

MICROBIAL QUALITY OF IMPORTED FROZEN *Sardinella* species AND *Micromesistius poutassou* OFFERED FOR SALE IN OYO STATE, NIGERIA.

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Abstract

Microbial quality of frozen fish offered for sale in Oyo state markets was investigated. The four Agricultural Development Programme (ADP) zones of the state were used for the study. *Sardinella* species and *Micromesistius poutassou* were selected among the commercially important imported frozen fish species in the state based on their availability in all zones. Bacterial and Fungal counts were determined using standard procedures. Data were analysed using descriptive statistics, percentages and t-test. Ten bacteria and three fungi and three yeast species were isolated from the fishes evaluated. Predominant bacteria isolates were *Shewanella putrefaciens* and *Streptococcus faecium*, fungi included *Penicillium notatum* and *Aspergillus niger* while yeast were *Cryptococcus laurentii* and *Torulasporea debrueckii*. Total viable counts for bacterial and fungal counts were 3.1×10^5 cfu/g and 1.8×10^5 cfu/g, respectively for *Sardinella* spp. and 3.2×10^5 cfu/g and 2.4×10^5 cfu/g for *M. poutassou*. Although all the fish samples were within acceptable limit, the number of bacteria isolated showed that the quality of frozen fish did not reach expected standard in Oyo state.

Keywords: Microbial quality; Frozen fish; Bacteria count; Fungi count, ADP zones

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Introduction

One-fourth of the world's food supply and 30% of landed fish are lost through microbial activities alone (Huis in't Veld, 1996 and Amos, 2007). Freezing is a widely used and accepted technology for preserving fish and fish products in their natural states (Tolstorebrov *et al.*, 2016). However, quality deterioration of stored fish is inevitable with length of storage period (Jeon *et al.*, 2002). Frozen fish displays third order biotic activity. It belongs to the class of foods in which the respiration process is suspended, but in which biochemical, microbial and other decomposition processes which must be taken into account still proceed (Huss *et al.*, 1992). Fish and bacteria exist in a state of equilibrium and it is only after death that bacteria can invade the tissue and spoil the fish (Clucas, 1990; Aberounmand, 2010). Bacteria according to Eyo (2001) secrete digestive juices and enzymes which breakdown the tissue and cause spoilage of fish. This results in loss of flavour and odour and is replaced by a sour and stale odour. Fish carry a flora of psychrotrophic bacteria, most of which survive freezing; and are ready to grow on thawing (Twiddy and Reilly, 1995). Fish may harbor a number of biohazards as well as chemical contaminations such as biogenic amines, biotoxins, pathogenic bacteria and viruses if not properly handled (Ashie *et al.*, 1996 and Gram *et al.*, 2000).

Contamination concern has been on high loads of unsuspected spoilage microorganisms like *Salmonella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Bramsnacs, 1999). During spoilage, microbes mainly bacteria spoil the fish to such an extent that it cannot be edible (Abbas *et al.*, 2009). Consumers are however becoming more aware of possible hazards, malpractices and mistakes arising from the food they consume and are individually and collectively becoming more demanding in respect of freshness, naturalness, microbial safety, freedom from pollutants, protection from damage and convenience. There is a need for determination of spoilage parameters to minimize losses, thereby increasing the quality of fish available for human consumption in many of the developing countries.

The objective of this study therefore is to evaluate the microbial load of frozen fish offered for sale in Oyo state markets and its effects on wholesomeness and safety of the product.

2. Materials and Methods

2.1 Microbiological Analysis

2.2 Sample collection

Frozen samples of *Sardinella* species (sardine) and *Micromesistius poutassou* (blue whiting) were collected from the retail depots at different zones on bi-monthly basis for a period of twelve months between January and

December. The two different samples were wrapped with aluminum foil and placed in sterile plastic containers with ice and conveyed to the laboratory for microbiological analysis.

2.3 Sample Preparation

The samples of *Sardinella* species and *Micromesistius poutassou* were aseptically removed from the plastic container and were placed on a sterile tray and with the aid of a sterile knife; cuts were made from the edible parts of the samples. 90ml peptone water was added to 10g of fish flesh and homogenized in a blender for a minute at high speed. 10ml of the original homogenate fluid was then taken for microbiological analysis.

2.4 Culture Media

Plate Count Agar (PCA), Mac Conkey Agar (MCA), Mannitol Salt Agar (MSA), *Salmonella Shigella* Agar (SSA), Potato Dextrose Agar (PDA), Blood Agar (BA) De Man, Rogosa Sharpe (MRS) Agar and Yeast Extract Agar (YEA) were weighed and distilled water was added according to Manufacturer's instruction. The solution was homogenised in a water bath for 10 minutes. The medium was then sterilized in an autoclave at 121°C for 15 minutes and allowed to cool to 45°C before use.

2.5 Enumeration and Isolation of Microorganisms

Serial dilutions were made from the samples using sterile pipette. This was done by mixing 1g of the sample thoroughly with 9ml of sterile distilled water to give 1:10 dilution. The dilution was made up to 10⁻⁶. Using sterile pipette of 1ml, appropriate dilutions were plated out using different culture media (Harrigan and McCance, 1976). The plates were inoculated in duplicates and allowed to set. After solidifying, MRS plates were incubated in a carbon-dioxide enriched jar at 37°C for 48 hours. Other bacterial plates were incubated aerobically at 30°C for 24 hours, while Potato Dextrose Agar plates were incubated for 3-5 days. At the end of incubation, representative colonies were selected at random and sub-cultured repeatedly to obtain pure cultures.

The culture media were used in the isolation and enumeration of the microbial loads of the fish samples. PCA for Total Viable Count, MCA was used for *Enterobacteriaceae* count, SSA for *Salmonella* and *Shigella* count, MSA for *Staphylococcus* count, MRS for Lactic acid bacteria, BA for haemolytic *Streptococci* count, PDA for fungi load and YEA for Yeast count using the pour plate technique. All isolated colonies were counted and expressed as colony forming units (CFU/g) per gram of fish.

2.6 Characterization of the Isolates

Characterization of the isolates was carried out by employing macroscopic, microscopic, biochemical and physiological tests such as Gram's staining, catalase test, use of API 20E and API 50 CH strips and medium for *Enterobacteriaceae* and lactic acid bacteria respectively (Willey *et al.*, 2008; Zwadyw *et al.*, 1977).

2.7 Characterization of *Staphylococcus* isolates

The tests employed in characterizing the isolates included Gram reaction, motility test, production of catalase, coagulase, starch hydrolysis, utilization of glucose, sucrose, lactose, mannitol, maltose, xylose, fructose and galactose. Identification to the generic level was done using Bergey's manual of determinative bacteriology (Holt *et al.*, 2000) as a reference of identification based on the result of the various biochemical tests obtained.

2.8 Cultural and Morphological Characterization of Fungal isolates

The isolates were characterized based on the pigmentation of the spores, nature of the mycelia and spores formed. Microscopic details were studied by performing a wet mount using lactophenol cotton blue mounting fluid. The preparation was examined under objective lens. The fungi were identified as detailed by Barnett and Hunter (1972), Rhode and Hartmann (1980), Kulwant *et al.* (1991) and Larone (2002).

2.9 Characterization and Identification of Yeast isolates

This was carried out employing standard morphological, physiological and sugar fermentation pattern tests. Identification was done using identification keys described by Barnett *et al.* (2000). Yeast isolates were further characterized by the conventional methods as described by Kreger Van Rij (1984).

2.10 Statistical Analysis

The statistical programme, (SPSS, 2003) VERSION 16.0 was used to analyze the result of the treatments. Descriptive statistics and percentages were used for the data collected while T-test was used to determine whether there was a difference in microbial load of the two frozen fish species examined.

3. Results

3.1 Microbiological Evaluation of Samples

The Total Bacteria Count of imported frozen fish across the four zones of the study area as shown in Table 1 indicates that the highest Total Viable Count (3.1×10^5 cfu/g) was from Ogbomoso zone in *Sardinella* spp. fish samples, while the lowest was from Oyo zone (1.2×10^5 cfu/g). In *M. poutasssou*, the highest TVC was also in Ogbomoso zone (3.2×10^5 cfu/g) and the lowest in Oyo zone (1.5×10^5 cfu/g). The highest Total *Salmonella-Shigella* Count (TSSC) in *Sardinella* spp. samples was recorded in Ogbomoso zone (6.9×10^3 cfu/g) and the least was from Oyo zone (4.1×10^3 cfu/g). The highest TSSC in *M. poutasssou* was recorded in fish samples collected in Saki zone (1.0×10^4 cfu/g) and the least (4.9×10^3 cfu/g) was from Oyo zone. Total Haemolytic Streptococci Count (THSC) in *Sardinella* species was highest in Oyo zone (3.5×10^4 cfu/g) and the least was in Saki zone (2.8×10^4 cfu/g). Ogbomoso zone (3.5×10^4 cfu/g) recorded the highest THSC in *M. poutasssou*, while Ibadan/Ibarapa recorded the least value of 3.1×10^4 cfu/g. Total Lactic Acid Bacteria Count (TLAB) in *Sardinella* species was lower in Ibadan/Ibarapa, Ogbomoso and Saki zones than in *M. poutasssou*. Oyo zone was the only zone that recorded a higher TLAB count of 6.8×10^3 cfu/g in *Sardinella* spp. than *M. poutasssou* (3.1×10^3 cfu/g).

Frozen samples collected from *M. poutasssou* had higher total *Enterobacteriaceae* Count (TEBC) in all zones compared to *Sardinella* species. The highest TEBC was recorded in Ogbomoso zone for both *M. poutasssou* (4.1×10^4 cfu/g) and *Sardinella* species (3.1×10^4 cfu/g) while the least was 2.3×10^4 cfu/g in Ibadan/Ibarapa zone and 1.2×10^4 cfu/g in Oyo zone respectively. Highest Total *Staphylococcus* Count (TSC) for *Sardinella* spp. samples was recorded in Oyo zone (3.0×10^3 cfu/g), while the least was in Saki zone (1.0×10^4 cfu/g). In *M. poutasssou* samples, highest TSC (6.6×10^3 cfu/g) was recorded in Ibadan/Ibarapa zone and the least (1.0×10^3 cfu/g) in Oyo zone. However, *M. poutasssou* samples had higher *Staphylococcus* count than *Sardinella* species in Ibadan/Ibarapa, Ogbomoso and Saki zones, while the case was different in Oyo zone where *Sardinella* species had higher counts than *M. poutasssou*.

The frequency of occurrence of bacteria isolated in *Sardinella* species showed that *Streptococcus faecium* was highest with 16.8% followed by *Shewanella putrefaciens* 16.1% and *Salmonella typhi* (14.8%), while the least bacteria count of 1.9% was recorded in *Leuconostoc mesenteroides*. In *M. poutasssou* samples, *Shewanella putrefaciens* also recorded the highest value of 16.5% followed by *Salmonella typhi*, 15.9% and *Streptococcus faecium* 14.1% while the least value of 2.9% was recorded in *Pediococcus damnosus*. All the 10 bacteria species recorded in this study showed their occurrences in both *Sardinella* spp. and *M. poutasssou* fish samples with *Shewanella putrefaciens*, *Streptococcus faecium* and *Salmonella typhi* showing higher frequencies of occurrence in both fish species. In Table 2, the result of the t-test conducted on the mean values of the TVC of the two fish species across zones showed significant difference ($p < 0.05$) in the two fish species.

Colonially, the size of the test isolates ranged from small to medium and their forms ranged from circular to irregular shapes. They had creamy, brownish to pinkish colouration with entire edges. Opacity ranged from translucent to opaque. They had raised elevation and consistency varied from friable, butyrous to viscid. The bacterial population of the frozen fish samples consisted of both Gram negative and Gram positive rods and cocci. The Gram negative bacterial isolates were catalase and oxidase negative, rod shaped, producing acid aerobically and anaerobically from glucose in Hugh-Leifson's medium and they were members of the family *Enterobacteriaceae*. The representative organisms identified were *Salmonella typhi*, *Shewanella putrefaciens*, *Enterobacter asburiae*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. Gram positive isolates were catalase and oxidase negative, non-endospore forming fermentative organisms recognized as members of the lactic acid bacteria group. They included *Lactobacillus acidophilus*, *Leuconostoc mesenteroides*, *Pediococcus damnosus* and *Streptococcus faecium* (Table 3). The *Staphylococcus aureus*, also isolated from the samples was found to be coagulase and catalase positive and did not ferment lactose, glucose and fructose, but were found to ferment only sucrose. The strains were also mannitol-positive, but found to be indole and motility negative and gram-positive cocci bacteria.

Table 1: Total Bacteria Count of imported frozen fish across the ADP zones

Fish sample	Zones	TVC (cfu/g)	TSSC (cfu/g)	THSC (cfu/g)	TLAB (cfu/g)	TEBC (cfu/g)	TSC (cfu/g)
<i>Sardinella</i>	Ibadan/Ibarapa	1.7×10^5	5.5×10^3	3.0×10^4	4.8×10^3	1.4×10^4	2.9×10^2
Species	Ogbomoso	3.1×10^5	6.9×10^3	3.1×10^4	3.7×10^3	3.1×10^4	1.0×10^3
	Oyo	1.2×10^5	4.1×10^3	3.5×10^4	6.8×10^3	1.2×10^4	3.0×10^3
	Saki	1.4×10^5	5.9×10^3	2.8×10^4	3.8×10^3	2.2×10^4	1.7×10^3
<i>Micromesistius</i>	Ibadan/Ibarapa	2.6×10^5	7.5×10^3	3.1×10^4	8.6×10^3	2.3×10^4	6.6×10^3
<i>Poutasssou</i>	Ogbomoso	3.2×10^5	9.5×10^3	3.5×10^4	9.5×10^3	4.1×10^4	1.5×10^3
	Oyo	1.5×10^5	4.9×10^3	3.5×10^4	3.1×10^3	2.6×10^4	1.0×10^3
	Saki	2.2×10^5	1.0×10^4	3.2×10^4	6.7×10^3	3.4×10^4	1.1×10^3

Table 2: T- Test for Mean values of Quality indices in *Sardinella* species and *Micromesistius poutassou* across the ADP zones

Parameter	Sample	N	$\bar{x} + SEM$	Df	t-value	P Level	(<0.05)
Log Total viable count	<i>Sardinella</i> spp. <i>M. poutassou</i>	30	0.2±0.10 0.29±0.06	58	-2.32	0.024	(<0.05)

Table 3: Frequency of occurrence of bacteria isolates in the fish samples across the ADP zones

Bacteria Species	Fish sample	
	<i>Sardinella</i> species (%)	<i>Micromesistius poutassou</i> (%)
<i>Salmonella typhi</i>	14.8	15.9
<i>Streptococcus faecium</i>	16.8	14.1
<i>Shewanella putrefaciens</i>	16.1	16.5
<i>Enterobacter asburiae</i>	10.3	8.2
<i>Pseudomonas aeruginosa</i>	9.0	12.4
<i>Proteus vulgaris</i>	12.9	13.5
<i>Lactobacillus acidophilus</i>	9.0	6.5
<i>Leuconostoc mesenteroides</i>	1.9	4.1
<i>Pediococcus damnosus</i>	3.2	2.9
<i>Staphylococcus aureus</i>	5.8	5.9
Total	100.0	100.0

3.2 Mould and Yeast Isolates in the Imported Frozen Fish Samples.

Based on the pigmentation of the spores, nature of the mycelia and spore formation, *Aspergillus niger*, *Penicillium notatum* and *Geotrichum* species were isolated from the two frozen fish samples. As shown in Table 4, Oyo zone has the lowest fungal count in both *Sardinella* spp. (7.9×10^4) and *M. poutassou* (1.4×10^5) samples respectively. In *Sardinella* spp. samples, the highest fungal load was in Ogbomoso zone (1.8×10^5) while *M. poutassou* samples recorded 2.4×10^5 in both Ibadan/Ibarapa and Saki respectively. In Table 5, frequency of occurrence of fungal isolates for *Sardinella* spp. showed that *Penicillium notatum* with the highest occurrence of 63.9%, *Aspergillus niger* (25%) and *Geotrichum* species with the least (11.1%). In *M. poutassou*, *Aspergillus niger* had the highest occurrence (39.1%), while both *Penicillium notatum* and *Geotrichum* species were 30.4% each respectively.

The yeast isolates were identified as *Pichia farinose*, *Cryptococcus laurentii* and *Torulaspora delbrueckii*. All yeast isolates fermented glucose, none was able to ferment xylose, arabinose and methanol while none utilize nitrate. In Table 6, yeast occurrence in *Sardinella* spp. samples shows *Pichia farinose* with highest (38.6%), while *Torulaspora delbrueckii* and *Cryptococcus laurentii* had 31.8% and 29.6% respectively. In *M. poutassou* samples, *Cryptococcus laurentii* has the highest (42.9%), *Torulaspora delbrueckii* (32.6%) and *Pichia farinose* (24.5%).

Table 4: Total Fungal count of imported Frozen Fish across the ADP zones

Fish sample	ADP zones	Yeast count (cfu/g)	Fungal count (cfu/g)
<i>Sardinella</i> species	Ibadan/Ibarapa	1.7×10^5	1.6×10^5
	Ogbomoso	1.8×10^5	1.8×10^5
	Oyo	2.2×10^5	7.9×10^4
	Saki	2.4×10^5	1.3×10^5
<i>Micromesistius Poutassou</i>	Ibadan/Ibarapa	2.1×10^5	2.4×10^5
	Ogbomoso	3.1×10^5	2.3×10^5
	Oyo	2.6×10^5	1.4×10^5
	Saki	2.8×10^5	2.4×10^5

Table 5: Frequency of occurrence of Fungi isolates in Frozen fish sample across the ADP zones

Fungi species	Fish sample	
	<i>Sardinella</i> species (%)	<i>Micromesistius poutassou</i> (%)
<i>Penicillium notatum</i>	63.9	30.4
<i>Aspergillus niger</i>	25.0	39.2
<i>Geotrichum</i> species	11.1	30.4

Table 6: Frequency of occurrence of Yeast isolates in Frozen fish sample across the ADP zones

Yeast species	Fish sample	
	<i>Sardinella</i> species (%)	<i>Micromesistius poutassou</i> (%)
<i>Pichia farinose</i>	38.6	24.5
<i>Cryptococcus Laurentii</i>	29.6	42.9
<i>Torulaspora Debrueckii</i>	31.8	32.6

4. Discussion

The presence of contaminating bacteria in seafoods could be attributed to cross-contamination from the environment, source, and handling along the distribution chain (Bryant *et al.*, 1988). The mean viable count of the organisms from the study was found to be within local and international standards (SON, 2004 and ICMSF, 2005) for frozen fish products which are between 5.0×10^5 and 1.0×10^6 CFU g⁻¹.

In this study, *Pseudomonas* species was isolated from the fish samples collected from the four zones. The isolation of *Pseudomonas* spp. from experimental samples is important because *Pseudomonas* is a potential pathogen and spoilage agent (Koutsoumanis and Nychas, 2000; Jeyasekaran *et al.*, 2006 and Yaqoub, 2009). *Pseudomonas* can survive freezing temperature and will resume growth when thawed (Frazier and Westhoff, 1988). This also corroborated Adebayo-Tayo *et al.* (2012), who isolated *Pseudomonas* spp. from frozen mackerel. *Pseudomonas* spp. also predominates in *Oreochromis niloticus* stored in ice (Farag, 2012).

Shewanella putrefaciens isolated from the fish samples is also a well-defined spoilage bacteria. It utilizes TMAO as the terminal electron acceptor in an anaerobic respiration resulting in off-odours and off- flavours due to formation of TMA (Dalgaard, 1995). Jorgensen (1988) and Jorgensen *et al.* (1989) also isolated *S. putrefaciens* in packed fish products and were observed to be capable of producing TMA and is indeed specific spoilage organism. *S. putrefaciens* and *Pseudomonas* were identified as specific spoilage organism of different types of fresh chilled fish when stored aerobically (Gram *et al.*, 1990). *Pseudomonas* spp. appeared responsible for sweet, fruity spoilage odours while *S. putrefaciens* was responsible for the H₂S production (Olafsdottir *et al.*, 2006). Lactic acid bacteria (LAB) were also isolated from the fish samples. As observed by Schroder *et al.* (1980), LAB occurs naturally in fish and they are easily outgrown by the Gram-negative bacteria during iced, aerobic storage. This could have accounted for why there were more *Enterobacteriaceae* isolates in the fish samples examined.

Staphylococcus aureus encountered in this study is in agreement with previous study by Eze *et al.* (2011) who isolated *S. aureus* in frozen mackerel. According to Adams and Moss (2000), *Staphylococcus aureus* is not a part of the normal flora of fish and fish products and the enumeration of *S. aureus* in food products is employed generally as a sanitation index. *Staphylococcus aureus* as an indicator of contamination of processed foods could come from the skin, mouth or nose of handlers (Clucas and Ward, 1996 and Acco *et al.*, 2003). *Staphylococcus aureus* causes many outbreaks of food poisoning resulting from hand contact (Bryant *et al.*, 1988). *Streptococcus faecium* was also isolated from the fish samples in agreement with FAO (1994) which observed that the organism is relatively resistant to freezing, which makes it potentially useful as an indicator organism for evaluating plant hygiene during processing of frozen food. However, many foods including fish products contain these organisms as normal part of their flora and they are also able to establish themselves and persist in a food processing plant.

The presence of other indicator organisms like *Enterobacter* and *Salmonella* was also reported by Okonko *et al.* (2008) who isolated *Enterobacter aerogenes* and *Salmonella* sp. in frozen seafood. *Salmonella* is normally not present on fish, but fish products may become contaminated during processing, storage, distribution or preparation for consumption (Huss *et al.*, 1987, Panisello *et al.*, 2000). The heat resistance of *Salmonella* is however very low, they are killed by pasteurization temperature and time (Bikek, 2001). The significance of the t- test on the mean values of the TVC between the two fish species implied that there was marked difference in the mean microbial load contents of both *Sardinella* species and *Micromesistius poutassou*. The presence of fungal isolates in the fish samples is in agreement with previous findings by Wogu and Maduakor (2011), who reported the presence of similar fungal species in fresh fish samples. Three fungi and three yeast species were isolated in each of the two frozen fish samples. This was fewer compared to bacteria isolates which were ten.

This corroborated earlier submission by Clucas (1990) that where water is abundant, bacteria grow much more rapidly and moulds are only of secondary importance and yeast are not important as far as sea foods are concerned but moulds, due to their ability to grow where water is limited, could be a problem on smoked or dried fish.

5. Conclusion

Total viable count (TVC), Total Staphylococcus Count (TSC), Total Haemolytic Streptococcus Count (THSC), Total Lactic Acid Bacteria count (TLAB) and Total Enterobacteriaceae Count (TEBC) were all within the Standard Microbiological Limit of 5.0×10^5 and 1.0×10^6 CFU g^{-1} across zones except for Total *Salmonella Shigella* count (TSSC) which was higher than the acceptable limit. However, for all the quality indices evaluated across zones, *Micromesistius poutassou* had higher values than *Sardinella* species, which implied that *M. poutassou* had higher pathogenic and spoilage potential than *Sardinella* spp. in the study area. Oyo zone had the highest quality of frozen fish, while Ogbomoso zone had the least quality amongst the four ADP zones of the state.

Although all the fish samples were within acceptable limit for consumption and their threshold values in terms of microbial load were not exceeded, the number of bacteria isolated showed that the quality of frozen fish did not reach expected standard in Oyo state. However, these products did not constitute any health risk or hazard since they would still be properly cooked before consumption.

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