analysis

Statistical analyses and graphical presentations were performed using the SPSS Statistics 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY) and Microsoft Excel spreadsheet software packages. One-way analysis of variance (ANOVA) was performed on the log-transformed bacterial count data after establishing the homogeneity of variances using Levene's test. The more robust Welch test was performed on data with unequal variances and indicated accordingly in the text.

Table 2 Bacterial species identified based on 16S rRNA gene and its acquired GeneBank accession numbers.

No.	Organism	GenBank Accession number
1	*Pseudomonas stutzeri* strain NA01	KJ563264
2	*Vibrio harveyi* strain NA02	KJ563265
3	*Psychrobacter faecalis* strain NA03	KJ563266
4	*Aeromonas salmonicida* strain NA04	KJ563267
5	*Psychrobacter faecalis* strain NA05	KJ563268
6	*Rahnella aquatilis* strain NA06	KJ563269
7	*Photobacterium damselae* strain NA07	KJ563270
8	*Hafnia* sp. strain NA08	KJ563271
9	*Pseudoalteromonas* sp. strain NA09	KJ563272
10	*Aeromonas* sp. strain NA10	KJ563273

3. Results

3.1. Environmental samples

Mean bacterial densities in seawater and sediment samples are depicted in the Fig. 2. Counts in seawater were highest in Area I (8.45, 1.91 and 3.8×10^5 MPN mL^{-1} for Mac, EMB and TCBS, respectively) and lowest in the control site (0.072, 0.066 and 0.094×10^5MPN mL^{-1}). Levene's test ($P > 0.05$) indicated homogeneity of variances for seawater between sites. Comparisons by ANOVA revealed significant differences ($P < 0.01$) in seawater bacterial count for Mac ($F_{(3,\ 8)} = 40.01$), EMB ($F_{(3,\ 8)} = 32.01$) and TCBS ($F_{(3,\ 8)} = 64.35$). Higher bacterial counts were also found in sediment from Area I (600, 412 and 526×10^5 CFU g^{-1} for Mac, EMB and TCBS, respectively) and lowest from the control site (0.38, 0.30 and 0.22×10^5 CFU g^{-1}). ANOVA also showed marked variations in sediment counts between sites for Mac ($F_{(3,\ 8)} = 129$, $P < 0.01$) and TCBS ($F_{(3,\ 8)} = 345$, $P < 0.01$), which both showed equality of variances (Levene's test, $P > 0.05$). For the EMB, which showed a significant Levene's test ($P < 0.05$), the robust Welch test showed significant difference in counts between stations ($F_{(3,\ 4.28)} = 64.54$; $P < 0.01$). The bacterial densities in seawater and sediment at the control site were significantly lower than at Areas I to III (Tukey HSD test, $P < 0.05$).

3.2. Fish samples

3.2.1. Counts on Mac agar

Mean bacterial counts in body parts on Mac agar are shown in Fig. 3, showing the highest loads for gills and muscles in *Mugil cephalus* from Area I (491×10^5 and 238×10^3 CFU g^{-1}, respectively), skin in *Cephalopholis argus* from market (615×10^3 CFU g^{-1}), and guts in *M. cephalus* from market (220×10^5 CFU g^{-1}). No bacterial load was detected in muscles of *Lethrinus lentjan* and *Variola louti* from Area II, market and field samples of *Chanos chanos,* and market samples of *Lutjanus bohar* and *Aphareus rutilans*. Bacterial counts on Mac agar differed significantly (Welch test) between species for muscles ($F_{(9,\ 41)} = 4.75$, $P < 0.01$), gut ($F_{(12,\ 19.5)} = 5.56$, $P < 0.01$) and gills, ($F_{(12,\ 20.1)} = 11.51$, $P < 0.01$) but not skin ($F_{(12,\ 19.4)} = 1.71$, $P > 0.05$).

3.2.2. Counts on EMB agar

On EMB agar, the bacterial counts (Fig. 4) between species were highest in *M. cephalus* from Area I for gills (146×10^5 CFU g^{-1}) and from market for muscles (41.2×10^3 CFU g^{-1}), *V. louti* from market for skin (308×10^3 CFU g^{-1}), and *L. bohar* from market for guts (32.3×10^5 CFU g^{-1}). The same species and sources as for Mac did not show bacterial growth on the EMB media. Bacterial counts on EMB significantly differed (Welch test) between species for all body parts: muscle ($F_{(9,\ 12.6)} = 8.03$, $P < 0.01$), gut ($F_{(12,\ 19.4)} = 7.61$; $P < 0.01$), gills ($F_{(12,\ 18.6)} = 5.41$; $P < 0.01$), and skin ($F_{(12,\ 19.5)} = 3.31$, $P < 0.01$).

3.2.3. Counts on TCBS agar

Bacterial counts on TCBS (Fig. 5) were consistently highest in *M. cephalus* for all body parts from all sources, except that the

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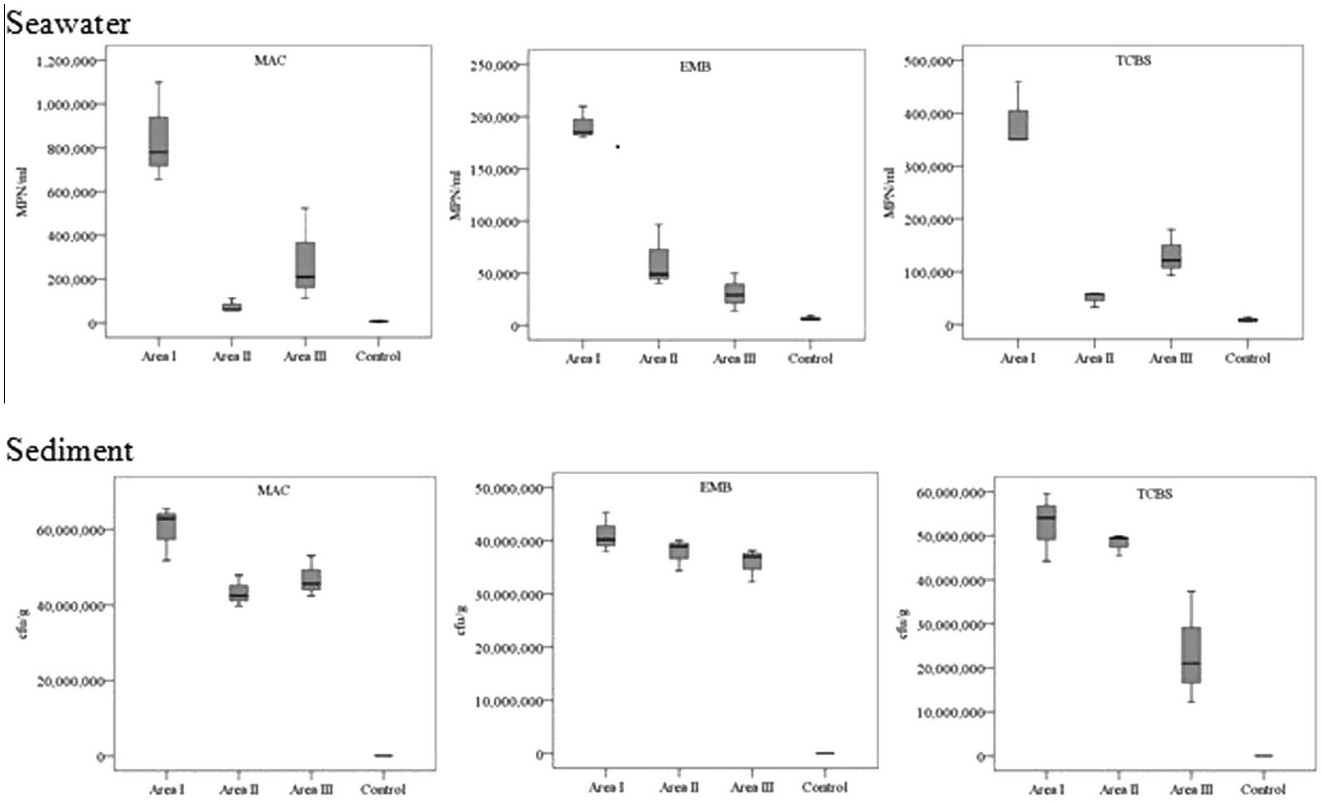

Figure 2 Boxplots of bacterial counts in sediment and seawater from the sampling sites isolated on three different culture media.

counts in gills were higher from markets than field samples. The highest counts of bacteria in *M. cephalus* were up to 351×10^5 CFU g^{-1} in gills, 696×10^3 CFU g^{-1} on skin, 61.5×10^3 CFU g^{-1} in muscles , and 206×10^5 CFU g^{-1} in guts. As for Mac and EMB, bacterial growth on TCBS varied significantly between species for all body parts. No bacterial growth on TCBS was detected for muscles in almost the same species and sources as for the other media. Welch test results on TCBS agar were as follows: skin ($F_{(12,\ 19.6)} = 4.33$, $P < 0.01$), gut ($F_{(12,\ 20.0)} = 200$; $P < 0.05$), gills ($F_{(12,\ 18.7)} = 10.4$, $P < 0.01$; $P < 0.05$), and muscle ($F_{(9,\ 13.7)} = 10.8$, $P < 0.01$; $P < 0.01$).

3.3. Locational differences

Bacterial counts in fish species grouped based on source of samples are shown in Fig. 6, with higher counts for gut, gills and muscle on Mac and TCBS in fish from Area I, whereas for skin from market. Counts for gut, gills and skin on EMB were higher from Area III, while that for muscle from Area I. Bacterial counts on all three media significantly differed between sources ($P < 0.01$) for skin, gut, and gills, but not muscle.

3.4. Feeding habits

By grouping the fishes according to feeding habit (Fig. 7), bacterial counts in gills and muscles showed higher values in detritivorous fish on all media. The same fish group showed higher bacterial abundances in skin and gut on Mac and TCBS, but not on EMB where the carnivorous and herbivorous species showed higher loads. Bacterial counts on TCBS significantly differed between feeding groups for skin (ANOVA $F_{(2,\ 72)} = 6.23$; $P < 0.01$), gut (ANOVA $F_{(2,\ 72)} = 3.98$; $P < 0.05$), gills (Welch $F_{(2,\ 19.1)} = 57.9$; $P < 0.01$) and muscle (Welch $F_{(2,\ 30.6)} = 44.5$; $P < 0.01$). On Mac agar, significant difference was detected between groups for gut (ANOVA $F_{(2,\ 72)} = 6.48$; $P < 0.01$), gills (Welch $F_{(2,\ 19.9)} = 45.5$; $P < 0.01$) and muscle (Welch $F_{(2,\ 24.85)} = 25.11$; $P < 0.01$), but not skin ($P > 0.05$). Counts on EMB markedly differed (Welch test) for muscle ($F_{(2,\ 28.7)} = 33.3$; $P < 0.01$) and gills ($F_{(2,\ 11.6)} = 6.16$, $P < 0.05$), but not skin and gut ($P > 0.05$).

3.5. Identification

16S rRNA gene fragment amplified using 27b F and 1492u R universal primers were around 1500 bp, covering 97% of the total. After sequence alignment with Vector NTI and inspected by eye, further manual editing was done to remove ambiguities based on PHRED scores and chromatogram. Fully aligned contig sequences were analyzed online with BLASTN for nucleotide similarity against 16S rRNA database. Bacterial species with hits of lowest *E*-value were identified and unique organisms were selected using clustal omega tool. These were submitted in GenBank/NCBI database (Table 2). Bacterial species of g-Proteobacteria group, constituting the families Vibrionaceae, Enterobacteriaceae, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae and Pseudoalteromonadaceae were identified. Phylogenetic analysis (Fig. 8) revealed *Rahnella aquatilis* (strain NA06) and *Hafnia* sp. (strain NA08) are

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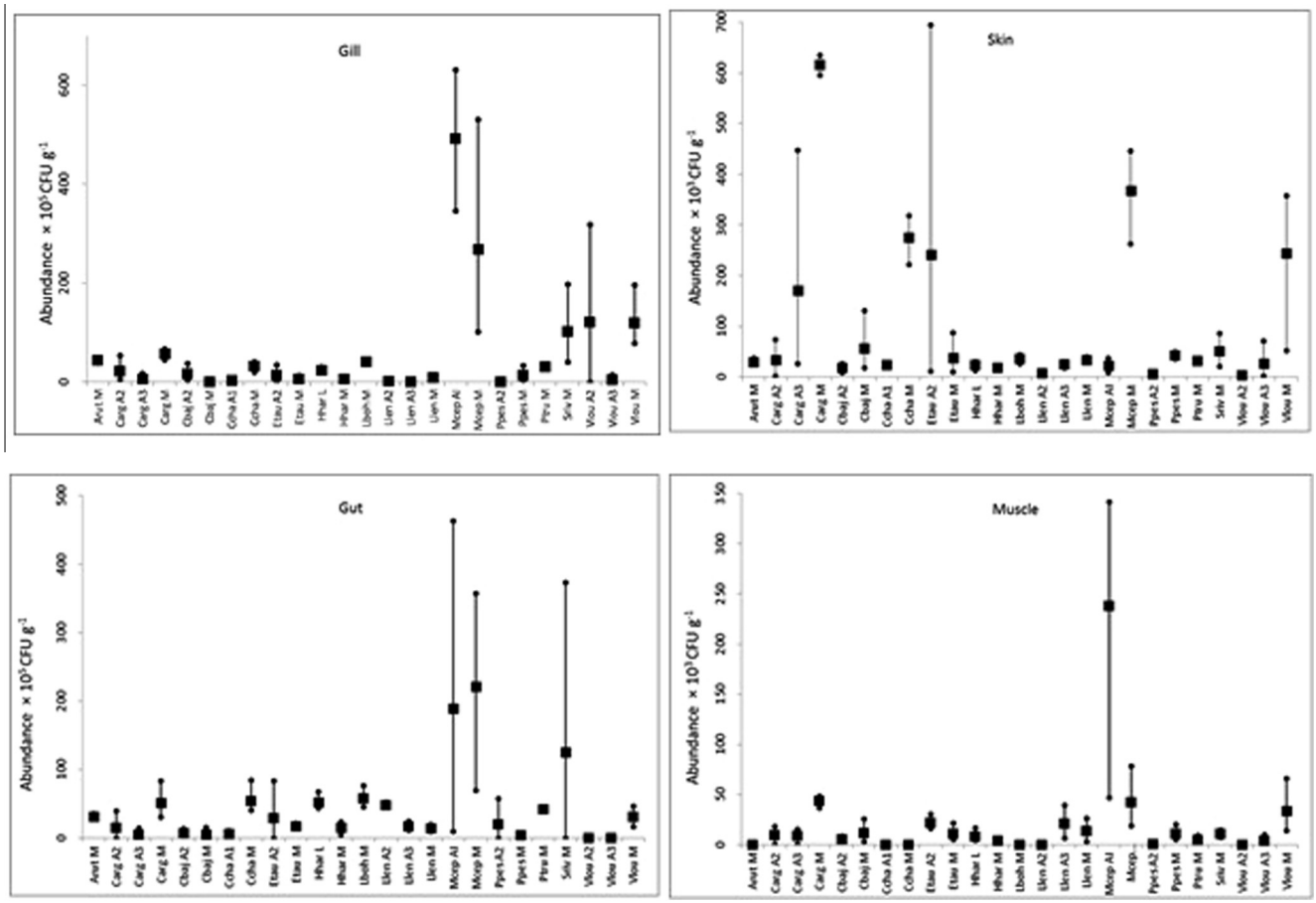

Figure 3 Bacterial counts on MacConkey (Mac) agar in gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of fish species from various sources in the Jeddah area. Data indicates mean and range of values; species is coded by genus initial and first three letters of specific name; source is coded by A1–A3, M, and L corresponding to Areas I–III, market, and landing site, respectively).

closely related bacterial species. The strains of *Aeromonas* sp. showed little differences in its branch length. However, the bootstrap values showed that the identified species were not very closely related.

4. Discussion

Investigations on bacterial loads in fish provide useful basis for assessing the level of contamination of coastal waters and the potential health risks to seafood consumers. Bacterial communities, however, are usually very complex in their association and thus very challenging for isolation. So far, only a small percentage of bacteria can be cultured under laboratory conditions and very few of them have been taxonomically identified. Although there is no single method to enumerate and estimate all bacterial population, culture-dependent heterotrophic bacterial population is conventionally estimated on solid media plates as pollution indicator in marine environment (Gonzalez and Moran, 1997; Pinhassi et al., 1997). Regardless of the paucity and methodological limitations in laboratory culture of bacteria, information on bacterial species that form colonies on media plates provide valuable insights into the prevalence of pathogenic microorganisms in test samples. Food contamination studies generally examine indicator bacterial groups, a method that is practically easy and cost-effective. Several indicator bacteria are not pathogenic themselves, but their abundance indicates potential risks of contamination or pollution.

This study quantified bacterial loads in seawater, sediment and 13 edible fish species collected from three different coastal water sites and the central fish market in the Jeddah area (Table 1). Bacterial counts in seawater and sediment from Area I, a semi-enclosed lagoon receiving discharges and surrounded by human activities, were significantly higher than the control site located farther from the city area. In general, the coastal sites had significantly more elevated bacterial abundances in seawater and sediment than the control site, which suggests anthropogenic impacts. These sites, especially Areas I and III, are located close to drainage outlets and sewage outfalls, which likely contribute to the high bacterial loads in the environmental media. In fact, a recent report cited severe bacterial contamination at coastal sites along the Jeddah coast due to untreated direct discharges to the sea (JEA, 2013). Bacterial load could be influenced by abiotic factors in the local environment (Madigan et al., 2000), which were not covered within the scope of this study. The lack of previous records constrains the evaluation of temporal trend in bacterial contamination at the examined coastal sites. A previous study at Al-Nawrus

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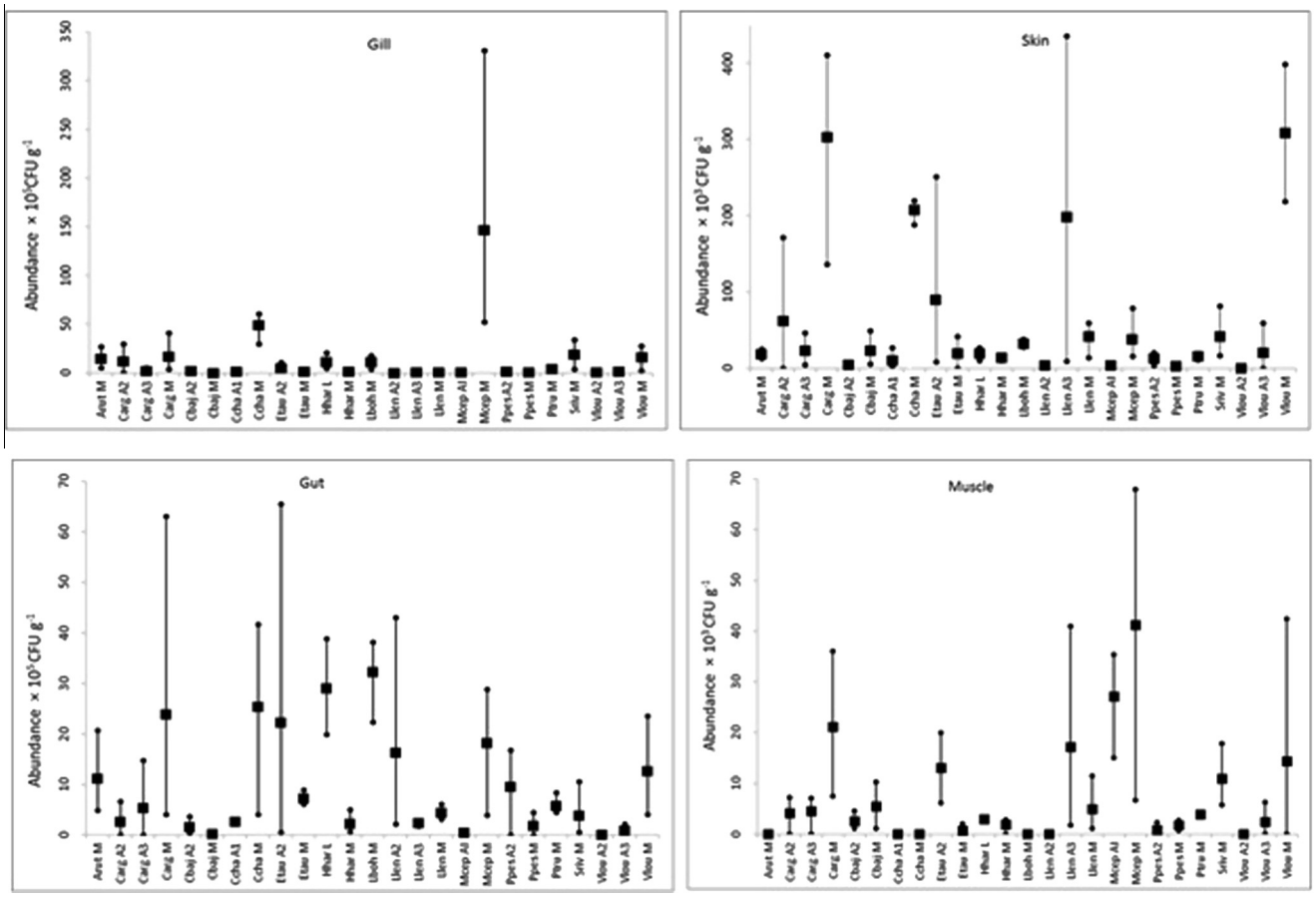

Figure 4 Bacterial counts on Eosin Methylene Blue (EMB) agar in gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of fishes from various sources in the Jeddah area. Data and sample codes are the same as in Fig. 3).

lagoon, located not far to the north of our study area, measured heterotrophic bacterial counts in seawater between 10^5–10^6 CFU mL^{-1} and total coliform counts of 10^2 CFU/100 mL (Turki and Mudarris, 2008), which are less than our present results. The findings of the previous study and with reference to the low bacterial loads at our control site located far from the Jeddah metropolitan area, our present study suggests increased bacterial contamination at coastal sites with greater concentration of human activities in the Jeddah area. Higher bacterial counts in sediment may be attributed to rich organic content and less stressors like sunlight and predation (Craig et al., 2002; Rodrigues et al., 2011; Rose et al., 2001; Rubentschik et al., 1936); however, the entry of pathogens may occur by way of the overlying seawater receiving animal and human wastes from drainage runoffs, infiltration and subsurface flows, sewage discharges and even episodic flood waters from inlands of the Jeddah region. Microbes entering the receiving water may sink to the sediments, which act as a potential reservoir for pathogens that could contaminate the resident fishes.

Our present results showed significant differences in bacterial counts between fish species. The tested agar media Mac, EMB and TCBS support the growth of total coliform, *Escherichia coli* and vibrio, respectively. Coliform and *E. coli* are associated with many chronic illnesses and often used as standard indicators of pollution in nearshore marine environments. Vibrios are dominant bacterial species in sewage discharges and have been linked with foodborne diseases, including pandemic diarrhea outbreaks (Austin, 2010). Total coliform, *E. coli* and vibrio in fish have been considered as indicator of bacterial contamination (Adebayo-Tayo et al., 2011; Akinyemi and Buoro, 2011; El-Zanfaljz and Ibrahim, 1982; Jha et al., 2010). We noted higher bacterial counts in *M. cephalus* (locally called *arabi*), especially from Area I, on TCBS (all body-parts), Mac (gills, muscle and gut) and EMB (gills and muscle). Interesting to note that a previous study from a lagoon in Lagos, Nigeria also showed higher bacterial counts in skin, buccal cavity and gills of *M. cephalus* than the other tested fishes (Akinyemi and Ajisafe, 2011). Fish may derive bacterial pathogens from seawater, sediment and food (Sugita et al., 1988); hence, the abundance of pathogens in fish is largely influenced by habitat conditions (Shewan and Hopps, 1967). In this study, the higher bacterial counts in fish from Area I coincided well with the high bacterial densities found in sediment and seawater at the same site (Fig. 6). The significant difference in bacterial counts for the skin samples on Mac and TCBS between sites indicates the likely effects of local environment. Bacterial counts on skin for all three isolation media were markedly correlated positively with the other body parts, which suggests that the surface microbial biota, probably due to conditions of the local environment, determines the overall quality of the fish. On the other hand, bacterial

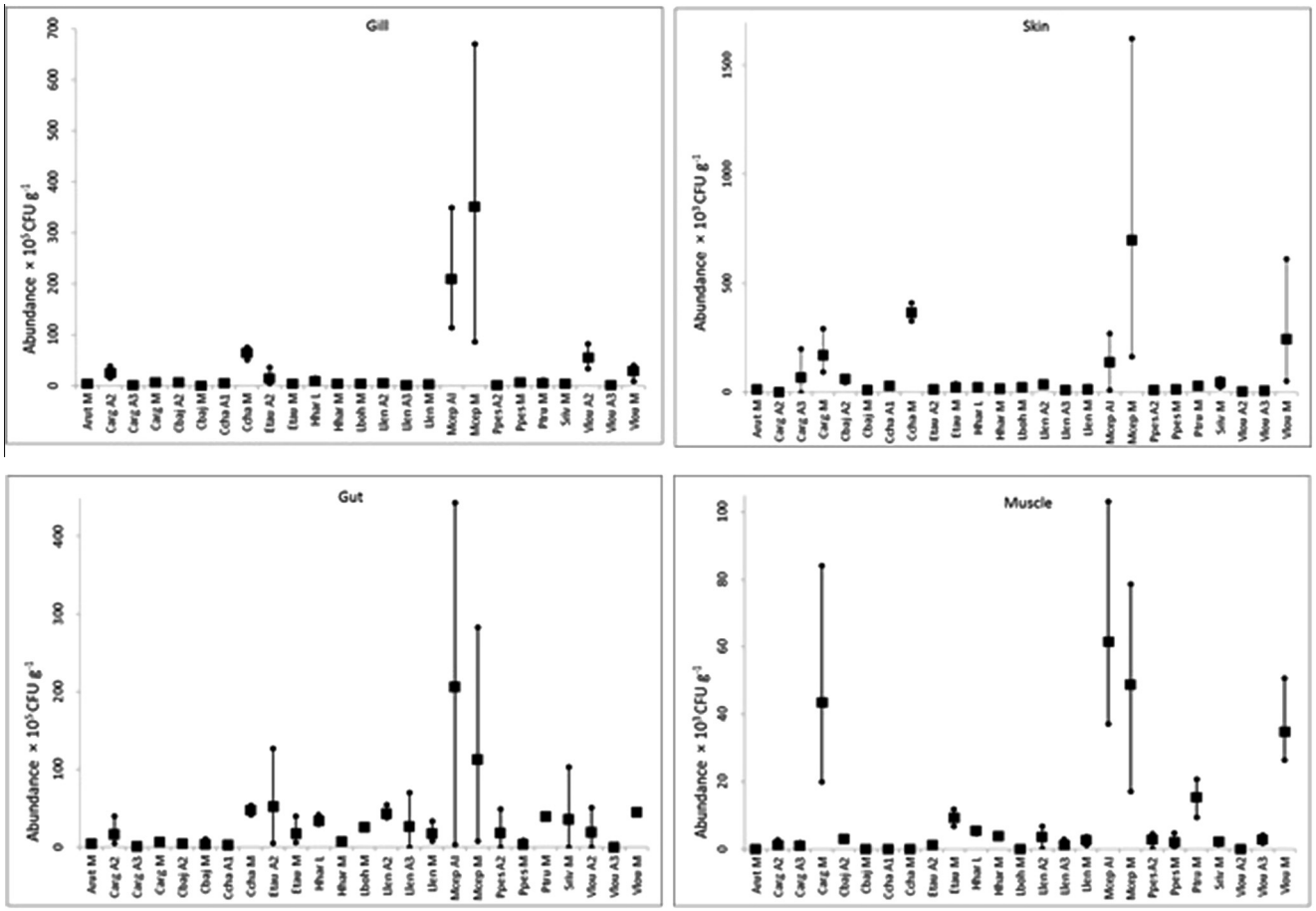

Figure 5 Bacterial counts on Thiosulfate-Citrate-Bile Salts (TCBS) agar in gills ($\times 10^5$ CFU g^{-1}), skin ($\times 10^3$ CFU g^{-1}), gut ($\times 10^5$ CFU g^{-1}), and muscles ($\times 10^3$ CFU g^{-1}) of fishes from various sampling sites. Data and sample codes are the same as in Fig. 3.

counts on TCBS and Mac for skin were higher in market than field samples, particularly for *C. argus*, *E. tauvia*, *M. cephalus* and *V. louti*. This points to potentially poor sanitary conditions at the market, including the quality of stalls, storage process and fish handling practices. Foodborne diseases are generally associated with poor hygiene practices and cross-contamination from equipment and food handlers during processing and storage. In addition, the bacterial loads of aquatic animals tend to multiply after death (Faghri et al., 1984). The examined fish samples from the market were obtained after several hours from capture; hence, such a delay could have induced substantial bacterial growth although fishes are commonly chilled with ice from the sea until reaching the market.

In general, we found that the detritivorous fish, which normally feed by ingesting organic matter along with sediment particles, have higher bacterial counts than the herbivorous or carnivorous species. Higher total coliforms counts in detritivorous fish have been noted in freshwater species (Rahman et al., 2010). The abundance of bacteria in gut is crucial as intestinal microbes are major causative agents of seafood spoilage (Kaneko, 1971). Our results for guts on Mac and TCBS revealed significant differences between fishes grouped by feeding habit, being higher in detritivores. This suggests that the intestinal bacterial loads in the examined fishes were due to their feeding habit, although a more definitive investigation is required to clarify such an assertion.

The dominant bacteria identified from the fish muscles (edible flesh) included well established primary pathogens, opportunistic pathogens, and also non-pathogenic species. The isolated species belong to the families Vibrionaceae, Enterobacteriaceae, Aeromonadaceae, Moraxellaceae, Pseudomonadaceae and Pseudoalteromonadaceae. Vibrionaceae was represented by *Photobacterium damselae* and *Vibrio harveyi*. The species *P. damselae* (formerly *Vibrio damsela*) is a dominant halophilic bacterium that is abundant in marine environments and capable of causing infections and fatal diseases in marine animals and humans. It is an established human pathogen with most reported infections through wounds inflicted during fish handling, exposure to seawater and marine animals, and ingestion of raw seafood (Asato and Kanaya, 2004; Morris et al., 1982;). In some cases, the infection leads to death (Yamane et al., 2004). *V. harveyi* is a free-swimming bacterium in tropical marine waters and known as dominant in gut microflora (Austin and Zhang, 2006). This species is categorized as a primary or opportunistic pathogen.

Enterobacteriaceae was represented by *Rahnella aquatilis* and *Hafnia* sp. The species *R. aquatilis*, recognized in 1976–1979 (Gavini et al., 1976; Izard et al., 1979), was isolated from open

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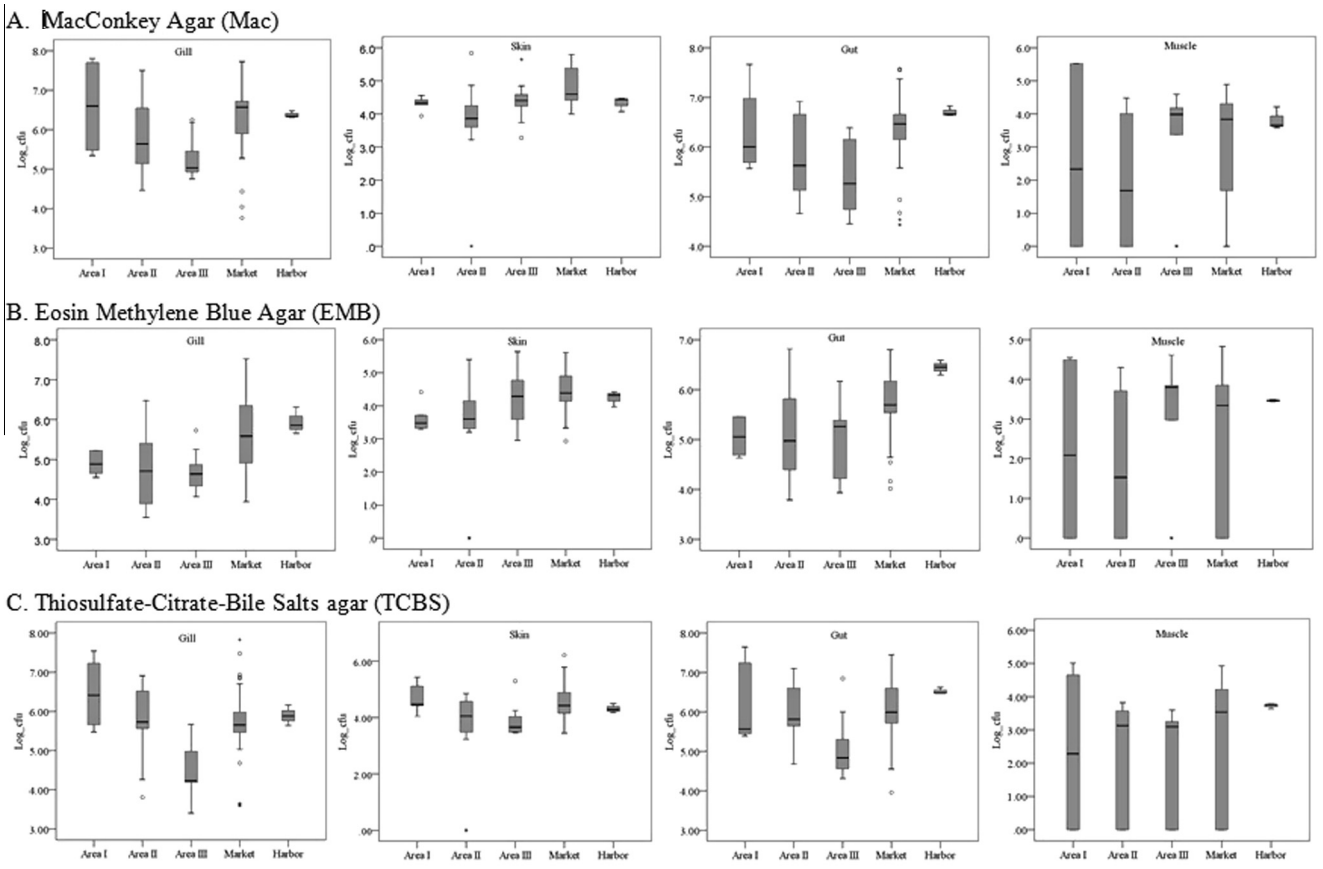

Figure 6 Bacterial counts on Mac, EMB and TCBS agar from body parts of fish species grouped by sample source.

water reservoirs, soil, clinical samples and food materials (Gras et al., 1994; Farmer et al., 1985; Funke and Rosner, 1995; Lindberg et al., 1998). They are opportunistic pathogens causing a wide spectrum of life-threatening infections in humans, mainly affecting the gastrointestinal tract, urinary tract, respiratory organs and cardiovascular system (Zdorovenko et al., 2004). Besides being highly pathogenic, *R. aquatilis* are naturally resistant to many commercial antibiotics (Stock et al., 2000). *Hafnia alvei* is the only species so far known under the genus *Hafnia*. Although, our sequence data does not confirm assignment to this species, the genus *Hafnia* is not normally pathogenic to humans. It has received attention from medical community due to its commensalism with human gastrointestinal tract and its resistance to antibiotics. *H. alvei* was originally isolated and identified at the International Centre for Diarrhoeal Disease Research, Bangladesh (Albert et al., 1991). Its strains have been validated by 16S ribosomal DNA sequencing and DNA–DNA pairing studies to be of *Escherichia*. A new species, *Escherichia albertii*, has been proposed instead of *H. alvei* (Huys et al., 2003). The role of this species as a gastroenteritis and diarrheal disease causing agent is yet to be confirmed.

Moraxellaceae had two morphologically different strains (NA03 and NA05) of *Psychrobacter faecalis*, although not associated with any foodborne illness. It is a relatively new species, first identified from bioaerosol generated by cleaning of room contaminated with pigeon faeces using MacConkey agar at a 36 °C (Kämpfer et al., 2002). This study also used the same inoculation media and incubation at almost the same temperature. Although *P. faecalis* is not reported from fish, the genus *Psychrobacter* has been isolated from the gills and skin of fish, as well as poultry, food and clinical sources (Juni and Heym, 1986).

The identified species of Aeromonadaceae includes *Aeromonas* sp. and *A. salmonicida*. Species of the genus *Aeromonas* are ubiquitous in aquatic environments and have been isolated from patients with gastroenteritis (Altwegg and Geiss, 1989; Joseph, 1996), thus indicating virulence in causing foodborne illness with the capability of producing extracellular toxins and enzymes (Gosling, 1996; Howard et al., 1996). Enterotoxin-producing *Aeromonas* spp. have shown significant correlations with diarrhea patients (Bloom and Bottone, 1990; Joseph, 1996). Although several species of *Aeromonas* are known pathogens, the species *A. salmonicida* has not been associated with human infection (USEPA, 2006) but it is a known fish pathogen.

Pseudomonadaceae was represented by *Pseudomonas stutzeri*, which is rarely found in humans but known to cause illness. Studies on cases of *P. stutzeri* associated with human illness were suspected to be mostly hospital-acquired infections during surgical procedures and/or due to a compromised immunity condition (Reisler and Blumberg, 1999). Hence, *P. stutzeri* is an opportunistic pathogen of low virulence and not associated with foodborne illness. Pseudoalteromonadaceae is represented by *Pseudoalteromonas* sp., formerly classified under the genus Alteromonas, is common in the

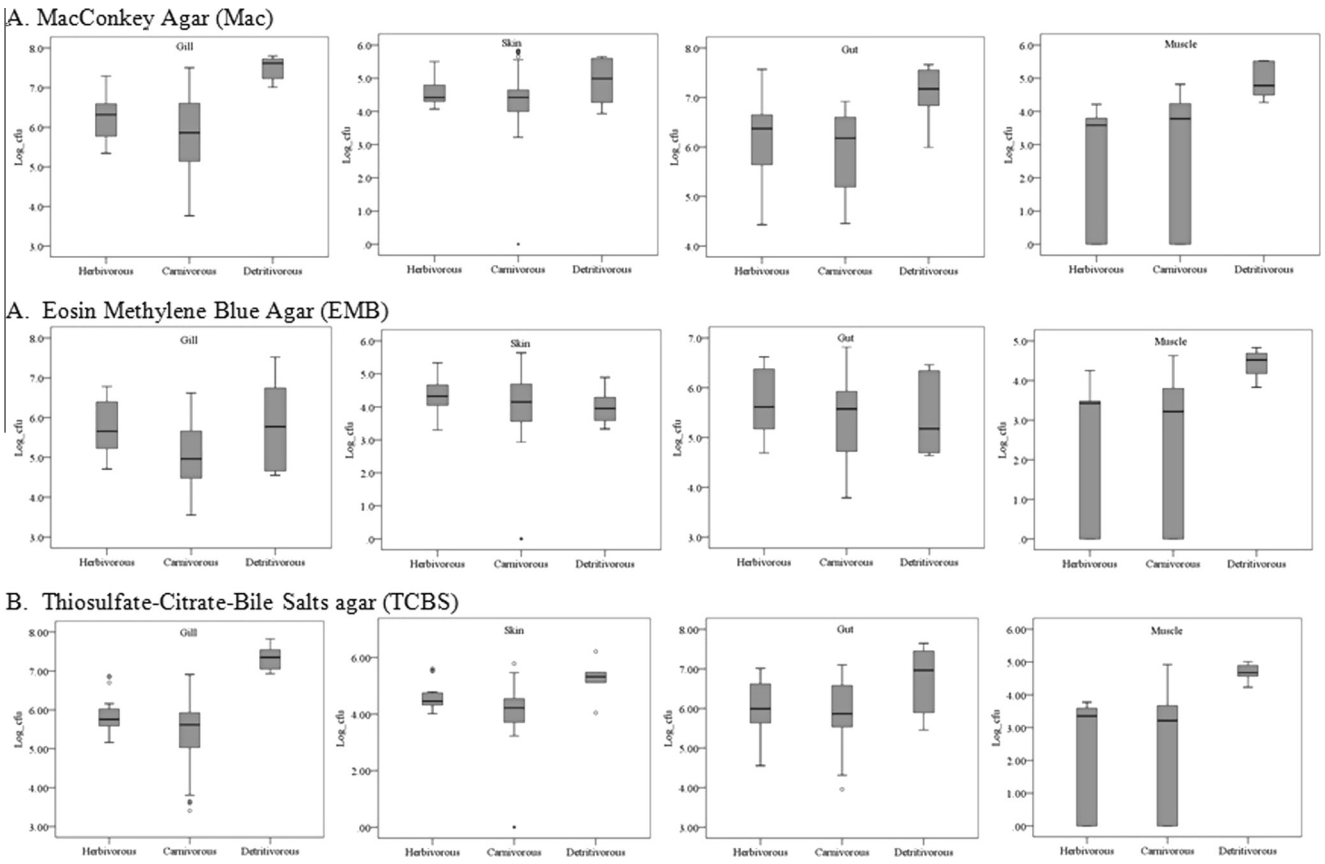

Figure 7 Bacterial counts on Mac, EMB and TCBS agar exhibited by various body parts of fish species grouped based on feeding behavior.

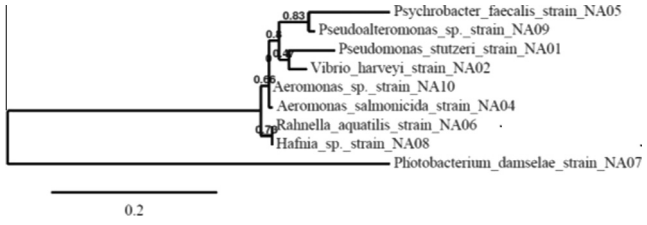

Figure 8 Phylogenetic tree constructed for the identified bacterial species isolated from muscles of fish species from the Jeddah area. Bar represents 2% sequence divergence.

marine environment in association with eukaryotic hosts (Gauthier et al., 1995) and is capable of producing the lethal tetrodotoxin (Simidu et al., 1990). However, no species of *Pseudoalteromonas* is currently known as human pathogen.

5. Summary

Bacterial contamination in commercial marine fishes from the Jeddah area was investigated by culture-dependent method and gene sequencing of isolates for taxonomic identification. Results revealed significant differences in bacterial loads between species, sources, and feeding habits of the examined fishes. The accepted limit of aerobic bacterial counts for fresh fish is between 5×10^5 and 5×10^7 CFU g^{-1} and that of *E. coli* between 1×10^1 and 5×10^2 CFU g^{-1} (ICMSF, 1986). Saudi Arabia Standards Organization (SASO) prescribes the maximum limit for bacterial load in meat products (No. 1556; SASO, 1998) at 10^6 CFU g^{-1}, and *E. coli* counts should be less than 10^2 CFU g^{-1}. This study observed higher bacterial counts than these limits in some fish species. 16S rRNA sequences identified pathogenic bacteria from *Vibrionaceae* and *Enterobacteriaceae* in the tested fish samples. Species such as *R. aquatilis* and *P. damselae*, are known human pathogens capable of causing foodborne illness with severe antibiotic resistance. *Opportunistic pathogens* such as *Hafnia* sp. (Enterobacteriaceae) and *Pseudomonas stutzeri* (Pseudomonadaceae) were also identified. The existence of these bacterial species in fish muscle samples are of concern. The present results indicate the potential risks of bacterial contamination of the fishes from the Jeddah area. Periodic monitoring, preferably including both microbial and chemical contaminants of concern, is thus important to keep track of any potential health risks to fish consumers.

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this paper are solely those of the authors and do not represent those of KAUST and the funding agency.

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