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LOADS AND VIRULENCE OF BACTERIAL ISOLATES ASSOCIATED WITH THE FINFISH (*Caranx hullianus)* HARVESTED FROM OKWANOBOLO RIVER ESTUARY.

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ABSTRACT

Loads and virulence of bacterial isolates associated with finfish, *Caranx hullianus*, from the estuarine water of Okwanobolo River in Eastern Obolo, Niger Delta, Nigeria was investigated. The total heterotrophic bacterial count (THBC), total coliform count (TCC), faecal coliform count (FCC), *Salmonella-Shigella* count (SSC), and Vibrio count (VC) of flesh, gills, and intestines of the fish samples were determined using standard bacteriological methods. Virulence factors of the selected bacterial isolates were also determined using microbiological techniques. The THBC and TCC of the flesh recorded mean log count of 5.12 CFU/g and 4.11 CFU/g respectively while FCC, SSC, and VC were not detected. The intestines had the highest bacterial counts while the flesh had the least. The THBC, TCC, FCC and SSC of the gills recorded mean values of 5.48 log CFU/g, 4.31 log CFU/g, 1.67 log CFU/g, and 1.82 log CFU/g respectively with no VC detected. The THBC, TCC, FCC and SSC of the intestines recorded mean value of 5.61 log CFU/g, 4.42 log CFU/g, 4.31 log CFU/g, and 2.62 log CFU/g respectively with no VC detected. The analysis of variance revealed significant differences among the physiological groups in the flesh ($p = 1.36E\ 32 < 0.05$); gills ($p = 7.18E\text{-}06 < 0.05$) and intestine ($p = 1.67E\text{-}10 < 0.05$). Of the eight bacterial species associated with the samples, *Proteus* sp. and *Staphylococcus aureus* were common in all the fish tissues. However, *Escherichia coli, Streptococcus* sp., *Bacillus subtilis, Klebsiella* sp., *Salmonella* sp. and *Shigella* sp. were isolated only from the fish gills and intestines. These isolates exhibited varying virulence potentials concerning DNase, Gelatinase, Lipase, Coagulase, and Urease markers. *Staphylococcus aureus* recorded the highest occurrence (100%) for all the virulence markers while *Escherichia coli, Klebsiella* sp. and *Shigella* sp. recorded the least occurrence (20%). Finfish from the estuarine environment habour potentially pathogenic microorganisms.

KEYWORDS: *Caranx hullianus*, estuary, bacteria, virulence, human health

INTRODUCTION

Fish constitutes an important source of protein intake of many people, particularly in the developing countries (Obiero *et al*., 2019; Golden *et al*., 2017). Fish is a major source of animal protein and an essential food item in the diet of Nigerians as it may be relatively cheaper than meat (Onyia *et al*., 2014). Fish contains most of the essential amino acids, particularly, lysine, methionine, and tryptophan that are lacking in plant proteins. It is also a major source of vitamins and minerals which are important for good living (Béné *et al*., 2015).

Fish is prone to spoilage, especially in hot climates and tropical areas where cold preservation techniques are often missing. Fish from natural environments are known to harbour various bacterial species (Novoslavskij *et al*., 2016). Fish skin and gills colonize bacterial isolates due to constant exposure to contaminated water, while the digestive tract is affected by contaminated natural water. Contamination of fish flesh is also possible when immunological resistance is compromised (Sheng and Wang, 2021). Generally, a small number of microorganisms are found on the fish's skin. Consumption of undercooked fish and fish products are often associated with human disease. The presence of different bacteria species including human pathogenic bacteria in fish are linked to direct contact with contaminated water, thus bacteria detected in fish reflect the condition and safety of the aquatic environments (Nkanang *et al.* 2023). Some of the bacteria species cause diseases in fish. An important example is *Yersinia ruckeri*, the causative agent of enteric red mouth (ERM) disease in rainbow trout, resulting in heavy commercial losses.

From the point of public health, the types of bacteria transmitted through fish that cause human diseases are important. The presence of human pathogenic microorganisms in fish and fish products are affected by various factors, including cultural practices, environmental conditions, processing, and distribution of products. The fish pathogens can be generally divided into two groups: those native to natural water habitats and those associated with water pollution. Along with human non-pathogenic bacteria species and natural micro flora of aquatic environments, pathogenic bacteria are also widely found in fish. According to the European Food Safety Authority, pathogens such as *Campylobacter*, *Salmonella*, *Yersinia*, *E. coli*, and *Listeria monocytogenes* are responsible for major food borne outbreaks worldwide (EFSA and ECDC, 2015). Not all pathogens are associated with food borne outbreaks through the consumption of contaminated fish and fish products.

Some seafood commodities are inherently riskier than others due to factors, including; the nature of the environment, mode of feeding, the harvest season, and preparation. Contamination of seafood by pathogens with a human reservoir occur when breeding areas are contaminated with

human sewage. Sources of seafood contamination includes; overboard sewage discharge into harvest areas, illegal harvesting from sewage-contaminated waters, and sewage runoff from the inland after heavy rains or flooding. Contributing factors include; storage and transportation at inappropriate temperatures, infected food handler, contact with contaminated seafood or seawater. Adequate cooking kills most pathogens; however, unlike other foods such as meat and poultry, that are often fully cooked, some sea foods are often prepared in ways that do not kill all the organisms (Dechet *et al.*, 2008).

Intensive growth of industry and agriculture cause contamination of natural and human-made aquatic environments, and will affect not only the health of fish, but also raise safety concerns with regard to fish used for human consumption. It is therefore pertinent to study the prevalence of pathogens in fish to ensure the safety of fish products and environments. Microbial assessment of fish also gives additional information about the hygienic status of environments, including lakes, rivers, ponds, and fish farms. Detection of pathogenic microorganisms or changes in natural micro flora in the water environment is an important indicator of possible contamination (Motlagh and Yang, 2019).

Studies on pathogens in fish have been conducted covering few pathogens over limited geographical areas (Sichewo *et al.*, 2013*;* Umana *et al*., 2017). Okwanobolo river estuary is a good fishing ground for fishermen and a beehive of other human activities like petroleum exploration and exploitation. Hence, the study of the bacterial loads and virulence of the isolates from *Caranx hullianus* samples harvested from Okwanobolo river estuary.

MATERIALS AND METHODS

Study Area

Okwanobolo is an estuarine fishing settlement located in Eastern Obolo Local Government Area of Akwa Ibom State in the Niger Delta. It lies within Latitude 4° 44′ 0.54″N, Longitude 8° 41′ 17.59″ E and Latitude 4° 42′ 55.29″N, Longitude 8° 42′ 46.63″E. The ecosystem provides an enabling environment for fishing by artisan fishermen and breeding of various kind of aquatic resources as well as sites for petroleum production and exploration activities (Udotong *et al.*, 2008).

Sample Collection

Five samples of finfish, *Caranx hullianus* from Okwanobolo estuarine environment were stored in ice-packed coolers and transported to the microbiological laboratory for analysis.

Isolation and Enumeration of Bacterial Isolates

Serial dilution of the samples was carried out to enhance the enumeration of the bacterial load of the samples. The flesh, gills and intestines of each fish samples were removed and grinded separately using sterile mortar and pestle. Each sample (1 g) was weighed out and a ten-fold serial dilution was carried out as described by Cheesbrough (2006).

The density of heterotrophic bacteria and potential pathogens was determined using standard analytical procedures as described by Harrigan and McCance (1990). Dilutions (10^{-4} – 10^{-6}) were plated in triplicates using pour plate technique on Nutrient Agar (NA), MacConkey Agar (MCA), Eosine Methylene Blue Agar (EMBA), *Salmonella-Shigella* Agar (SSA), Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) and Mannitol Salt Agar (MSA) for isolation and enumeration of heterotrophic, total coliform, faecal coliform, *Salmonella* and *Shigella*, *Vibrio* and *Staphylococcus* species respectively. The inoculated plates were incubated at 28±2°C for 24 to 48 hours. Discrete colonies that appeared on the culture plates were enumerated and expressed as log value of the colony forming units per gram (CFU/g).

Characterization and Identification of Bacterial Isolates

Discrete colonies were sub-cultured on freshly prepared Agar plates and purified by repeated sub-culturing. Bacterial isolates were characterized and identified by subjecting the isolates to morphological, biochemical and fermentative tests such as: Gram's reaction, Catalase, Citrate Utilization, Motility Test (Stab Method), Oxidase, Spore Staining, Voges Proskauer (VP), Methyl-Red (MR), Coagulase and Urease test. Identification of bacterial isolates was based on their morphological and biochemical characteristics as describe by Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Determination of Virulence Markers of Bacterial Isolates

Determination of the virulence markers of bacterial isolates was carried out employing various test such as lipase, haemolysis, gelatin hydrolysis and DNase. The data collected for the various isolates were collated and identified based on the determinative scheme bacteriology.

RESULTS AND DISCUSSION

The bacterial load of the *Caranx hullianus* flesh samples are presented in Fig 1. Analysis of the total heterotrophic bacteria (THBC) and total coliform (TCC) loads of the *Caranx hullianus* flesh revealed that the THBC ranged from 5.07 $\log_{10}$ cfu/g to 5.26 $\log_{10}$ cfu/g with a mean count value of 5.11 $\log_{10}$ cfu/g while the TCC ranged from 4.07 $\log_{10}$ cfu/g to 4.23 $\log_{10}$ cfu/g with a mean count value of 4.10 $\log_{10}$ cfu/g. Faecal coliform (FCC), *Salmonella – Shigella* (SSC) and *Vibrio* (VC) were not detected. The analysis of variance revealed significant difference among the physiological groups in the flesh (P = 1.36E 32 <0.05).

The microbial count of the different physiological groups in *Caranx hullianus* gill samples are presented in Fig 2. The THBC ranged from 5.26 $\log_{10}$ cfu/g to 5.46 $\log_{10}$ cfu/g with a mean count value of 5.48 $\log_{10}$ cfu/g. The TCC ranged from 4.25 $\log_{10}$ cfu/g to 4.38 $\log_{10}$ cfu/g with a mean count value of 4.31 $\log_{10}$ cfu/g. The faecal coliform count (FCC) ranges from 4.14 $\log_{10}$ cfu/g to 4.20 $\log_{10}$ cfu/g with a mean count value of 1.67 $\log_{10}$ cfu/g. SSC recorded a mean count value of 1.81 $\log_{10}$ cfu/g respectively. The analysis of

variance revealed significant difference among the physiological groups in the gills (P = 7.18E-06 <0.05).

The microbial count of the different physiological groups in *Caranx hullianus* intestine samples are presented in Fig 3. The THBC ranged from 5.57 log_{10} cfu/g to 5.65 log_{10} cfu/g with a mean count value of 5.61 log_{10} cfu/g. The TCC ranged from 4.36 log_{10} cfu/g to 4.50 log_{10} cfu/g with a mean count value of 4.42 log_{10} cfu/g. The faecal coliform count (FCC) ranges from 4.25 log_{10} cfu/g to 4.36 log_{10} cfu/g with a mean count value of 4.30 log_{10} cfu/g. SSC ranged from 3.17 log_{10} cfu/g to 3.32 log_{10} cfu/g with a mean count value of 2.61 while VC was not detected. The analysis of variance revealed significant difference among the physiological groups in the intestine (P = 1.67E-10 <0.05).

The occurrence and distribution of the bacterial isolates among the samples are presented in Fig. 4. The incidence and distribution of the bacteria isolates among the studied finfish parts revealed that the intestine had the highest bacterial load harboring most of the isolates associated with the sample while the flesh had the least bacterial load.

The occurrence and distribution of the bacteria isolates among the samples are presented in Figure 4. The incidence and distribution of the bacteria isolates among the studied finfish parts revealed that the intestine had the highest bacterial load harboring most of the isolates associated with the sample while the flesh had the least bacterial load. The result of the virulence attributes of bacterial isolates associated with the *Caranx hullianus* is presented in Table 1.

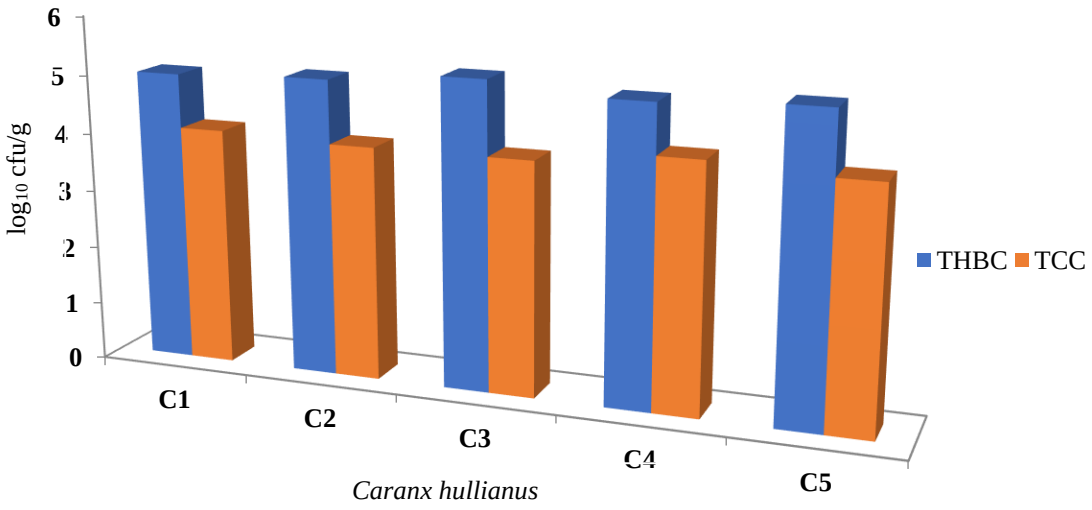

Fig. 1 Bacterial Load of the *Caranx hullianus* flesh Samples. (THBC-Total heterotrophic bacterial count; TCC- Total coliform count)

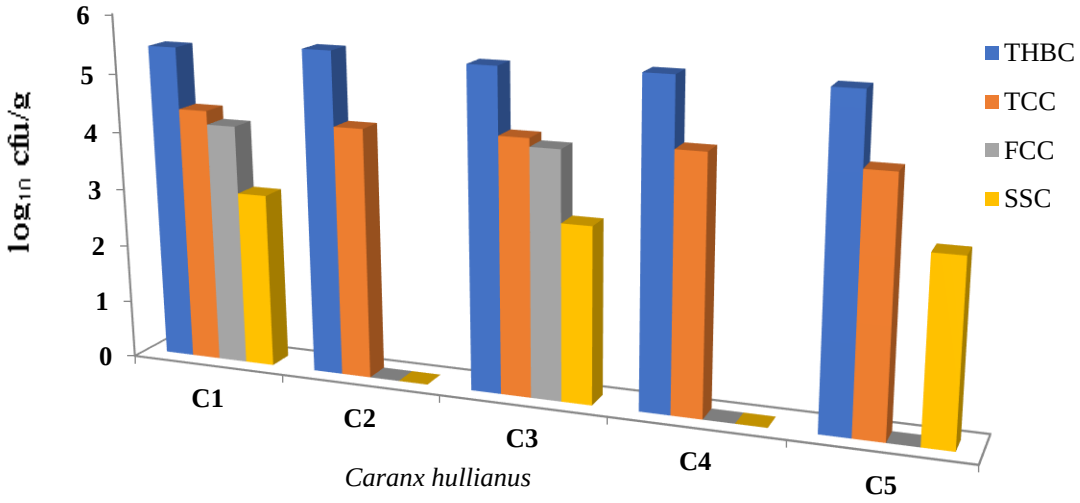

Fig. 2 Bacterial Load of the *Caranx hullianus* Gills Samples. (THBC-Total heterotrophic bacterial count; TCC- Total coliform count. FCC –Feacal coliform count; SCC-*Salmonella- Shigella* Count)

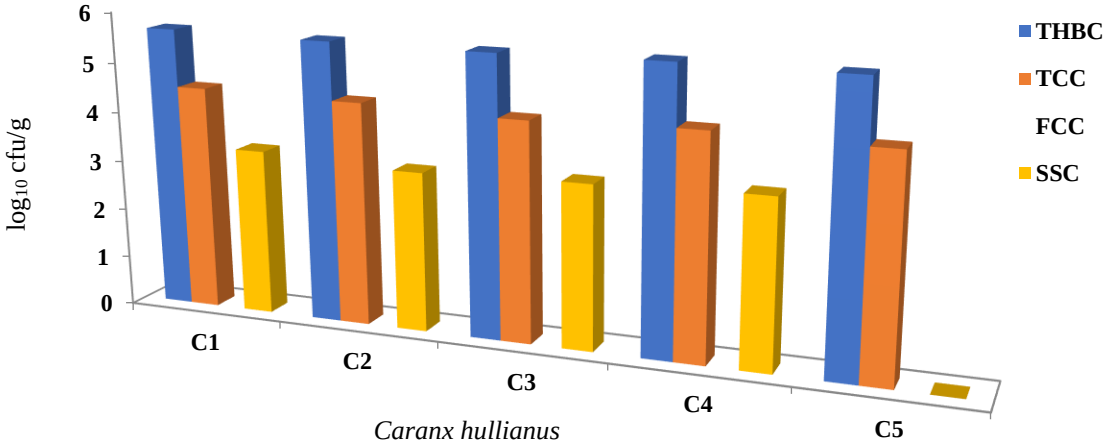

Fig.3: Bacterial Load of the *Caranx hullianus* Intestines Samples. (THBC-Total heterotrophic bacterial count; Total coliform count; FCC –Feacal coliform count; SCC-*Salmonella- Shigella* Count)

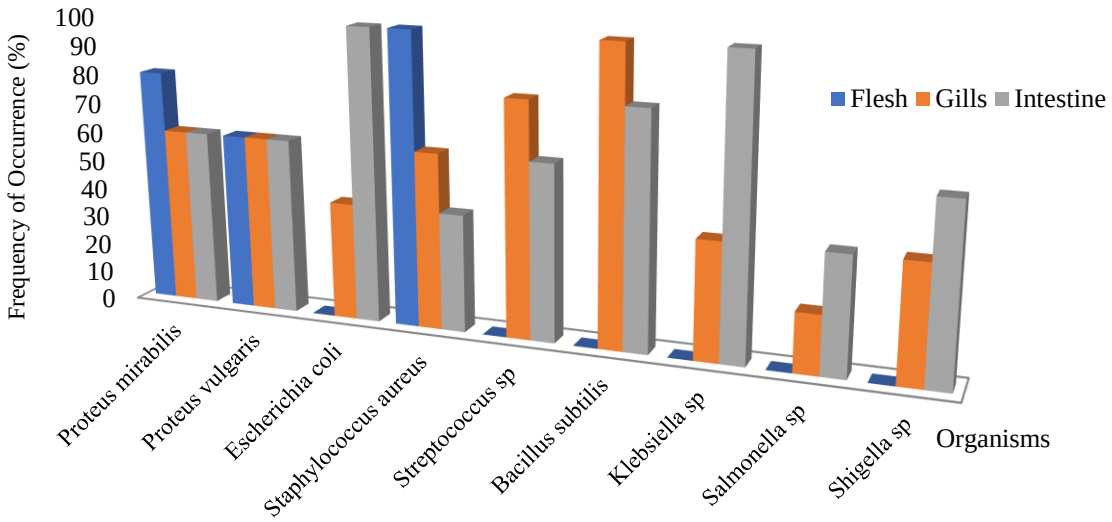

Fig. 4. Occurrence and Distribution of Bacterial Isolates on the *Caranx hullianus*

The result of the virulence markers of the bacterial species associated with the finfish, *Caranx hullianus* samples revealed that the isolates exhibited varying virulence markers.

Table 1. Virulence Markers and Percentage Occurrence of Bacterial Isolates

Isolates	Virulence Markers						Percentage occurrence (%)
	DNase	Gelatinase	Lipase	Coagulase	Urease	Haemolysis	
Proteus mirabilis	+	+	+	-	+	α	80
Proteus vulgaris	+	+	+	-	+	α	80
Escherichia coli	-	-	+	-	-	β	20
Staphylococcus aureus	+	+	+	+	+	β	100
***Streptococcus* sp.**	-	-	+	-	+	β	40
Bacillus subtilis	-	+	+	-	-	β	40
***Klebsiella* sp.**	-	-	-	-	+	ϒ	20
***Salmonella* sp.**	+	-	+	-	-	β	40
***Shigella* sp.**	+	-	-	-	-	α	20

Key: + = Positive, - = Negative, α = Alpha haemolysis, β = Beta haemolysis, ϒ = Gamma haemolysis.

The result showed that the intestines of the *Caranx hullianus* had the highest level of bacterial load with a THBC mean count value of 5.61 $\log_{10}$ cfu/g; TCC mean count value of 4.42 $\log_{10}$ cfu/g; TCC mean count value of 4.30 $\log_{10}$ cfu/g and SSC mean count value of 2.61 $\log_{10}$ cfu/g of the fish sample (Figure 3). The gills recorded a lesser bacterial load with a THBC mean count value of 5.48 $\log_{10}$ cfu/g; TCC mean count value of 4.31 $\log_{10}$ cfu/g; FCC mean count value of 1.67 $\log_{10}$ cfu/g; SSC mean count value of 1.81 $\log_{10}$ cfu/g of the fish sample (Figure 2). However, the flesh of the *Caranx hullianus* recorded a much lesser bacterial load with THBC mean count value of 5.11 $\log_{10}$ cfu/g and a TCC mean count value of 4.10 $\log_{10}$ cfu/g only (Figure 1). Identified isolates included *Proteus mirabilis, Proteus vulgaris, Escherichia coli, Staphylococcus aureus, Streptococcus* sp., *Bacillus subtilis, Klebsiella* sp., *Salmonella* sp. and *Shigella* sp.

The virulence attributes result showed that the different bacterial isolates demonstrated varying responses to the virulence factors; DNase, Gelatinase, Lipase, Coagulase, and Urease. *Staphylococcus aureus* recorded 100 % occurrence as it was positive for all the determined virulence factors, while *Escherichia coli, Klebsiella* sp. *and Shigella* sp. recorded 20 % occurrence for the determined virulence factors (Table 1).

The isolation of pathogenic bacteria isolates from *Caranx hullianus,* is an indication that finfish is a potential carrier of pathogenic organisms therefore confers dangerous health implications if not properly prepared before consumption. This agrees with the findings of Marijani (2022) who reported that fish is a potential source of bacterial pathogens for infection and intoxication in human beings. The presence of *Escherichia coli* is an indication of contamination by human or animal faeces. Most of the bacterial species isolated in the study are known etiologic agents of human infections. *Proteus* sp., a known opportunistic pathogen causes complicated urinary tract, nosocomial, and wound infections in people with impaired or compromised immune systems (Devi *et al*., 2014*).*

Although most strains of *E. coli* are relatively harmless causing brief diarrhea, pathogenic strains is implicated with severe abdominal cramps, bloody diarrhea, nausea, and vomiting (Ara *et al.*, 2007). *Bacillus* sp. is widely distributed in the environment being part of the normal flora in the soil, opportunistic *Bacillus subtilis* contaminates food causing food poisoning (Ara *et al.*, 2007; Tewari and Abdullah (2015). The occurrence of *Streptococcus* sp. in the finfish sample agrees with the findings of Emikpe *et al*. (2011) who reported that bacteria, *Pseudomonas angulluseptica* and *Streptococcus* sp. encountered in different fish, are potentially pathogenic.

Consumption of infected fish causes diseases, some of which are associated with pathogens that are resistant to antibiotics (Adebayo-Tayo *et al.*, 2012). Pal and Maiti (2019) stated that the contamination of fish often occurs from human and animal sources, and thus, fish and seafood are involved in the transmission of pathogenic microorganisms and toxins.

In this study, it was observed that the flesh of the *Caranx hullianus* fish recorded only three (3) bacterial species; *Staphylococcus aureus*, *Proteus mirabilis* and *Proteus vulgaris* with incidence rates of 100%, 80% and 60% respectively across the five *Caranx hullianus* samples (Figure 4).The isolation of bacteria species from the flesh of the fish samples disagrees with the reports of Karunasagar, (2015) and Novoslavskij *et al.* (2016) who reported that fish is considered a safe food in general, and the flesh of healthy fish are considered sterile.

The *Caranx hullianus* gills harbored all the isolated bacterial isolates with *Bacillus subtilis* recording the highest occurrence rate (100%) and *Salmonella* sp. *recording* the lowest incidence rate (20%) across the five samples (Figure 4). In the *Caranx hullianus* intestines, *Escherichia coli* and *Klebsiella* sp. recorded the highest occurrence rate (100%), while *Staphylococcus aureus* and *Salmonella* sp. recorded the lowest occurrence rate (40%) (Figure 4). This agrees with Galaviz-Silva *et al*. (2009) who reported that microorganisms are commonly present on fish surfaces, such as skin and gills, as well as inside the fish in areas; the digestive tract and internal organs; the kidney, liver, and spleen.

CONCLUSION

Conclusively, based on the findings of this study, the finfish, *Caranx hullianus* harvested from Okwan Obolo estuarine environments has been shown to harbor high bacterial density and diversity in its tissues. The bacterial species associated with the fish tissue samples had varying virulence markers that are known tools the microbes use to cause human diseases. Although the sources of the bacterial pathogens were not determined, it is recommended that the fin fishes harvested from Okwan Obolo estuary should be properly cooked before consumption**.**

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