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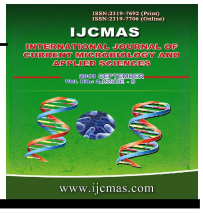

Original Research Article

Assessment of the microbial load of smoked sardine (*Sardina pilchardus*, Walbaum, 1792) sold in Makurdi Markets

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Keywords

smoked fish; *Sardina pilchardus;* makurdi; bacteria; mycoflora.

A B S T R A C T

The microbial load of smoked sardine (*Sardina pilchardus*) sold in five markets in Makurdi was investigated. A total of 45 samples were collected, three samples from each location, and the experiment was conducted three times, amounting to nine replicates per location. Sampling was randomly done and Pour-plate method was adopted for inoculation of all media plates. The isolates were characterized using NA, MSA, EMBA, SDA, MCA and SSA media plates and growth from the plates were subjected to Gram staining test, motility test and Bio-chemical (Coagulate, Catalase, Citrate utilization test and Indole) tests, to confirm their identity. Bacteria flora implicated with the samples included *Staphylococcus aureus, Salmonella sp,p E.coli* and the mycoflora included *Aspergillus niger, Mucor ssp, Yeast ssp* and *Alternaria ssp.* the mean count of bacteria load ranged from $2.632 \times 10^3 \pm 0.978 \times 10^3$ cfug^{-1} (Wurukum Market) to $0.988 \times 10^3 \pm 0.272 \times 10^3$ cfug^{-1} (Modern Market). There was a strong correlation between water activity and Bacterial load.

Introduction

Fish provides a high source of protein required in the diets of man as it contains essential nutrients such as vitamins, fats and minerals which help in the maintenance of life (Ashano and Ajayi, 2003). In spite of these valuable nutrients derivable from fish, it is, however highly perishable especially in the hot climatic regions, where average temperature is warm enough to enhance proliferation of most decomposing microorganisms and specifically in Nigeria, where non-sanitary environment and poor handling practices worsen the situation, thereby making it susceptible to spoilage by a great number of microorganisms (George, 1999). fish is an extremely perishable food commodity as it is highly susceptible to autolysis (the destruction of a cell through the action of its own enzymes), rancidity [which occurs especially in oily fish such as herring, mackerel and sardines as a result of oxidation of fat which creates unpleasant odours and flavours (FSA, 2004)], oxidation, hydrolysis and microbial spoilage, therefore requiring prompt processing and preservation to avoid spoilage.

Fish smoking which involves the application of smoke from smoldering wood for the preservation of fish dates back to civilization. The bacteriostatic, bactericidal and antioxidant function of smoke and dehydration effect of the process were used inadvertently by the early fish processors in the preservation of fish (Eyo, 2001). Smoke contributes greatly to fish preservation by acting as an effective antioxidant, bacteriostatic and bactericidal agent, as well as providing a protective film on the surface of the smoked fish. The smoke compounds implicated in the bacteriostatic effect are the acids, aldehydes and phenolic compounds (Barylko-Pikielna, 1977). However the resistance of microbes to the inhibiting effect of smoke varies. Micrococcus species are repoted to be more sensitive to the inhibiting effect of smoke than lactobacillus species and streptococcus species while Yeast and mould are more resistant to inhibiting influence of smoke (Clucas, 1996). Smoke drying of fish is one of the conventional procedures for reducing water activity (a_w) which expresses the moisture requirement of micro-organisms (Clifford *et al.,* 1980). This is effective in fish preservation as the nature and extent of fish spoilage depends proportionally on water activity, an important constituent of biological systems (Ihekeronye and Ngoddy, 1985). Fish smoking helps in slowing down fish deterioration thereby giving the commodity a longer shelf-life.

However, investigations have shown the presence of microbial contaminants even on smoked fish (Wogu and Iyayi, 2011; Nyarko *et. al.,* 2011). Most of the post processing microbial contaminants such as bacteria and fungi originate from poor handling practices while some could be from the air, the source of the fish, or from other degrading substances. In humid tropical conditions, very dry smoked fish with low moisture contents are prone to insect infestation, while others not so dry, having medium to high moisture contents suffer from both bacterial and mould contaminations (Banwart, 2004). Contamination by these microorganisms can cause human infections. Fungal contaminants found in fish, which are known to cause disease in humans include: *Aspergillus spp* which produce aflatoxins that causes heptoma (cancer of the liver), acute hepatitis, reduced red blood cell and decreased immune system in man; *Fusarium sp*. which is reported to produce fumonisin toxin and; *Penicillium spp* which produces penicillic acid (Wogu and Iyayi, 2011). Bacterial pathogens, which may be transferred from fish to human beings include: *A. hydrophila* (septicemia, diarrhea), *Campylobacter jejuni* (gastroenteritis), *Clostridium botulinum* type E (botulism), *Edwardsiella tarda* (diarrhea), *Leptospira interrogans* (leptospirosis), *Mycobacterium spp.* (mycobacteriosis), *Plesiomonas shigelloides* (gastroenteritis), *Pseudomonas aeruginosa* (wound infections), *Salmonella sp.* (food poisoning) *Vibrio parahaemolyticus* (food poisoning) (Austin and Austin, 1989) and *Streptococcus iniae* (meningoencephalitis) (Bercovier *et al.,* 1997). Some bacteria that come in contact with fishes, capable of producing toxic chemicals (such as, *Bacillus cerus, Bacillus subtilis, Bacillus lieheniformis* and B*acillus megaterium)* can survive certain preparatory processes such as heating and drying due to their endospores and are thus found even on dried foods (Collins *et al.,* 1999). Prolonged intake of smoked fish with these metabolites may constitute potential public health hazard. Sardines are generally imported frozen into Nigeria and

smoking is hence conducted by retailers (Clucas, 1981). Smoked sardine is a common sight in Makurdi markets, Nigeria. This study is thus tailored towards investigating the types and level of microbial contamination of smoked sardine (*Sardina pilchardus*) sold in makurdi markets so as to ascertain the potential danger in which the consumers may be exposed to.

Materials and Methods

Sample Collection and Preparation

A total of forty five (45) Smoked Sardine (*Sardina pilchardus*) were randomly collected from the five different Markets (North-bank, Wurukum, High Level, Wadata and Modern Markets) in Makurdi, Nigeria. Nine (9) samples were collected randomly between January and March, 2010 from each market on three sampling occasions and wrapped aseptically. Samples were transported from the Markets to the veterinary microbiology laboratory of the University of Agriculture, Makurdi in sterile polythene bags for microbial analysis.

Portions of the skin surface, gills and tissue of all the samples were cut and 1g of each sample was weighed out and homogenized in sterile test tube containing 9ml of distilled water which became the stock solution (1:10 dilution). Serial dilutions were then made up to 10^{-9} and 10ml.

Media Used

The media used included: nutrient agar (NA), which is suitable for the growth of most bacteria and was used for routine work in culturing microorganisms; Manitol salt agar (MSA) was used for *Staphylococcus aureus;* Eosin Methylene Blue Agar (EMBA) was for E. Coli; Sabauroude dextrose agar(SDA) was used for Mycological analysis; Mackonkey Agar (MCA) was used to enhance the growth of Gram-negative organisms and as a multipurpose agar; Salmonella shiggella agar (SSA) was used for culturing salmonella and shigella species.

All media for isolation of the organisms were prepared aseptically according to manufacturer's specification.

Inoculation of media plates

Pour-plate method was adopted for inoculation of all media plates. A loopful of each stock solution was picked with a well flamed wire loop and inoculated on each of the media plates by streaking across the surface of the media plates. The inoculums were spread by swirling the plate gently. The plates were then subjected to 24 hours incubation at 37^{o}C after which all plates were read. Each discrete colony observed was picked and streaked on fresh media plates to obtain pure cultures. All plates and test tubes used were properly labeled according to dilution, agar used and Market from which the sample was obtained. Bacterial growths from the plates were subjected to Gram staining test, motility test and Bio-chemical (Coagulate, Catalase, Citrate utilization test and Indole) tests, to confirm their identity.

The total viable bacteria count of each sample was estimated using the method described by Collin and Lyne (1970).

The mould count method of Harrigan and Maccance (1976) was adopted in enumerating yeast and mould.

The colony forming unit per gram (cfu/g) was calculated using the formular:

$$\text{total viable counts per gram} = \frac{\text{average number of colony counted per dilution}}{\text{dilution factor}}$$

($Cfug^{-I}$)

Water Activity (a_w)

Water activity was determined using the formular:

Where: $a_w = p/p_o$

p = vapour pressure of water in the substance at a certain temperature,

P_o = vapour pressure of pure water at the same temperature.

Result and Discussion

Table.1 shows the analysis of bacteria isolates. Samples from Wurukum market recorded the highest total viable count ($2.632 \times 10^3 \pm 0.978 \times 10^3$ $cfug^{-1}$), while samples from Modern Market recorded the least ($0.988 \times 10^3 \pm 0.272 \times 10^3$ $cfug^{-1}$), although, this difference was not statistically significant. A total of three species of bacteria (*Staphylococcus aureaus, Salmonella spp and Escherichia coli)* were isolated. There was no significant difference in the plate count of *S. aureus* and *Salmonella spp* between locations . However, a significant difference in plate count of *E. Coli* was observed, with Wurukum market recording the highest ($1.0 \times 10^3 \pm 0.407 \times 10^3$) and High level market recording the lowest ($0.00 \times 10^3 \pm 0.00 \times 10^3$).

Table.2 indicates the Biochemical Characteristics of the bacterial Isolates. All the bacteria species isolated were catalase positive and none of the isolates was oxidase positive. coagulase test was only positive for *staphylococcus aureus,* while only *E. coli* indicated indole positive.

Table 3 indicates the Morphological and Cultural Characteristics of the bacterial Isolates. Two gram negative rod shaped bacteria species and one gram positive coccus bacteria species were isolated. The gram positive coccus bacteria isolated was non-motile while all the gram negative bacteria were motile. Each bacteria species showed different growth characteristics.

Table 4 shows the percentage frequency of bacteria isolated from the five (5) markets sampled. A total of three bacteria in seventy eight (78) occurrences were recorded.

Table.5 shows Mycological Analysis using Sabarode dextrose Agar (SDA). A total of four (4) species of fungi were isolated. All the fungi isolated from the various markets had different growth characteristics and shapes with the exception of samples from High Level Market and the North Bank Market having *Aspergillus spp* which exhibited similar shades of greenish colonies. *Aspergilus niger* was isolated from samples of High Level Market and North Bank Market. *Alternaria Spp* was isolated from samples of Modern market, *Mucor spp* from samples of Wadata market and *Yeast spp* was found in samples of Wurukum market.

Table 6 shows the Mean water activity (a_w) of all samples used, ranging from 49.00 ± 0.26 (high level market) to 48.20 ± 0.57 (Modern market). The difference between mean water activity of all the samples was not statistically significant.

Table.1 Bacteriological Analysis of smoked *Sardina Pilchardus*

Isolates/ Market location	*S. aureus* (cfu/g)	*Salmonella spp* (cfu/g)	*E. coli* (cfu/g)	Mean Total viable count (cfu/g)
High Level	$1.344 \times 10^3 \pm 0.218 \times 10^3$	$0.550 \times 10^3 \pm 0.196 \times 10^3$	$0.000 \times 10^3 \pm 0.000 \times 10^{3b}$	$1.899 \times 10^3 \pm 0.414 \times 10^3$
Modern	$0.877 \times 10^3 \pm 0.161 \times 10^3$	$0.100 \times 10^3 \pm 0.100 \times 10^3$	$0.011 \times 10^3 \pm 0.011 \times 10^{3\,b}$	$0.988 \times 10^3 \pm 0.272 \times 10^3$
North Bank	$0.522 \times 10^3 \pm 0.254 \times 10^3$	$0.455 \times 10^3 \pm 0.253 \times 10^3$	$0.766 \times 10^3 \pm 0.020 \times 10^{3\,a}$	$1.743 \times 10^3 \pm 0.527 \times 10^3$
Wadata	$1.300 \times 10^3 \pm 0.306 \times 10^3$	$0.233 \times 10^3 \pm 0.130 \times 10^3$	$0.944 \times 10^3 \pm 0.720 \times 10^{3\,a}$	$2.477 \times 10^3 \pm 1.156 \times 10^3$
Wurukum	$0.977 \times 10^3 \pm 0.270 \times 10^3$	$0.655 \times 10^3 \pm 0.301 \times 10^3$	$1.000 \times 10^3 \pm 0.407 \times 10^{3\,a}$	$2.632 \times 10^3 \pm 0.978 \times 10^3$

Mean values in the same column with the same superscript are not significantly different ($p>0.05$)

Table.2 Biochemical Characteristics of the bacterial Isolates of smoked *Sardina Pilchardus*

S/N	Microorganisms	Oxidase test	Catalase test	Coagulase test	Indole test
1	*Salmonella Spp*	-	+	-	-
2	*Staphylococcus aureaus*	-	+	+	-
3	*Escherichia coli*	-	+	-	+

\+ = Positive ; - = Negative

Table.3 Morphological and Cultural Characteristics of the bacterial Isolates of smoked *Sardina Pilchardus*

S/No	Microorganisms	Gram reaction	Shape	Culture medium	Description of colonies
1.	*Salmonella spp*	-	Straight rod	SSA	Creamy brown, 2– 3mm in diameter at 24 hours, With black or brown centre.
2.	*Staphylococcus aureus*	+	Clustered cocci	MSA	Deep yellow colonies of about 0.75mm diameter having uniform colouration
3.	*Escherichia coli*	-	Straight rod	EMBA	It show blue-black by transmitting light and have metallic sheen incident light

\- = Gram negative ; += Gram positive

Table.4 Frequency and Percentage occurrence of bacterial Isolates of smoke-dried *Sardina Pilchardus*

S/N	Isolates	Frequency	Percentage Occurrence
1.	*Staphylococcus aureaus*	38	48.7%
2.	*Salmonella spp*	17	21.8%
3.	*Escherichia coli*	23	29.5%
		$\Sigma f = 78$	

Table.5 Mycological Analysis of smoke-dried *Sardina Pilchardus* using Sabarode dextrose Agar (SDA)

Sampling Sites	Pathogen Isolated	Growth rate	Morphological Appearance	Microscopic Appearance of Colonies in Lacto phenol Cotton blue Preparation
High Level Market	*Aspergilus niger*	1 - 2 days (fast)	Initially white to yellow but later becomes distinctly black as colony develops	The vesicle of the condiosphore is large and globose, bearing 2 series of sterigmata over its entire surface. The conidia are brown to black and rough walled.
Modern Market	*Alternaria SPP*	2 - 4 days (slow)	They grow thick colonies which are black or gray in colour. The phialides are clustered on a club shaped or spherical vesicle.	Individual conidiophores arise directly from substrate forming bushy heads consisting of 4-8 large catenate conidia chains.
North Bank Market	*Aspergilus niger*	1 - 2 days (fast)	Initially white to yellow but later becomes distinctly black as colony develops	The vesicle of the condiosphore is large and globose, bearing 2 series of sterigmata over its entire surface. The conidia are brown to black and rough walled. Spores are black and green.
Wadata Market	Mucor Spp	1 - 3 days (fast)	Cotton-like or white in colour to beige or gray and are fast growing	Simple or branched and form apical, globular sporangia that are supported and elevated by a column-shaped columella.
Wurukum Market	Yeast spp	(2-5days) slow growth	Creamy white	Are bi-polar budding cells with "lemon" – shaped mother tips.

Table.6 Mean water activity (a_w) of smoke- dried *sardina pilchardus*.

Market Location	Water activity (a_w)
High Level	49.00 ± 0.26
Modern	48.20 ± 0.57
North Bank	48.73 ± 0.43
Wurukum	48.46 ± 0.58
Wadata	48.24±0.23

Values were not significantly different ($p>0.05$).

Figure.1 Linear Relationship between Water Activity and *S. Aureus* count (cfu/g)

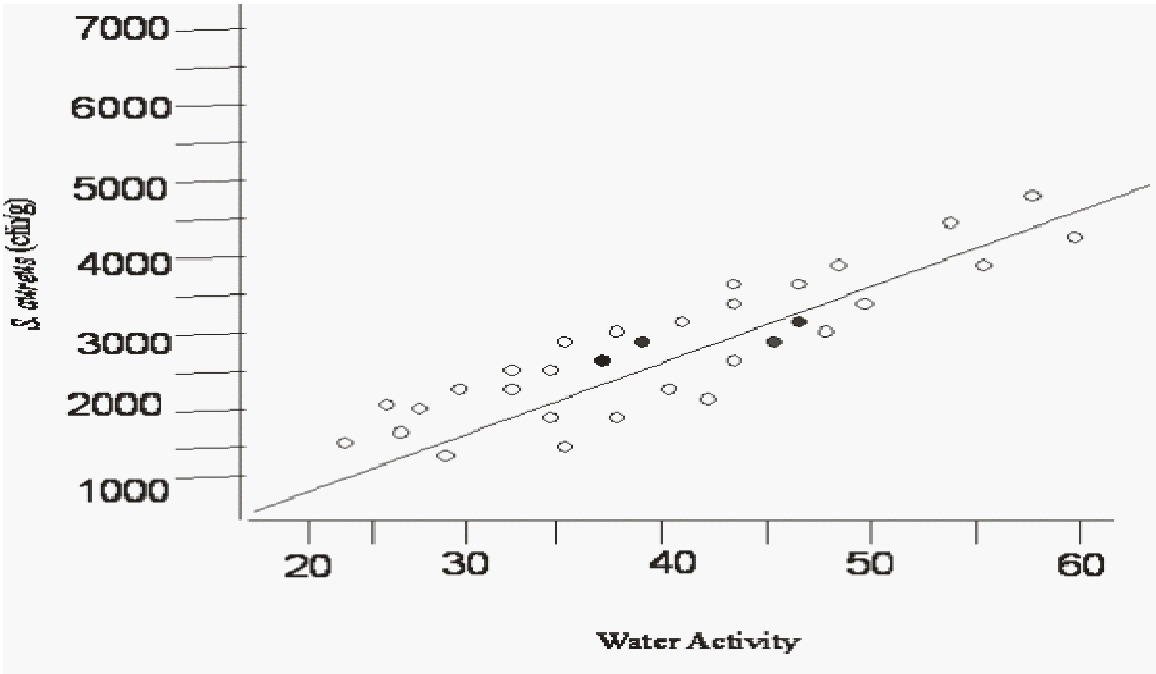


Figure.2 Linear Relationship between Water Activity and *Salmonella spp* count (cfu/g)

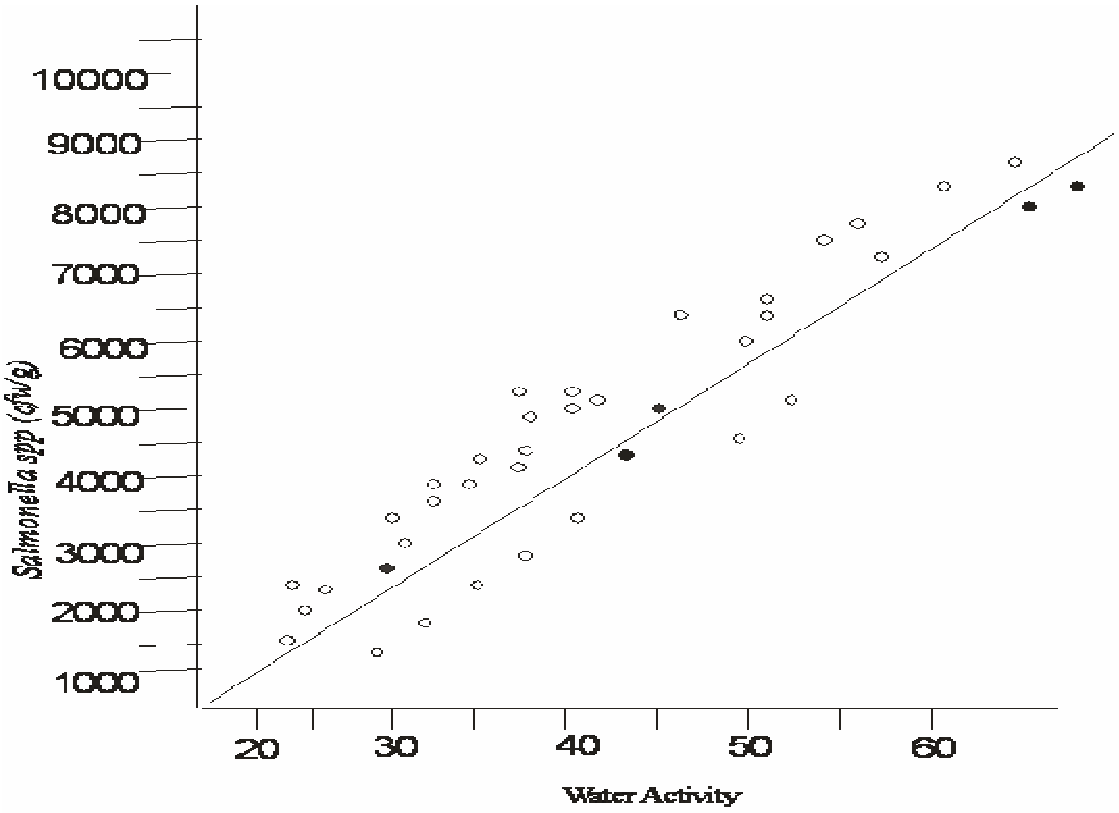
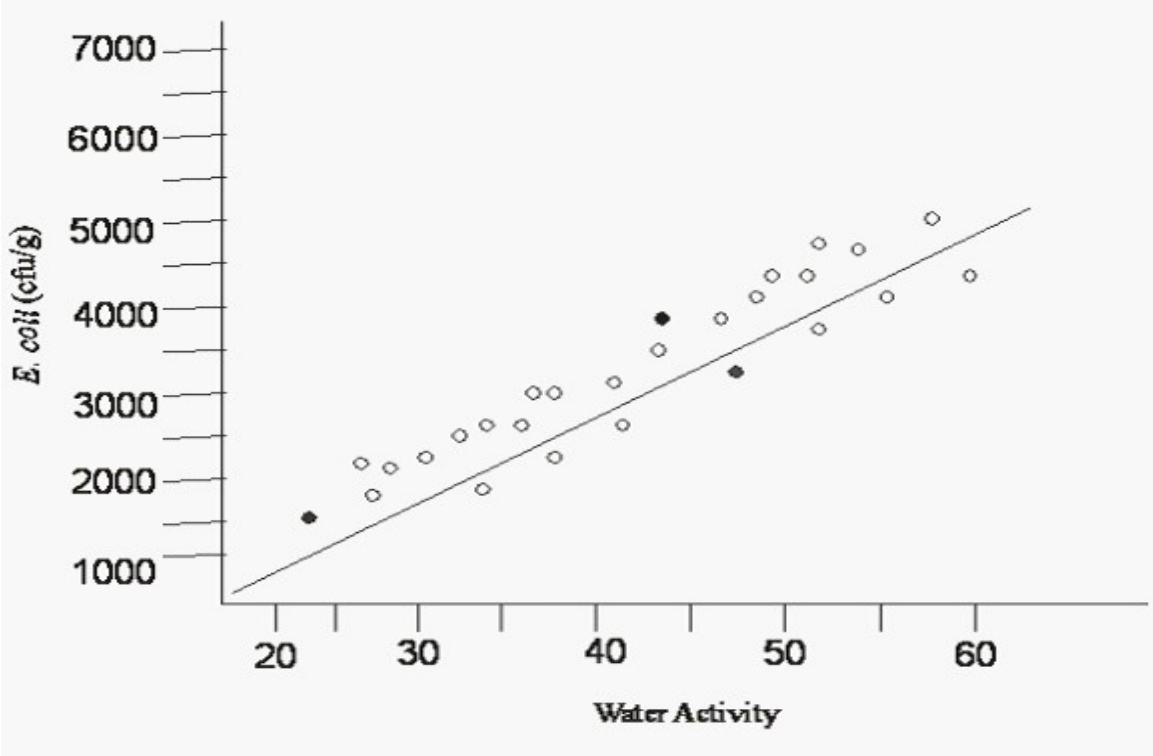


Figure.3 Linear Relationship between Water Activity and *E. Coli* count (cfu/g)



There was a positive linear relationship between bacterial load and water activity as observed in figures 1 - 3.

Results obtained in this study reveals that a total of seven (7) types of microbes [three species (3) of bacteria and four species of fungi (4)] were isolated from forty five (45) samples of smoked sardine collected from five markets in Makurdi metropolis. The isolated bacteria flora included: *Staphylococcus aureaus, Salmonella spp, Escherichia coli,* while the mycoflora included: *Aspergilus niger, Alternaria Spp, Mucor spp* and *Yeast spp.* This finding corroborates the report of Nyarko, *et. al.,* (2011) who reported the presence of *Salmonella typhi,* coliform bacteria, yeast and moulds in smoked sardine *(Sardinella aurita)* at smoking sites and market centres in Ghana. In line with this, Adegunwa, *et. al.,* (2013) also isolated fungal and bacterial contaminants from smoked *Sardinella eba.*

Similarly, the occurence of contaminating micro-organisms have been documented for other smoked fish species. Omojowo *et al.* (2009), reported that bacteria flora (*Bacillus coagulans, B. cereus, Klebsiella ozanae, Proteus vulgaris, Escherichia coli, Staphylococcus aureus, Streptococcus* spp) and fungi (*Aspergillus niger, A. candidus, A. flavus* and *A. nidulan*) were isolated in potassium sorbate untreated and smoked tilapia. He also reported the occurences of bacteria flora (*Bacillus coagulans, B. cereus, Klebsiella ozanae, Proteus vulgaris, Escherichia coli, Staphylococcus aureus,* and *Streptococcus sp*) and fungi (*Penicillium verrucosum, Aspergillus niger, A. candidus, A. flavus* and *A. nidulan*) in smoked Catfish (*Clarias gariepinus*). An experiment on fungal infestation and nutrient quality of smoke-dried *Clarias gariepinus,*

Chrysichthys nigrodigitatus, Sarotherodon galilaeus, Heterotis niloticus, Heterobranchus bidorsalis, Synodontis schall, Synodontis clarias and *Clarias anguillaris* revealed that isolates of fungi (*Fusarium* spp., *Aspergillus* spp., *Rhizopus* spp., *Mucor* spp. and *Penicillium* spp.) and bacteria (*E. coli, S. aureus* and *Salmonella* spp.) were encountered and *Aspergillus* spp. which was found in all the fish samples ranked as the most prevalent species(Fafioye *et. al.,* 2008). Another study revealed that smoked dried Stock (*Gadus morhua*), Skip jack tuna (*Katsuworus pelamis*), Croaker (*Pseudotolithus typhus*), Sting ray (*Dasyatis margarita*), Cat (*Arius hendelot*i), Bonga (*Ethalmosa fimbriota*), Ribban fish (*Triuchurius trichurius*), Stark (*Carchanas faunis*), Thread fin (*Pentanemis qumquarius*), Sole (*Cynoglossus browni*), Spade (*Drepane africana*) were highly contaminated by aflatoxigenic fungi (*Aspergillus flavus, A. tereus, Aspergillus fumigatus, Absidiasp., Rhizopus sp., A. niger, Mucorsp., Cladosporium sp., Penicillium italiculum, Penicillium viridatus, Candida tropicalis* and *Fusarium moniliformis*) (Adebayo-Tayo, *et. al.,* 2008).

In line with this, a study on the mycoflora of smoked fish species of *Ethmalosa fimbriata* (bonga fish), *Tilapia sp*. (Banda mangala), *Gadus morhua* (stock fish), *Pseudotolithus typhus* (croak*er), Arius hendeloti* (cat fish) and *Drepane africana* (spade fish) revealed the presence *of Aspergillus niger, A. flavus, Penicillium sp, Fusarium sp. Rhizopus sp.* and *Trichoderma sp.* in their order of decreasing frequency in all the fish samples (Wogu and Iyayi, 2011). These reports from various authors indicate that these microbial contaminants are not peculiar only to this fish species under study, as contamination by bacteria and fungi cuts across various smoked fish species .

The result of this study shows that Wurukum market had the highest level of microbial contamination. This could be due to the unhygienic environment of the market, as the market was observed to be highly congested and surrounded by dumping sites, waste water and dirty gutters. Exposure and improper hygiene handling seems to be other explainable reasons for the high bacterial contamination as the commodity was observed to be displayed in open trays, exposing it to contact by insects, dust particles and even consumers who were observed to be making direct contact with the commodity with bare hands while bargaining. Adebayo-Tayo, *et. al.,* (2008) observed a similar scenario in Uyo, Nigeria, where retailers displayed smoke-dried fish samples in open trays beside gutters or refuse heaps. Wurukum market was closely followed by Wadata market in terms of high microbial contamination. This could be associated with the proximity of Wadata market to River Benue which was observed to be highly polluted due to anthropogenic activities, such as defecating and refuse dumping. This is in contrast with Modern market whose environment was the most hygienic of all the markets under study and this translates to its relatively low microbial load. Similar report was given by Nyarko, *et. al.,* (2011) who reported that smoked sardine from marketing centres had higher microbial counts than those from smoking sites due to the better sanitary conditions of the latter. This conforms to the findings of Adegunwa, *et. al., (*2013) who reported that smoked fish collected at a camp location in Odeda, Ogun, Nigeria had higher microbial load than other locations

as a result of handling, frequent exposure, poor environmental and sanitary conditions. The disparities in contamination levels between location have been observed to be influenced by one or more of the factors enumerated by Tatcher and Clark (1973) as follows: Source of the raw fish; Additional contamination introduced by handlers; Temperature of food during storage and processing; Severity of freezing process in terms of lethality to microorganisms; Contamination after the fish had already been processed. For instance, apart from the afore mentioned explanatory reasons, the regular power fluctuation within Makurdi metropolis may make the freezing process of sardines (*Sardina pilchardus*) inefficient and that may result in high contamination even before the fish are smoked.

It could also be deduced that *S. aureus* was more predominant in all the locations (Markets) except in Wurukum which had more occurrances of *E.coli*. This is still attributable to insanitary conditions of Wurukum Market. Although the average total bacterial count of all locations was within the acceptable range of Aerobic Plate count of 5.0×10^5 cfu/g as stipulated by International Commission on Microbiological Specification for Food (ICMSF, 1986), the count of *salmonella spp, E. Coli* and *Staphylococci aureus* for all the locations had exceeded the recommended level of 0, 11 and 10^3 cfu/g respectively. The presence of *Salmonella spp* indicates poor food preparation and handling practices (Yusuf and Tengku Abdul Hamid, 2012) and it is associated with food borne diseases. *E. Coli* is an enteric bacteria causing gastroenteritis and the contamination of fish or fish products with pathogenic *E. coli* is associated with improper handling of fish (Ayulo *et al.,* 1994; Asai *et al.,* 1999) and fecal contamination (Feng, 2002). *Staphylococci aureus* are capable of producing enterotoxins that cause gastroenteritis (Novotny, *et. al.,* 2004). Thus, food poisoning and food bourne diseases could occur as a result of intake of this commodity. Similarly, the mycoflora contaminants (*Aspergilus niger, Alternaria Spp*, *Mucor spp* and *Yeast spp*) isolated in this study are associated with the production of toxins which are hazardous to human health. This research is therefore instructive as consumption of contaminated smoked fish could pose serious health problems to the consuming masses.

The result of this study shows a strong correlation between water activity and Bacterial load, indicating that a high water activity connotes a high bacterial load.

Thus, fish processors and retailers have to take extra care in ensuring proper processing and handling of fish and fish products by observing sanitary and hygiene rules. Appropriate authorities should enforce sanitary and hygiene rules in the market and ensure constant power supply in order encourage effective preservation of fish prior to smoking. Fish marketers should showcase the commodity in closed transparent containers instead of displaying it in open trays to minimize contamination. Consumers bargaining for the commodity should avoid making direct contact with the commodity using bare hands to curtail cross contamination.

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