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Food-borne bacterial load in fresh and frozen fish sold in Mauritius

Pohoroo, A and *Ranghoo-Sanmukhiya, V.M.

Faculty of Agriculture, The University of Mauritius, Reduit, Mauritius

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Abstract

Microbial load of fresh *Lethrinus nebulosus* (locally known as Capitaine) and *Siganus sutor* (locally known as Cordonnier) was compared during summer and winter in Mauritius. Fresh fish samples were collected from different regions of Mauritius during summer and winter. The bacterial load of the fresh fish was found to be higher (1.2x 10^5CFU/g) during the hot season ($p < 0.05$). Significant differences in staphylococcal count were also observed among the different fish species ($p < 0.05$) though the values abided by the limits. Furthermore, frozen *L. nebulosus* showed a significant difference in the total bacterial load by 100 fold and 10 fold for staphylococcal count compared to the fresh samples of the same fish species. Emphasis was also placed on the isolation of the probable presence of the main food pathogens encountered on fish namely *Enterococcus* and *Vibrio* species. 16S ribosomal DNA Sequencing revealed the presence of *Vibrio alginolyticus* and *Enterococcus cloacae.* Improper handling practices by the fish mongers and sellers/vendors and inadequate storage temperature affected the bacterial load of fresh fish. Both fresh and frozen fish in Mauritius have an acceptable total microbial load and can be assumed to be safe for consumption.

Introduction

Mauritius, being an island surrounded by the sea, has developed the fishery sector into a well-organized "seafood hub" to better exploit its resources and lower its economic stress. The Ministry of Fisheries estimated that the turnover of the Seafood Hub increased by 2% as compared to 2012 and estimated to Rs 12 billion in 2013 (Business Mega, 2013). Furthermore, the average annual fish consumption per individual in Mauritius was found to be approximately 20 kg which is higher than that of the world mean being 16.3 kg (HRDC, 2007; Business Mega, 2012). Fish consumed in Mauritius can be categorized as fresh, frozen or canned and the sale of these products is mostly based on its availability, quality and price. Costal people tend to buy fresh fish since it is easily available and is believed to have higher nutritive value than the frozen and the canned fish. The microbiological safety of the fish is however ignored since fresh fish sold in the coastal regions by local fishermen, or middlemen rarely adopt proper storage and handling practices. Worldwide, 43% of food-borne illnesses reported were assumed to be related to storage of seafood products at room temperature which is the optimum temperature for the pathogens (Forsythe, 2010).

Freezing and thawing the fish to be sold as fresh is a common practice by fish merchants which is harmful for the consumer. Since fish is highly nutritious, it renders the latter highly perishable and spoilage-prone and in addition varying temperature could favor the growth of microorganisms affecting the gastrointestinal tract. Microorganisms such as *Clostridium perfringens, Clostridium botulinum, Vibrio cholerae, Vibrio parahaemolyticus, Salmonella* species, *Escherichia coli, Enterococcus* species and *Staphyloccoccus aureus* can be most commonly isolated from fresh seafood (Novotny *et al.*, 2004; Eze *et al*., 2010; Adebayo-Tayo *et al*., 2012). The major symptoms caused by the pathogens are vomiting, fever, nausea, headache and diarrhea which can also be bloody (Forsythe, 2010). In Mauritius, cases of food-borne illness have been observed to have increased since 1990 with 445 cases reported in 2011 (Gaungoo and Jeewon, 2013). Analysis of frozen Mackerel fish in Nigeria had a microbial load of 1.135x10^6 CFU/g which was beyond the acceptable limits (5.0 x10^5 CFU/g) (Eze *et al*., 2011). A comparison between the microbial load of fresh and frozen fish samples in Croatia revealed a count greater than 10^5 for the fresh samples and a count within the range of 10^2 to 10^3 for the frozen fish (Topic *et al.*, 2010). An aerobic plate count ranging from 10^5 to 10^7CFU/g has been considered as acceptable in fresh and frozen fish by the International Commission on Microbiological Specifications for Foods (ICMSF, 1986). Another important factor that could play a role in the incidence

*Corresponding author.
Email: *m.sanmukhiya@uom.ac.mu*

of the microorganisms would be the weather or more specifically the temperature and humidity. A surge of 8% in diarrhoeal cases associated with food was reported during the hot season in Peru (Tirado *et al*., 2010).

Therefore, the aim of this study was to investigate the microbial load and quality of fresh and frozen *Lethrinus nebulosus* (common name: Capitaine) and *Siganus sutor* (common name: Cordonnier) which were acquired from different regions during the summer (November) and winter (August) season in Mauritius. Moreover the precise identification of the bacterial contaminants was also carried out using biochemical and molecular methods.

Materials and Methods

Sample collection

A sample of three fresh fish samples with weight ranging from 400 g to 600 g (both Capitaine and Cordonnier) were twice collected from the Northern, Eastern, Southern and Western-Central regions of Mauritius during the summer and winter seasons (2014/2015). Five frozen fish samples (only Capitaine) were taken from the Western-Central region. The samples were collected and transported to the laboratory in sterile plastic bags within a polystyrene box filled with ice packs to keep the fish at low temperature and minimize the risk of contamination.

Isolation and enumeration

25 g of each fish was aseptically homogenized in 225 ml of buffered peptone water (BPW) using a stomacher for 1 minute and serial dilution was carried out using 0.1% BPW (1:10 dilution) and inoculated on duplicate plates of the appropriate agar for enumeration of Staphylococcus aureus and total viable count.

Total viable count

The diluted samples were inoculated on Plate Count agar and incubated at 30°C for 72 ± 2 hrs. The colony forming units per gram for each sample was then calculated using the following formula according to ISO 7218 then the data was analyzed using the Kruskal Wallis test and presented using Minitab 17:

$$CFUs/g = c / (d * v [N1 + (0.1* N2)])$$

c = Sum of colonies of two successive dilutions (duplicate)

d = 1st dilution factor

v = Volume of inoculum

N1 and N2 = No. of plates for 1st and 2nd dilution factors respectively

Isolation of suspected Enterococcus *species*

1 ml of homogenized sample was streaked on blood agar plates which were incubated for 24 ± 2 hrs at 37°C in anaerobic jars using AnaeroGen sachets. The suspected colonies were subjected to biochemical tests such as gram stain, glucose and lactose fermentation tests.

Isolation of suspected Vibrio *species*

25 g of sample were homogenized in 225 ml alkaline peptone water (APW) and were incubated at 37°C for 8 ± 2 hrs followed by inoculation on thiosulfate citrate bile salt sucrose agar. The plates were then incubated for 24 hrs at 37°C and suspected *Vibrio* colonies were retained for biochemical tests namely gram stain, oxidase, Methyl red-Vogues Proskauer, Triple Sugar Iron test and lysine decarboxylase test and grown on HiCrome™ Vibrio agar for cultural confirmation.

Isolation of suspected Staphylococcus aureus

The selected dilution factors were inoculated on Baird Parker agar supplemented with egg tellurite emulsion and incubated for 24 ± 2 hrs at 37°C. Suspected *Staphylococcus* colonies were then subjected to gram stain, catalase, mannitol fermentation and coagulase test.

Identification of bacterial strains using the PCR technique

DNA extraction was carried out as specified by Cheng and Jiang (2006). The obtained genomic DNA was amplified by PCR with specific markers of each microorganism. The PCR reactions were carried out using 25µl of PCR mix containing 1X DreamTaq buffer, 2.5mM dNTPs, 2µl of forward and reverse primers, 1U Taq DNA polymerase and 2 µl of template DNA. The amplification was done using the Applied Biosystems 2720 thermal cycler with the specific cycling parameters for each pair of primers (Table 1) and was loaded together with the O'GeneRulerTM 1kb Plus DNA ladder on a 1.5% agarose gel for 2 hrs. The gel was immersed in ethidium bromide for 5 minutes and then washed for few seconds in water. Then, the gel was placed on the UV trans-illuminator following which the resulting data was obtained. Identity of the suspected and biochemically similar microorganisms was confirmed by sequencing the 16S ribosomal DNA whereby the forward and reverse sequences were used to make a consensus

Table 1. List of primers and the cycling parameters

Microorganisms /No.of cycles	Primer Sequence	Cycling Parameters
	Coa-FP	94 °C- 5 mins
S.aureus	5'ATAGAGATGCTGTACAGG3'	94 °C-1 min
(30 cycles)		56 °C-30 sec
	Coa-RP	72 °C - 1 min
	3'GCTTCCGATTGTTCGATGC5'	72 °C- 4min
	16S -F	95 °C- 3 mins
16SrRNA	AGTTTGATCATGGCTCAG	95 °C- 1 min
(25 cycles)		52 °C- 20
	16S -R	secs
	TTACCGCGGCTGGCA	72 °C- 1 min
		72 °C- 5 mins

sequence using BioEdit. The consensus sequences were subjected to a BLAST search using BLASTN for a comparative analysis with the National Center for Biotechnology Information (NCBI) database.

Results

Total viable count

Significant variation can be seen between both seasons ($p < 0.05$) with the highest mean, 985.46 x10^4 CFU/g occurring during summer and 279.61 x10^4 CFU/g during winter. A relatively high mean, 1423.64 x10^4 CFU/g can be observed for the central Cordonier sample during summer and a low mean, 138.05 x10^4 CFU/g was obtained for eastern samples during same season. *Capitaine* and *Cordonier* species did not bear any difference concerning the TVC values ($p > 0.05$) (Figure 1). Furthermore, a low TVC value of 5.0x 10^4 was recorded for the frozen *Capitaine* species as compared to a value of 6.0x10^6 CFU/g in the fresh samples.

Identification of Staphylococcus aureus

Shiny black, round colonies with smooth margin and surrounded by a clear zone were selected and subcultured from Baird Parker medium supplemented with egg tellurite emulsion for both fish species. Gram stain revealed gram-positive cocci, arranged in grapelike clusters. The fresh *Staphylococcus* cultures were found to be catalase positive, mannitol fermenters and only the culture for the northern *Cordonnier* samples during the winter and eastern *Cordonnier* during summer gave positive for the coagulase test.

Furthermore, a significant difference can be seen while comparing the seasons ($p < 0.05$) with the highest mean being 123.32 x10^3 CFU/g for

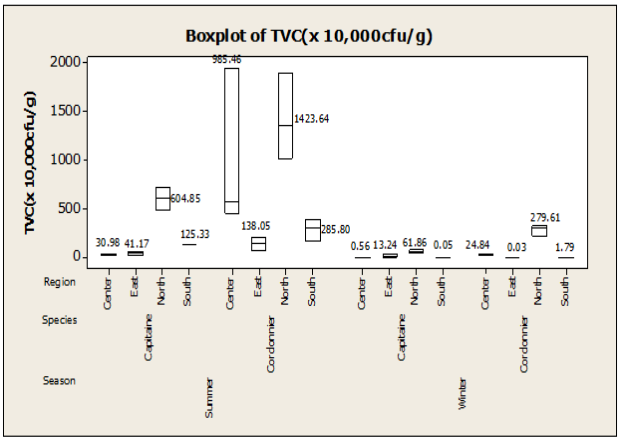

Figure 1. Boxplot of TVC (x 10, 000 CFU/g) during winter and summer

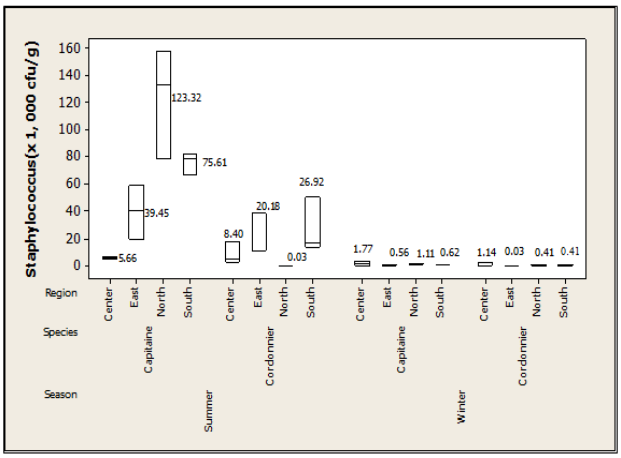

Figure 2. Boxplot of Staphylococcal (x 1,000 CFU/g) count during winter and summer

Capitaine during summer. Species-wise ($p < 0.05$), a higher count can be observed for *Capitaine* in both seasons (123.32 x10^3 CFU/g in summer and 1.77 x10^3 CFU/g in winter) as compared to *Cordonnier* (26.92 x10^3 CFU/g in summer and 1.14 x10^3 CFU/g in winter). On the contrary, no significant difference was found for different regions ($p > 0.05$) (Figure 2). The fresh Capitaine had 10 times more colony forming units (1.3x10^5) as compared to the frozen *Capitaine* (1.3x10^4). A band of approximately 600 bp was obtained from the PCR amplification of the coagulase gene of *Staphylococcus aureus* isolated from eastern *Cordonnier* during the hot season confirming the identity of the strain as reported by (Iyer and Kumosani, 2010).

Identification of suspected Enterococcus *and* Vibrio *species using 16S rRNA*

The northern, eastern *Capitaine* samples collected during winter and northern *Capitaine* samples collected during summer suspected to belong to Enterococcus were sent for sequencing and the 16S rRNA analysis showed 99% query coverage

for *Enterococcus casseliflavus* strain ATCC 25788 (accession no.: KC510229.1) and 97% coverage with *Enterococcus casseliflavus* isolate F00440 (accession no.: DQ343839.1) for the winter samples. The summer sample gave a 99% homology and 93% query coverage with *Enterobacter cloacae* strain SP30 (accession no.: JX317636.1) and *Enterobacter hormaechei* strain R11 (accession no.: KF843700.1).

BlASTN results for the eastern *Capitaine* species for summer season revealed that the isolate was related to the family genera *Vibrio*. The sample from *Capitaine* gave a percentage homology of 98% for *V. parahaemolyticus* (accession no.: CP007004.1) and *V. alginolyticus* (accession no.: CP006718.1).

Discussion

Total microbial count

The total viable or aerobic plate counts were highest during the hot season. The highest mean recorded was 1.4 x 10^7 CFU/g for the northern *Cordonnier* and the lowest value recorded was 3.1 x10^5 CFU/g for the central *Capitaine* species. The total viable count for the winter ranged from 10^2 to 10^5 CFU/g though the northern *Cordonnier* samples gave a high mean of 2.8x10^6 CFU/g. The Kruskal Wallis test revealed a p-value of 0.00 for both staphylococcal and total viable count and being less than 0.05, it indicates a significant variation between the staphylococcal count in the fish during summer and winter. Increase of microbial load during summer had also been reported by the USDA (2013). A high heterotrophic count, 6.3x 10^5 CFU/g was also obtained from Mackerel fish (Adebayo-tayo *et al*., 2012). Study involving microbial analysis of raw oysters also showed an increase in the total bacterial count during summer (Prapaiwong *et al*., 2009).

A significant level of *S. aureus* was found in the fresh *Capitaine* and *Cordonnier* samples during summer with the highest staphylococcal mean being 1.2x 10^5 CFU/g for *Capitaine* from the northern region. The other *Capitaine* and *Cordonnier* summer samples showed an incidence level of 10^4 CFU/g. All the winter samples had an incidence level of 10^3 CFU/g henceforth, it can be reported that the incidence of *Staphylococcus* in both fish species increased to a hazardous level during summer. The staphylococcal count obtained was more or less within the acceptable range established by ICMSF and USFDA however, it would be completely rejected by the Eight Schedule (regulation 62 (2) (b)) of the International Commission on Microbiological Specifications for Foods (ICMSF, 2011). A clear difference between the two seasons can be seen as in Figure 2.

Species-wise comparison showed higher values of *Staphylococcus* obtained for *Capitaine* in both seasons; 1.2 x10^5 CFU/g in summer and 1.77 x10^3 CFU/g in winter whereas *Cordonnier* gave highest values 2.7 x10^4 CFU/g in summer and 1.14 x10^3 CFU/g in winter. The statistical analysis revealed that there was a significant difference between the incidence level of *Staphylococcus* in both species (p-value < 0.05). The variation could be due to the physico-chemical properties of the fish. Fish have a high water activity of 1.00 to 0.95 and a pH range of 6.6 to 6.8 however variation in the fish chemical composition could easily affect those factors. *S. aureus* requires a substratum with a pH range of 4 to 10 and water activity of 0.830 to grow (Argudin *et al*., 2010; Forsythe, 2010; FAO, 2014). It can be assumed that the two fish species have varying physico-chemical properties that may play a role in the incidence of *S. aureus*. On the other hand, the total viable count bear no differences between the fish species (p-value > 0.05). Despite that approximately 100% of the selling points were displaying and assorting the fish by species, there could have been a possibility that no significant difference was obtained because the fish were transported in a single container.

There was no noticeable difference that was obtained when the staphylococcal count was analyzed region-wise (p-value > 0.05) unlike for the TVC which had a p-value less than 0.05. For example, there was a count of 1.4 x10^7 CFU/g in the summer *Cordonnier* sample and 1.4 x10^6 CFU/g in the summer southern Cordonnier sample. According to the checklist results, 20 to 40% of the selling points allowed their customers to handle to fish by themselves thus increasing the chances of contamination. One important factor that could easily favor the growth of pathogens would be storage conditions of the fresh fish. It could be possible that ice used to keep the fish chilled or the surfaces where the fish are kept or the weighing balance were contaminated and improper cleaning could lead to high bacterial counts. Observational study at the selling points allowed conclusively that there was no proper cold temperature condition for the storage of fish in 60% of the selling points during both seasons. In addition to this, 60% and 40% of the selling points were handled by a single person thus; the individual had to handle the cash and fish products at the same time. This may have played a major part in the high bacterial incidence and variation between the different regions. An important aspect contributing to the variation could be cleanliness of the fishing vessels, storage facilities within the fishing vessels,

loading and unloading of the fish at the selling points and storage temperature during land transportation (Chen and Chang, 2004).

Comparison between the fresh and frozen *Capitaine* indicated a higher staphylococcal count and TVC value in the fresh fish being 1.3x105 CFU/g and $6.0x10^6$ CFU/g respectively. The counts for frozen fish were $1.3x10^4$ CFU/g for *Staphylococcus* and $5.0x10^4$ CFU/g for TVC. The incidence level of frozen sample fell within the acceptable limits specified by ICMSF and USFDA. Chang *et al.* (2008) studied the frozen swordfish fillets and stated an aerobic plate count of 10^5 to 10^6 CFU/g in all the samples. Another work on frozen Mackerel fish gave a bacterial load of $1.135x10^6$ CFU/g which was considered as unacceptable by Nigerian National Agency for Drug Administration and Control (Eze *et al*., 2011). The low level of bacterial count found in the frozen fish in this study is mainly due to the storage temperature which tends to inactivate the enzymes of the bacteria. Therefore, they are unable to maintain a proper cell activity and division.

Identification of Staphylococcus aureus

Most of the fresh *Capitaine* and *Cordonnier* samples from the North, East, Center and South and frozen *Capitaine* tested positively for *Staphylococcus* species during the summer and winter season. All shiny black colonies with a clear zone in Baird Parker medium were assumed to *Staphylococcus aureus.* The clear zone occurs when the lecithin in the egg yolk emulsion is digested by the enzyme lecithinase and the black coloration is due to reduction of tellurite (Bennett and Lancette, 2001; Corry *et al.*, 2003; Tavakoli *et al.*, 2008; Ezzeldeen *et al*., 2011; Vazquez-Sanchez *et al.,* 2012). All isolates were identified as Staphylococcus species as per the biochemical characteristics giving gram-positive cocci clustered in grapelike structures, being able to ferment mannitol and being catalase-positive (Tavakoli *et al*., 2008; Ezzeldeen *et al.*, 2011; Rohinishree and Negi, 2011). Around 100% and 97.30% of the isolates from Baird Parker medium were catalase-positive and mannitol fermenter however, only 65.5% was coagulase-positive thus was confirmed to be *S. aureus* (Ezzeldeen *et al*., 2011). Another study showed that only 17.17% out of 728 isolates from Baird Parker were confirmed to be *S. aureus* (Vazquez-Sanchez *et al.,* 2012). In this work, only 4.17% of the isolates were confirmed to be *S. aureus* through biochemical properties. Confirmation of the strain was carried out using PCR amplification. An amplicon of size 500-600 bp was obtained for the eastern *Cordonnier* sample during summer and as mentioned by Iyer and Kumosani, (2010), the primers coa-FP and coa-RP targeting the coagulase gene in *Staphylococcus aureus* would give an amplicon of size 500 - 600 base pairs, implying that the isolate was *S. aureus*.

Identification of Enterococcus *species*

The isolate from the northern *Capitaine* during summer was the only one that gave gram-positive bacilli upon observation under the microscope. The microorganism was observed to be gram-positive coccus-shaped bacteria. Further biochemical tests revealed that the isolate was a lactose and glucose fermenter and gave white colonies with gamma-hemolysis on sheep blood agar. Sequencing data of the isolated gave a 99% and 93% query coverage similarity with *Enterobacter cloacae* strain SP30 (accession no.: JX317636.1) and *Enterobacter hormaechei* strain R11 (accession no.: KF843700.1). The microorganisms are part of the enteric bacteria and considered as important nosocomial pathogens. The microorganism is most likely to be *E. cloacae* since the latter has been most frequently isolated from fish product. For example, it was identified from *Oreo chromisniloticus, Mugilcapito, Dicentrachus labrax* and Stingray fish fillets (Hassan *et al*., 2012; Ghanem *et al.*, 2014).

The northern, eastern *Capitaine* samples during winter were also sent for sequencing and the results revealed a 99% query coverage for *Enterococcus casseliflavus* strain ATCC 25788 (accession no.: KC510229.1) and 97% coverage with *Enterococcus casseliflavus* isolate F00440 (accession no.: DQ343839.1). Both isolates gave the same results implying that they are the same microorganism. The data obtained corresponded to the gram stain that revealed facultative anaerobic, gram-positive cocco-bacilli/cocci (Corry *et al*., 2003). The isolate was most likely to be *E. casseliflavus* strain ATCC 25788 with a 99% of query coverage. However, the bacterium is rarely isolated from fish and since it is known to be an enteric bacterium responsible for urinary tract infection or abdominal infection, there was a high probability that those samples were contaminated by improper handling of the fish by either the sellers or the customers (Moore *et al*., 2006; Trivedi *et al.*, 2011; Fraser *et al*., 2012).

Identification of suspected Vibrio *species*

Yellow round colonies on TCBS agar were suspected to be *V. cholerae.* However, other *Vibrio* species such as *V. alginolyticus, V. furnissi, V.metschnikovii* among others also give yellow colonies on TCBS agar being sucrose fermenter. The greenish colonies can be represented by *V.*

parahaemolyticus that is frequently isolated from marine samples (Corry *et al.*, 2003; Thompson *et al*., 2005; Forsythe, 2010; Hassan *et al.*, 2012). All the *Vibrio* species are oxidase positive except for *V. metschnikovii* and this helps to differentiate them from Enterobacteriaceae (Nicholas and Alireza, 2000; Corry *et al*., 2003; Jayasinghe *et al*., 2008; CDC, 2012). The yellow and green colonies were present in most of the samples except for the sourthern *Capitaine* and *Cordonnier* samples during summer, central Siganus sutor samples during winter and the frozen *Capitaine* and all the selected yellow isolates were oxidase positive thus giving the first evidence of being *Vibrio* species. *Vibrio* species are known to enter the viable but not culturable state when exposed to cold, salinity and nutrient deprivation therefore making it difficult to culture them on agar medium (Wong and Wang, 2004; Oliver, 2005) which can explain the absence in the frozen samples.

Gram-negative cocco-bacilli and comma-shaped rods were observed under the microscope. The cocco-bacillary shape can be explained by the VBNC state whereby the microorganisms tend to undergo morphological changes (dwarfing) due to stress for survival purposes which may be either due to cold due transportation or nutrient depletion in plates (Oliver, 2005;2009). The isolates from eastern *Capitaine* species during summer and the southern *Siganus sutor* during winter were considered to be *V. cholerae, V. parahaemolyticus* or *V. alginolyticus* based on the biochemical results obtained. Both species were isolated from freshwater prawn *(Macrobrachium rosenbergii)* hatcheries (Hoa *et al*., 2000). The two samples suspected to be *V. cholerae, V. parahaemolyticus* or *V. alginolyticus* after the biochemical tests were sent for sequencing and accordingly the isolate from eastern summer *Capitaine* species was found to bear a 98% homology with *V. parahaemolyticus* (accession no.: CP007004.1) and *V. alginolyticus* (accession no.: CP006718.1). However, considering the biochemical properties of the isolate, the microorganism was most likely to be *V. alginolyticus* being VP-positive as *V. cholerae.* This can be further supported by the fact that *V. alginolyticus* strains were found to possess homologues of the virulence genes for *V. cholerae* and *V. parahaemolyticus* (Xie *et al*., 2005).

Conclusion

In this work, the targeted microorganisms were identified using the conventional cultural methods and molecular techniques such as the PCR and DNA sequencing. It was observed that only one of the isolate for *S. aureus* gave positive results using the molecular methods. The PCR identified *V. cholerae* isolate was found to be *V. alginolyticus* upon receiving the sequencing data. It can henceforth be concluded that the biochemical tests used in this study were not sensitive enough to give a confirmation of the targeted microorganisms and that the molecular methods are more accurate at giving the identity of a specimen. Molecular data showed that there was a 98% homology with *V. alginolyticus* (accession no.: CP006718.1) for the *Vibrio* isolate and 99% *Enterobacter cloacae* strain SP30 (accession no.: JX317636.1). Even though no *V. cholerae* or *C. perfringen*s was isolated, the presence of *V. alginolyticus* having the virulence genes of *V. parahaemolyticus* and *V. cholerae* could still be hazardous to human health. *E. cloacae* also being an important nosocomial pathogen could be the cause of illness in human. Regarding the microbial load, variations among the season, fish species, storage and regions were observed and being of high incidence, 1.2x 10^5 CFU/g in fresh fish, *S. aureus* surely might cause illnesses in the consumers if not properly handled. It would therefore be recommended that the local fish seller/vendors are familiarized with the GHP and HACCP for better services. For further work, it would be interesting to analyze different fresh and frozen fish species during summer whereby a high microbial load was obtained. Samples to be analyzed can be collected from different critical control points to have a better grasp of where the contamination occurred and also implement proper hygienic practices to minimize the bacterial load.

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