International Scholars Journals

African Journal of Fisheries Science ISSN 2375-0715 Vol. 8 (9), pp. 001-005, September, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Isolation and identification of pathogenic bacteria associated with frozen mackerel fish (*Scomber scombrus*) in a humid tropical environment

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Accepted 18 July, 2020

Aquaculture products can harbour pathogenic bacteria which are part of the natural micro-flora of the environment. An *in-vitro* assay was carried out to ascertain and identify major bacterial contaminants of frozen fish, which hitherto had constituted an important dietary intake of the people of Nsukka, Nigeria. Fish samples collected were identified and bacterial load of the samples determined using agar plate method. Differentiations and characterizations of various isolates were based on biochemical reactions and gram-staining technique. Frozen mackerel fish (*Scomber scombrus*) was used for the study. *In-vitro* assay result revealed that the samples were predominantly contaminated by three bacteria species viz: *Staphylococcus aureus, Escherichia coli* and *Lactobacillus plantarum*. Incidences of the various isolates in the culture were found to be 60, 20 and 15%, respectively. The mean bacteria load of the isolates was 1.135 x 10^6 CFU g^{-1}. This value was found to be markedly higher than the recommended public health and safety standard value of between 5.0 x 10^5 CFU g^{-1}, approved by Nigerian National Agency for Drug Administration and Control (NAFDAC).

Key words: Isolation, identification, pathogenic bacteria, *Scomber scombrus*.

INTRODUCTION

Fish constitute the cheapest source of animal protein in Africa (Clucas and Ward, 1996). It is one of the main food components of humans for many centuries and still constitutes an important part of the diet of many countries. The advantage of fish as a food resulted from its easy digestibility and high nutritional value. Since 70% of the earth's surface is covered by water, there are plenty sources to harvest fish from. Fishes are found in different waters. Some are found in fresh water while some are found in salt water (sea and oceans). However, the type of microorganism found associated with a particular fish depends on the water it was found (Thatcher and Clark, 1973; Clucas and Ward, 1996). Freshly harvested aquaculture products, particularly those from tropical regions may harbour pathogenic bacteria, which form part of natural micro-flora of fish ponds (Clucas and Ward, 1996). It was further reported by these workers that harmful microorganism could also enter seafood processing chain because of inadequate process control, poor standards of hygiene and sanitation in processing plants and post-production contamination during incorrect handling or storage.

Kvenberg (1991) and Rodeick (1991) classified the bacteria pathogens associated with fish into two: the non-indigenous bacteria pathogen and the indigenous bacteria pathogens. The non-indigenous pathogen contaminate fish or fish's habitat in one way or the other and the pathogens include *Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus, Salmonella* species, *Shigalla* species, *Escherichia coli*, etc. The indigenous bacteria pathogens are those naturally living in the fish's habitat. They are the *Vibrio* species, *Aeromonas* species etc. Clucas and Ward (1996) also listed some organisms likely to cause food-borne diseases when present in ready-to-eat sea foods. The list included *S. aureus, Salmonella, Vibrio parahaemolyticus,*

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Monocytogenes, Shigalla, Aeromonas, Yersenia and *Pseudomonas.*

Fishes have high water content and freeze between temperatures of 0 and 3°C with an average of about 2°C (Desrosier, 1978). Freezing kills some bacteria, but the ones not killed will grow upon thawing (Frazier and Westhoff, 1988). Some bacteria that grow on fish, like *Pseudomonas* species, *Moraxella* species, *Alcaligenes* species, *Flavobacterium* species, etc can survive freezing temperature and will resume growth when thawed (Frazier and Westhoff, 1988). At a temperature of 3°C or above, species of *Clostridium botulinum* can survive freezing and may grow and produce toxins (Frazier and Westhoff, 1988). FAO (1989) report showed that fishes become contaminated at sea prior to freezing due to difficulty in designing the plant that would be able to operate satisfactorily at all time in adverse weather conditions at sea. The report further indicated that the method of catching fish contributes to the bacterial load of frozen fish and observed that trawling of fish net along the bottom sediments of water for a long time could result in exposing the fish to a high bacterial contamination.

In view of the various ways fishes could be contaminated with microorganisms, the present research was therefore aimed at finding out if frozen fish consumed in this locality is contaminated with bacterial pathogens, and if so, to identify the major bacteria contaminants. The specific objectives of this study therefore were:

(1) To ascertain and identify the type of pathogenic bacteria associated with frozen fish commonly consumed by people in this locality, and
(2) To assess the incidence of these bacterial pathogens in the fish sample.

MATERIALS AND METHODS

Location of study

The study was carried out at Nsukka, Nigeria. Nsukka is located at latitude 06°52´N and longitude 07°24´E and at an altitude of 447.26 m above mean sea level. It is under a humid tropical environment. Rainfall is bi-modally distributed with peaks around July and September of each year. Annual rainfall is approximately 1700 mm and spread between April and November of each year. The fish species used was identified to be *Scomber scombrus* in the Department of Zoology, University of Nigeria, Nsukka, Nigeria; while the assays on the fish samples were carried out at the Department of Crop Science Laboratory of the same University. The ambient laboratory temperature during the study was 30 ± 3°C with an average solar radiation of 88.7 watts m^{-2} (max) and 3.7 watts m^{-2} (min.).

Collection of fish samples

Frozen fish samples were bought from twenty different sellers at Nsukka main market during the early morning hours of the day (between 7:00 and 8:00 h local time) when the fish was being brought out from cold room. They were packed in ice boxes, which were later transferred to the laboratory for identification and biological assays. The laboratory is situated about four kilometers from the market.

Sterilization of materials

All the glass wares used were washed, dried and sterilized in hot air oven at a temperature of 160°C for 1 h according to the method described by Adibe and Eze (2004). Culture media used were sterilized in an autoclave at a temperature of 121°C for 15 min. The wire loop was also sterilized using spirit lamp.

Sample preparation

Sample preparation was made using the method described by Obi and Krakowiaka (1983). About 10 g of the fish sample was cut from the head, middle and tail regions with a sterile knife. The cut samples were crushed into small pieces in a sterile mortar with about 10 ml sterile water. From the crushed sample, 1 ml aliquot volume was measured out and homogenized in a clean, dry sterile beaker containing 9 ml of distilled water giving a 1:10 dilution. This was done for the 20 fish samples.

Estimation of bacteria load

The bacteria load was estimated using the method described by Collins et al. (1989) for microbial count of frozen foods and involves the following:

Preparation of serial dilution

Nine milliliters of sterile water was poured aseptically into five tubes each and 1 ml of the original crushed fish sample was added to the first test tube and mixed thoroughly. Another 1 ml was taken from the first tube and added to the second test tube and mixed very well. From the second test tube, another 1 ml was taken and introduced into the third test tube and mixed very well. This procedure continued until the fifth test tube. The crushed sample was therefore diluted from 10^{-1} to 10^{-5} for each fish sample.

Inoculation of plates

Duplicate plates of nutrient agar were inoculated with 0.1 ml of the diluted solution (10^{-2} to 10^{-5}) using glass spreader technique. All plates were incubated at a temperature of 37°C for 24 h before colony enumeration and isolation. The temperature was chosen to differentiate the mesophiles which constitute most medically important pathogenic bacteria (Baker and Silverton, 1985).

Calculation of bacteria counts

The method described by Collins et al. (1989) for estimating bacteria counts was used to enumerate the total viable counts of the isolates. Countable plates showing 1 to 32 colonies were selected and counted. The mean colony count on the nutrient agar plates of each given dilution was used to estimate the total viable count for the samples in colony forming units per gram (CFU g^{-1}).

Calculation of mean colony forming unit per gram (CFU g^{-1})

The mean colony forming unit per gram (CFU g^{-1}) denoted by (x)

was calculated as $\Sigma fx/\Sigma f$, where Σfx is the sum of the products of number of colonies and the colony forming unite per gram; while Σf is the summation of the number of colonies.

Identification of isolates

All isolates were sub-cultured and gram-staining was carried out. Identification of isolates was carried out based on the method described by Sakazaki and Shimad (1986), Collins et al. (1989) and Cheesbrough (2002). The gram staining was aimed at differentiating gram reactions, sizes, shapes and arrangement of cells of the isolates. For the gram-staining of the various isolates, glass slides were washed and air dried. A drop of normal saline was placed on the slide. Using a flame inoculation wire loop, a small amount of inoculum was taken and smeared on the drop of normal saline on the slide. The smear was allowed to air dry and heat fixed by passing over flame three times. The fixed smear was flooded with crystal violet for a minute and then rinsed with clean water. Lugols iodine was added for another one minute and this served as a mordant. This was later rinsed and cleaned with distilled water. Acetone-alcohol was added as decolorizer and rinsed immediately with clean water. A counter stain, sefranin, was added and allowed to stand for a minute before being rinsed with clean water. This was allowed to dry before observing under oil immersion objective microscope.

RESULTS

Total viable bacterial counts

The result of the total viable bacterial count of the frozen fish samples expressed in colony forming unit per gram (CFU g^{-1}) is shown in Table 1. The result shows that for the twenty crushed frozen fish samples represented by S_1 to S_{20}, the dilution factor with visible clear colonies showing in each plate ranged from 10^{-5} to 10^{-2} dilution. On the other hand, the mean number of colony per dilution factor ranged from 1 to 32 colonies. The colony forming unit per gram of each dilution factor ranged between 3.2×10^6 in fish sample S_{10} and $2 \times 10_{11}$ in fish sample S_5. The mean colony forming unit per gram (CFU g^{-1}) for the crushed sample was calculated as $\bar{x} = \sum fx / \sum f$, where $\bar{x}$ is mean, $\Sigma f\chi$ is the summation of product of x and f and Σf is the summation of f as shown in Table 1.

Biochemical reactions, gram-staining and inoculation tests

S. aureus, E. coli and *Lactobacillus plantarum* were the predominant pathogenic bacteria species found associated with the fish sample (Table 2). The *S. aureus* isolated was also found to be coagulase and catalase positive and do not ferment lactose, glucose and fructose, but were found to ferment only sucrose. The strains were also manitol-positive but found to be indole and motility negative gram-positive cocci bacteria.

The result also revealed that *E. coli* strains sampled displayed different strains with differential results with respect to sucrose fermentation. Generally, they were coagulase and catalase negative and fermented lactose, glucose or fructose. They also exhibited positive reactions to manitol, indole and motility. They reacted negatively to gram-strains and were therefore characterized as bacilli bacteria species. The strains of *L. plantarum* isolated exhibited differences in fermentation result with respect to sucrose. They were obviously coagulase or catalase negative strains and with negative indole and strain motility reactions. They reacted positively to manitols but fermented lactose not glucose or fructose.

Frequency of occurrence of the various isolates

S. aureus was isolated from twelve out of twenty inoculated plates (Table 3). This represented 60% of the isolated samples. *E. coli* were isolated from five of the inoculated plates and therefore represent 25% of the isolated microorganisms. The least was *L. plantarum*, which represented 15% of the isolated organism having been isolated from five of the inoculated plates.

DISCUSSION

S. aureus, *E. coli* and *L. plantarum*, in this order, were the common pathogenic bacteria found associated with frozen fish in this environment. The presence of *S. aureus* was attributed to the contamination of the fish samples by man. Clucas and Ward (1996) recorded that *S. auerus* seldom, if ever, occurs as natural micro-flora of fish and shellfish; its main habitat is humans and animals and is found mostly in the nose, throat and skin of healthy individuals. This suggests that frozen fish with this pathogen post-harvest must have been contaminated through handling. Contamination of frozen fish with *E. coli* also suggested that one or more of enteric pathogens have gained access to the fish. This organism (*E. coli*) is particularly useful as an indicator of contamination when appeared in small numbers or as an indicator of mishandling when appeared in large numbers (Silliker and Gabis, 1976). Although some species of *Lactobacillus* are normal flora, which are beneficial to the host tissue where they are found, other species are part of the normal intestinal flora and maybe predominant in infants and others having a high intake of sugars, especially lactose (Burrow et al., 1969). The isolation of the enteric bacteria, *L. plantarum* from the frozen fish samples which may be enteric supports the thesis that the fish sampled were contaminated by faecal materials. The co-association of *E. coli* and *L. plantarum* in the fish samples is also normal as both of them are known to co-exist commensally in their natural micro-floral environment . The *E. coli* bacteria isolated from the

Table 1. Colony counts and colony forming units of the various fish samples at different dilution factors.

S/n	Fish sample	Dilution factor	Average number of colonies (f)	Colony forming unit of 0.1 ml aliquot	Colony forming unit of 1ml aliquot	Colony forming unit of ml/g (x)	fx
1	S_1	10^{-3}	20	$2x10^4$	$2x10^9$	$2x10^6$	$4x10^6$
2	S_2	10^{-3}	16	$1.6x10^4$	$1.6x10^8$	$1.6x10^5$	$2.56x10^6$
3	S_3	10^{-3}	10	$1x10^4$	$1x10^9$	$1x10^6$	$1x10^6$
4	S_4	10^{-3}	50	$5x10^4$	$5x10^9$	$5x10^6$	2.5×10^7
5	S_5	10^{-4}	20	$2x10^5$	$2x10^{11}$	$2x10^8$	$4x10^8$
6	S_6	10^{-3}	30	$3x10^4$	$3x10^9$	$3x10^6$	$9x10^6$
7	S_7	10^{-3}	40	$4x10^4$	$4x10^9$	$4x10^6$	$1.6x10^7$
8	S_8	10^{-4}	8	$8x10^3$	$8x10^7$	$8x10^4$	$6.4x10^5$
9	S_9	10^{-3}	20	$2x10^3$	$2x10^7$	$2x10^4$	$4x10^5$
10	S_{10}	10^{-2}	32	3.2×10^3	$3.2x10^6$	3.2×13^3	$1.0243.2\times 10^5$
11	S_{11}	10^{-3}	20	$2x10^4$	$2x10^9$	$2x10^6$	$4x10^6$
12	S_{12}	10^{-4}	10	$1x10^5$	$1x10^{11}$	$1x10^8$	$1x10^8$
13	S_{13}	10^{-3}	40	$4x10^4$	$4x10^9$	$4x10^6$	1.6×10^7
14	S_{14}	10^{-2}	60	$6x10^3$	$6x10^7$	$6x10^4$	3.6×10^5
15	S_{15}	10^{-3}	30	$3x10^4$	$3x10^9$	$3x10^6$	$9x10^6$
16	S_{16}	10^{-3}	20	$2x10^4$	$2x10^9$	$2x10^6$	$4x10^6$
17	S_{17}	10^{-2}	60	$6x10^3$	$6x10^7$	$6x10^4$	$3.6x10^5$
18	S_{18}	10^{-3}	8	$8x10^3$	$8x10^7$	$8x10^4$	$6.4x10^5$
19	S_{19}	10^{-3}	7	$7x10^3$	$7x10^7$	$7x10^4$	$4.9x10^5$
20	S_{20}	10^{-2}	30	$3x10^5$	$3x10^9$	$3x10^6$	$9x10^6$
			$\sum f = 531$				$\sum fx = 6.025524 \times 10^8$

$\bar{X} = \sum fx / \sum f = 6.025524x10^8/531 = 1.135x10^6$ CFU per ml g^{-1}.

Table 2. Biochemical reactions, gram-staining and inoculation results of the various isolates.

Isolates	Gram reaction	Biochemical reaction								
		COA	IND	MOT	LACT	MAN	GLU	SUC	FRUCT	CAT
S. aureus	Positive cocci	+	-	-	-	+	-	+	-	+
E. coli	Negative bacilli	-	+	+	+	+	+	D	+	-
L. plantarum	Positive bacilli	-	-	-	+	+	-	D	+	-

Where + = positive; - = negative; D = different strains given different results; IND = indole; MOT = motility; Lac = lactose; Man = manitol; Glu = glucose, SUC = sucrose; FRUCT = Fructose, CAT = catalase; COA = coagulase.

Table 3. Incidences of the various isolates in the culture.

Isolates	Number of colonies	Occurrence of the colonies (%)
S. aureus	12	60
E. coli	5	25
L. plantarum	3	15
Total	20	100

samples were the most reliable indicator of faecal pollution. Besides, the co-existence of *E. coli* and the gram-positive *S. aureus* which cause food poisoning could have been transferred through contact with skin of handlers. The mean viable count of the organisms from the study was found to be higher than the Nigerian Agency for Drug Administration and Control (NAFDAC) recommended official public health standard of between

5.0×10^5 and 1.0×10^6 CFU g^{-1}. The mean viable count although cannot be taken as an absolute figure. This is because the number and type of bacteria found on frozen fish is dependent on many factors, of which, source of the fish contributes the major factor and supports Thatcher and Clark (1973) earlier report which stated that the kind and number of micro organisms found on frozen fish is dependent on the source of the fish, additional contamination introduced in the fishing boat, freezing temperature during storage, severity of freezing process with respect to lethality to microorganisms and contamination by handlers and market sellers. Brooks et al. (2004) however, concluded that one of the sources of infection is contaminated food. It is therefore recommended that proper processing of frozen fish samples be carried out before consumption.

Conclusion

The determination of bacteria contamination of frozen mackerel (*S. scombrus*) fish carried out in this study was necessary in safeguarding public health. This is because frozen fish which is contaminated with faecal material before or during harvest, may cause outbreak of intestinal infectious disease such as typhoid fever.

The bacteria isolation assay result revealed the presence of commensal bacterial of intestinal origin, viz: *E. coli* and *L. plantarum.* The *S. aureus* however is of external sources. The microbial population was found to be higher than the approved safety standard by NAFDAC. Since these microorganisms could contaminate fish and therefore a source of food poisoning; harvesting, handling and cooking of frozen fish especially mackerel (*S. scombrus*) should be done properly so as to reduce the bacterial load. In this case, eating of frozen *S. scombrus* raw or half-boiled should be discouraged to eliminate zoonotic infections from fish especially in this area. This study therefore is intended to provide basic information about these microorganisms likely to cause food-borne disease when present in frozen fish that is ready-to-eat.

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