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Original Research Article

Evaluation Of Microbial Pathogens In Biofilms Of Deteriorating Fish In A Suburb of Ogun State, South-western Nigeria

ABSTRACT

The experiment was carried out to identify bacteria pathogens that causes fish spoilage and consequently reduces their shelf-life in the environment and invariably found out basis for controlling and preventing their activity.

Thirty fish samples comprising of fifteen Titus (*Scomber scombrus*) and fifteen Kote (*Trachurus trachurus*) were collected from five major markets in Ogun state, Nigeria. Samples were cultured and processed using the pour plate and streak plate technique. Samples from the skin scrapings were cultured in five media consisting of four selective media and a basal media; Maconkey agar, Eosin Methylene Blue agar, Mannitol Salt agar, Salmonella Shigella agar and Nutrient agar. Differentiation and isolation of various isolates were based on gram-staining technique and biochemical reactions using OXOID MICROBACT^{TM} identification kits. On examination, in vitro assay showed the presence of six bacteria species in the surface biofilms namely; *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Salmonella typhi, Proteus vulgaris* and *Proteus stuartii*. Prevalence of the various isolates in the culture were found to be 25.2%, 20.6%,18.7%, 14.9%, 11.2% and 9.3% respectively. The highest colony count (140) was obtained from samples obtained from Ijebu-Ode while the lowest colony count was obtained from Sagamu. The mean bacteria load of the isolates was $7x10^6$ CFU ml^{-1}. The high microbial load in biofilms of the samples obtained from the market especially those of enteric bacteria showed the need for enforcement of high hygiene standards for food handlers and food storage companies.

Keywords: Pathogen, biofilms, nutrient agar, enteric bacteria, fishes, colony count

INTRODUCTION

Fish and fish products are the most important source of protein and it is estimated that more than thirty percent of fish for human consumption comes from aquaculture (Hastein *et al*., 2006). Fish constitute the cheapest source of animal protein in Africa (Clucas & 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

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and high nutritional value. Since 70 percent of the earth's surface is covered by water, there are plenty sources of fish to harvest from. Fishes are found in different waters. Some are found in fresh water while some are found in salt water (seas and oceans). Ponds have a wide variety of microbial life. According to a study in 2000, ponds have an average of 184.5 different types of microbe (Microbewiki.kenyon.edu, 2008).

The bacteria pathogens associated with fish have been classified into two: the non- indigenous bacteria pathogen and the indigenous bacteria pathogens. The non- indigenous pathogen that contaminate fish or fish's habitat are *Clostridium botulinum, Listeria monocytogenes*, *Staphylococcus aureus, Salmonella species, Escherichia coli,* etc. The indigenous bacteria pathogens are those naturally living in the fish habitat (Herrera *et al*., 2006).

Fishes have high water content and freeze between temperatures of 0 and 3^0C with an average of about 2^0C. Freezing kills some bacteria, but the ones not killed will grow upon thawing (Behravesh *et al*., 2012). Some bacteria that grow on fish, like *Pseudomonas species, Moraxella species, Alcaligenesspecies, Flavobacteriumspecies* can survive freezing temperature and will resume growth when thawed (Behravesh et *al*., 2012). The method of catching fish contributes to the bacterial load of frozen fish and also the 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.

The major psychrotrophic bacteria are found in milk, meats and poultry and fish and other seafood (Vanderzant & Splittstoesser, 1992). A biofilm is a well-organized, cooperating community of microorganisms. Microbial cells attach to the surfaces and develop a biofilm. Biofilm associated cell is differentiated from suspended counterparts by reduced growth rate, up and down regulation of gene and generation of extracellular polymeric matrix (Donlan, 2002)

Genetic studies have revealed that biofilms are formed through multiple steps. They require intracellular signaling and transcribe different set of genes different from planktonic cell. Therefore, biofilm formation can be viewed as a developmental process, which shares some of the features of bacterial developmental processes (Watnick *et al*., 2000)

MATERIALS AND METHODS

Location of Study

This study was carried out in Ogun State, South-western Nigeria. Ogun State is located at latitude $07^0$00'N and longitude $03^0$35'E and at an altitude of 77m above mean sea level.

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Collection of Fish Samples

A total of 30 deteriorating fish samples comprising of fifteen Horse Mackerel (Kote) and fifteen mackerel (Titus) were bought from five major markets (Ilaro, Abeokuta, Ijebu- Ode, Sagamu and Sango- Ota) representing the five major zones in Ogun state, Nigeria. They were collected and packed aseptically in ice boxes and were later transferred to the laboratory for identification and biological assays.

Sterilization of Materials

All glass wears used were washed, dried and sterilized in hot air oven at a temperature of 160^0C for 1 hour according to the method described by (Adibe and Eze, 2004). Culture media used were sterilized in an autoclave at a temperature of 121^0C for 15 minutes. The wire loop was sterilized using a bunsen burner.

Sample Preparation and Culturing of the Samples

Sample preparation was made using the method described by Obi and Krakowiaka (1983). About 1g of the superficial skin layer of middle segment between the head and the tail region of the fish samples was scraped with a scalpel blade. The scraped samples were crushed into smaller pieces in a sterile pestle and mortar with about 10ml peptone water.

Culture of Fish Sample

All the fish samples were cultured in five different media; Nutrient agar, MacConkey agar, Eosin Methylene Blue agar, Mannitol Salt agar and Salmonella Shigella agar. For the colony count, 1.0ml of the diluents (10^{-5}) were pipetted into petri dishes in duplicates and then nutrient agar was added and rocked on the bench and incubated at 37^0 C for 24 hours.

Inoculation of Plates

Plates of nutrient agar were inoculated with 1.0 ml of the diluted solution (10^{-5}) using pour plate technique. All plates were incubated at a temperature of 37^oC for 24 hours before colony enumeration and isolation. The temperature was chosen to differentiate the mesophiles which constitute most medically pathogenic bacteria (Baker and Silverton, 1985).

Pour Plate Technique

1ml of the dilutions was dispensed into petri dishes using sterile syringes in duplicates. Already prepared nutrient agar and MacConkey agar were poured in the plate respectively and rocked

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gently allowing to solidify and incubated at 37^0 C for 18- 24 hours. The plates were inspected for growth and total colony count of all organisms was done.

Streak Plate Technique

The colonies were purified by aseptically picking characteristic discrete colony and streaked on selective media, Eosin methylene blue agar (EMBA), Salmonella shigella agar (SSA), Mac Conkey Agar (MA), Manitol Salt Agar (MSA). And prepared according to the manufacturer's instructions and incubated at 37^0C for 24 hours.

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 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 ml^{-1})

Calculation of mean Colony Forming Unit per Gram (CFUml^{-1})

The mean colony forming unit per gram (CFUml^{-1}) denoted by (x) was calculated as $\sum fx/\sum f$, where $\sum fx$ is the sum of the product of number of colonies and the colony forming unit per milligram; while $\sum f$ is the summation of the number of colonies.

Isolation of *Escherichia coli*

To yield a pure discrete colony of *E. coli*, a colony of suspected *E.coli* which appears pink on Mac Conkey agar was picked using a sterile inoculating loop and streaked on Eosin Methylene Blue Agar (EMBA) which had been prepared according to the manufacturer's instructions and incubated at37^0C for 12-18h. Green metallic sheen on the line of streaking on EMBA plate shows *E.coli.*

Isolation of *Staphylococcus aureus*

A colony of suspected *S.aureus* which appeared off- white on nutrient agar was picked and inoculated using a sterile inoculating loop on Manitol salt Agar plate and incubated at 37^0C for 12-18 h. Yellow appearance on the plate depicts *S. aureus*.

Isolation of *Salmonella*

To yield a discrete colony of *Salmonella*, a colony of a colourless growth was picked on MacConkey agar and streaked using a sterile inoculating loop on Salmonella Shigella agar

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preapared according to the manufacturer's instructions and was incubated at 37^0C for 24 hours. Black colonies on the line of streaking show the growth *Salmonella.*

Isolation of *Klebsiella* sp,

Klebsiella sp formed typical pink colonies which were wet and raised on MacConkey. Colonies suspected to be for *Klebsiella* were subjected to biochemical tests for confirmation.

Biochemical tests

Indole Test

Using a sterile inoculating needle, an isolated colony from MacConkey agar plate was inoculated into peptone water prepared according to the manufacturer's instruction and incubated for 12-18 hours at 37^0C. Then Kovac's reagent was added and shaked properly red- pink coloration confirms *Escherichia coli*.

Catalase Test

3ml of hydrogen peroxide was dispensed into a test tube and a loop full of a colony of suspected *Staphylococcus aureus* was picked and introduced into the test tube bubbles of Oxygen confirms it.

Citrate Utilization Test

Simmon's citrate agar slants which had been prepared according to the manufacturer's instructions were inoculated by aseptically streaking the slanted region with an isolated colony from MacConkey agar plate. The tubes were incubated at 37^0C for 24 h. Blue coloration of the slant confirms the presence of *Klebsiella sp*.

Identification and characterization.

Identification of gram negative organisms was done using the Oxoid MICROBACT 12A.

After 18-24 hours pure cultures of organism have been obtained. 1-3 isolated colonies was picked from an 18-24 hours culture and emulsify in prepared peptone water and mixed properly to make a homogenous suspension and incubate for 2-3 hours.

An identification number was written at the end of the tag. The strip was placed in the holding tray and four drops of the suspension was dropped in each of the wells.

It was incubated at 37^0C for 24hours, before reading the colour change, mineral oil was added into the first 3 wells for 12A. Other reagents like Nitrate A, Nitrate B, reagent VP I and VP II, Kovac's reagents were added when necessary.

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The seal tape was removed and reactions are evaluated as positive or negative by comparing with the colour chart.

RESULTS

A total number of 107 isolates were obtained from 30 samples of fish from five different markets Abeokuta, a suburb in Ogun state from which skin scrapings were obtained, visible clear colonies showed in double plates of 10^{-5} dilution (Tables 1-5). The percentage prevalence for isolated and identified organism in the state was highest for *S. typhi* (27.27%) in Sango-Ota and *S. aureus* (27.27%) both in Sango-Ota and Ijebu- Ode and simultaneously least in Sango-Ota with percentage prevalence for *E.coli* and *Proteus vulgaris* at both 4.54% respectively (Table 7).

This research also showed that the highest percentage prevalence of *E. coli* and *S. aureus* was 23.8% while the least prevalent is *P. vulgaris* (9.52%) in Abeokuta. In Ijebu-Ode, the most prevalent organism was *S.aureus* (27.27%) while the lowest was *Proteus vulgaris* (9.09%). In Ilaro, *S. typhi* and *S aureus* (23.80%) were the most prevalent while *E. coli* and *P.stuartii* (9.52%) were the least prevalent. In Sango-Ota, *S. typhi* and *S. aureus* (27.27%) were the most prevalent organisms while *E. coli* and *P. vulgaris* (27.27%) were the least prevalent with percentage (4.54%). Sagamu has *S. aureus* (23.09%) as the most prevalent and *E. coli* (9.52%) as the least prevalent. The mean number of colony for the dilution factor used ranged from 19 to 125 colonies. The mean colony forming unit per ml (CFU/ml) in the various biofilm assays of the samples was calculated as:

$$\text{Mean} = \sum fx / \sum f = 7339743.6$$

$$\text{Mean} = 7 * 10^{6} \text{ CFU/ml}$$

Where mean was calculated from $\sum fx$ which is the summation of product of (x) and (f) and $\sum f$ is the summation of (f) as shown in Table 6.

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179 **RESULTS OF THE COLONY COUNT**

180 **TABLE 1- Colony Counts From Duplicate Cultures From Abeokuta**

Sample location	Dilution^{-5}	Dilution $^{-5}$	Average
Abeokuta T1	130	110	120
Abeokuta T2	110	100	105
Abeokuta T3	50	55	52.5
Abeokuta K1	140	122	131
Abeokuta K2	120	115	117.5
Abeokuta K3	148	130	139

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182 **TABLE 2 - Colony Counts From Duplicate Cultures From Ijebu- Ode**

Sample location	Dilution^{-5}	Dilution $^{-5}$	Average
Ijebu-Ode T1	43	39	41

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Ijebu- Ode T2	130	120	125
Ijebu- Ode T3	60	50	55
Ijebu - Ode K1	45	39	42
Ijebu - Ode K2	50	30	40
Ijebu - Ode K3	60	40	50

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184 **TABLE 3 - Colony Counts From Duplicate Cultures From Ilaro**

Sample location	**Dilution^{-5}**	**Dilution $^{-5}$**	**Average**
Ilaro T1	85	80	82.5
Ilaro T2	120	100	110
Ilaro T3	110	100	105
Ilaro K1	137	128	132.5

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Ilaro K2	150	130	140
Ilaro K3	80	70	75

TABLE 4 - Colony Counts From Duplicate Cultures From Sagamu

Sample location	Dilution^{-5}	Dilution $^{-5}$	Average
Sagamu T1	60	70	65
Sagamu T2	40	32	36
Sagamu T3	50	24	37
Sagamu K1	43	11	27
Sagamu K2	75	29	52
Sagamu K3	20	18	19

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190 **TABLE 5- Colony Counts From Duplicate Cultures From Sango- Ota**

Sample location	Dilution $^{-5}$	Dilution $^{-5}$	Average
Sango-Ota T1	65	45	55
Sango -Ota T2	40	22	31
Sango -Ota T3	50	32	41
Sango-Ota K1	80	65	72.5
Sango -Ota K2	100	80	90
Sango-Ota K3	102	78	90

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192 **TABLE 6: Results Showing the Colony Forming Unit per ml of the Organisms**

LOCATION	Colony Count 1	Colony Count 2	Dilution Factor	Average Number Of Colonies (f)	Colony Forming Unit per ml (x)	Fx
Abk T1	90	87	10^{-5}	88.5	8,850,000	796500000
Abk T2	82	86	10^{-5}	84	8,400,000	705600000
Abk T3	40	20	10^{-5}	30	3,000,000	90000000
Abk K1	100	110	10^{-5}	105	10,500,000	1102500000
Abk K2	92	76	10^{-5}	84	8,400,000	705600000

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Abk K3	100	90	10^{-5}	95	9,500,000	902500000
IL T1	28	20	10^{-5}	24	2,400,000	57600000
IL T2	13	30	10^{-5}	21.5	2,150,000	46225000
IL T3	40	30	10^{-5}	35	3,500,000	122500000
IL K1	30	28	10^{-5}	29	2,900,000	84100000
IL K2	40	30	10^{-5}	35	3,500,000	122500000
IL K3	50	40	10^{-5}	45	4,500,000	202500000
IJ T1	72	58	10^{-5}	65	6,500,000	422500000
IJ T2	100	90	10^{-5}	95	9,500,000	902500000
IJ T3	90	84	10^{-5}	87	8,700,000	756900000
IJ K1	120	100	10^{-5}	110	11,000,000	1210000000
IJ K2	135	115	10^{-5}	125	12,500,000	1562500000
IJ K3	80	60	10^{-5}	70	7,000,000	490000000
Sag T1	50	50	10^{-5}	50	5,000,000	250000000
Sag T2	34	22	10^{-5}	28	2,800,000	78400000
Sag T3	45	25	10^{-5}	35	3,500,000	122500000
Sag K1	30	11	10^{-5}	20.5	2,050,000	42025000
Sag K2	50	30	10^{-5}	40	4,000,000	160000000
Sag K3	20	18	10^{-5}	19	1,900,000	36100000
Sango T1	40	30	10^{-5}	35	3,500,000	122500000
Sango T2	25	15	10^{-5}	20	2,000,000	40000000
Sango T3	40	22	10^{-5}	31	3,100,000	96100000
Sango K1	60	40	10^{-5}	50	5,000,000	250000000
Sango K2	80	50	10^{-5}	65	6,500,000	422500000

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Sango K3	92	58	10^{-5}	75	7,500,000	562500000
				1698		12465150000
				∑f= 1696.5		∑fx= 12451875000
				Mean = $7*10^{6}$ CFU/ml		Summation fx = $1.2*10^{10}$

TABLE 7:Percentage prevalence of individual organism per location

Organisms	Abeokuta	Ijebu- Ode	Ilaro	Sango-Ota	Sagamu
Escherichia coli	23.8%	18.18%	9.52%	4.54%	9.52%
Klebsiella pneumonia	14.28%	13.63%	14.28%	22.72%	19.04%
Proteus stuartii	14.28%	18.18%	19.04%	13.63%	14.28%
Proteus vulgaris	9.52%	9.09%	9.52%	4.54%	14.28%
Salmonella typhi	14.28%	13.63%	23.80%	27.27%	19.04%
Staphylococcus aureus	23.80%	27.27%	23.80%	27.27%	23.80%

DISCUSSION

This study showed the presence of 107 bacterial isolates comprising of *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli*, *Salmonella typhi, Proteus vulgaris, Proteus stuartii*, in their order of prevalence respectively 25.2%, 20.6%, 18.7%, 14.9%, 11.2% and 9.3%. The

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organism with the highest prevalence was *S.aureus* (25.2%) and the least prevalent is *P.stuartii* (9.3%).These bacteria have been previously reported as been largely responsible for deterioration and spoilage of seafood especially fish (Herrera *et al*., 2006).

Five of the isolated organisms were gram negative while *S. aureus* was the only gram positive which was similar to previous studies in which majority of fish spoilage bacteria were gram negative, while the presence of few gram positive bacteria was reported.

S.aureus, S.typhi and *Klebsiella pneumoniae* in this order, were the common pathogenic bacteria found to be most prevalent in the biofilm of deteriorating fish in Ogun state.

S. aureus was the most implicated bacteria responsible for fish spoilage due to its highest percentage prevalence in Ogun state. The presence of *S. aureus* was attributed to the contamination of the fish samples by man. Clucas and Ward (1996) recorded that *S. aureus* seldom if ever occurs as natural microflora of fish and shell fish; its main habitat is human and animals and was found mostly in the skin, nose and throat of healthy individuals. This suggests that the deteriorating fish must have been contaminated post- harvest with this pathogen through handling.

The isolation of *S. typhi* and its high percentage prevalence in the biofilm of deteriorating fish also supports the theory that the contamination of the fish samples derives from terrestrial sources and that fish may serve as a vector for Salmonella species since fish and shell fish appear to be passive carrier of the organism (Novotny *et al*., 2004).

The isolation of *Klebsiella pneumoniae* in significant amount correlates with the work of (Taylor *et al*., 1979; Diana and Ramulu, 2010) showing that *K. pneumoniae* belonging to the family of *Enterobacteriaceae* is a predominant pathogen isolated from most fish samples.

Evidently, the prevalence level of enteric bacteria in the biofilm of spoiling fish in Ogun State as indicated by the presence of *Klebsiella pneumoniae* suggests that the hygiene level of food handlers in the state is questionable.

REFERENCES

Adibe N.C., Eze E.L. (2004). General Laboratory Techniques for Tertiary Institutions, Mike Social Publishers, Enugu, Nigeria.

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Baker F.J, Silverton R.E. (1985). Introduction to Medical Laboratory Technology. 6th Edition Butterworths, Toronto.

Behravesh C.B., Williams IT., Tauxe R.V., (2012). Emerging foodborne pathogens and problems expanding prevention efforts before slaughter or harvest.

Clucas I. J, Ward A R (1996). Post-harvest Fisheries Development: A Guide to Handling, Preservation, *Processing and Quality*. Charthan Maritime, Kent ME4 4TB, United Kingdom.

Collins C.H., Lyne P.M., Grange G.M. (1989). Collins and Lyne Microbiological Methods, 6th Ed. Butterworth, London.

Diana, T. C., and K. S. Ramulu, 2010. Changes in the protein content of liver and intestine in relation to *Klebsiella* infection in Nemipterus japonicas from the coast of Visakhapatnam, India Asian J. Amin. Sci., 4: 143-145

Donlan R. M, (2002). Biofilms: Microbial life on surfaces, *Emerg Infect. Dis*,(8): 881-890

Hastein, T. B., Hjeltnes, J., Lilehaug, U., Skare, M., Berntssen, H. and Lundebye, A. K. (2006): Food safety hazards that occur during the production stage: Challenge for fish farming and the fishing industry. Review in *Science and technology* 25; 607-625

Herrera T C, Santos J A, Otero A, Garcia-Lopez ML (2006 Mar): occurrence of foodborne pathogenic bacteria, in retail prepackaged portions of marine fish In Spain. The society for Applied Microbiology, *Journal of Applied Microbiology* 100: 527-536

Microbewiki.kenyon.edu,2008

Novotny L., Dvorska L., Lorencova , V.Beran, L Pavlik (2004): Fish: a potential source of bacterial pathogen . Vet. Med- Czech, 49, 2004(9): 343-358

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Obi and Krakowiaka A (1983). Theory and practice of Food Microbiology(unpublished)

Tayor, S. L., Guthertz L. S., M. Leatherwood and E. Lieber, 1979. Histamine production by *Klebsiella pneumonia* and an incident of Scromboid fish poisoning. Applied Environ. Microbiol. 37:274-278.

Vanderzant, C. and Splittstoesser D., 1992. Compendium of methods for the microbiological examination of Foods, American Public Health Association, 153-156.

Watnick, P and Kolter, R. (2000). Biofilm, city of microbes,*J Bacteriol*, (182) :2675-2679.