Bacterial load, composition and succession in the African catfish, *Clarias gariepinus* (Burchell, 1822) held at ambient temperatures.

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Abstract: Microorganisms, especially bacteria are the major causes of spoilage in fresh fish. In the coastal areas of developing tropical countries where ambient temperatures are usually high and access to modern preservation and processing technologies is low, fish is often left under ambient conditions for long periods after capture, resulting in quality deterioration and spoilage with attendant increases in post harvest losses. This study was carried out to identify, characterize and estimate the number of colony forming units (CFU) of microorganisms associated with freshly slaughtered African catfish, *Clarias gariepinus* held at ambient temperatures. Microorganisms were isolated from the flesh, gills, guts, mouth and skin of fish samples at successive 6-hour intervals post-slaughter for 42 hours when the fish were adjudged spoilt by a 5-member trained assessment panel. Determination of viable bacterial count was carried out by introducing aliquots of three dilutions of samples into nutrient agar plates, incubating at 37^{o}C for 24hrs and counting the number of CFU. Microorganisms were thereafter identified using colonial and morphological characteristics and biochemical tests. Organoleptic assessment of fish samples were carried out by a 5-man trained panel. Results showed that a total of eleven microorganisms of pathogenic and/or spoilage importance were isolated at various intervals from fish samples, including *Acinetobacter* spp*, Bacillus subtilis, B. megaterium, Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, K. pneumoniae, Proteus mirabilis, Pseudomonas lundensis* and *Staphylococcus aureus*. Generally the number of CFU from various parts of fish increased significantly ($P < 0.05$) as time interval increased.
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Keywords: *Clarias gariepinus,* microbial load, spoilage, postharvest losses, food safety, preservation.

Introduction

Clarias gariepinus, commonly called Clarias belongs to the family *Clariidae*, and the group of prominent cultivable Clarias species found in Nigeria (Cowx, 1992; Olaosebikan and Raji, 1998). *C. gariepinus* is a valuable food fish with high aquaculture and nutritional importance. Besides its high quality flesh, distinctive taste and texture, *Clarias* commands good market price possibly due to its relatively low fat and absence of intramuscular spines compared to other common cultivable species (Eyo, 2001). Fish is a major source of nutrients for humans, providing a significant portion of the protein intake in the diets of a large proportion of people in developing countries where it represents one of the cheapest sources of animal protein. However, freshly harvested fish is highly perishable; and depending on harvesting techniques and handling, may deteriorate and spoil within six hours of landing (Narain and Nunes, 2007). Agbon *et. al.*, (2002) and Saliu (2008) reported that fish spoilage in Nigeria is influenced to a large extent by high ambient temperatures, considerable distances of landing ports to points of utilization and poor as well as inadequate infrastructures for post-harvest processing and distribution. Apart from the high perishability of fish, consumer safety is an issue to be considered because fish is a good medium for rapid bacteria multiplication particularly when processed under unsanitary conditions. Fish is processed mainly by smoke-drying in Nigeria, however, smoking may not commence immediately after capture as fresh fish is usually left at ambient temperatures where bacterial proliferation is encouraged. Shewan (1977) and Austin (2002) observed that microorganisms associated with freshly caught fish are principally a function of the environment where it is caught. According to Lima dos Santos (1978) tropical freshwater fish have a microbial flora comprising 54% gram negative and 43% gram positive bacteria; while the flora of tropical marine fish species are 60% gram negative and 37% gram positive. Adebona (1981) reported a range of microbial load of 10^2 - 10^3 CFU/cm^2 on the skin of

fresh *Chrysichthys* species caught in Nigerian waters, while Al-Harbi and Uddin (2008) observed levels ranging from $4.3 \pm 2.9 \times 10^6$ to $1.6 \pm 3.9 \times 10^7$ cfu/g in gills filaments and $8.7 \pm 4.1 \times 10^9$ to $5.4 \pm 3.2 \times 10^{10}$ cfu/g in intestines of the common carp, *Cyprinus carpio* cultured in ponds in Saudi Arabia. Generally, microbial load increases on freshly caught fish where appropriate preservation techniques are not employed immediately after catch. As the natural defenses of fish break down as a result of death, available nutrients are used by microorganisms to sustain their life processes and support rapid multiplication. With an increase in bacterial flora and load, decomposition of the fish is rapid. This study was undertaken to assess microbial proliferation in various parts of freshly slaughtered *Clarias gariepinus* and is indicative of spoilage pattern in the fish, microorganisms responsible for spoilage in this species and possible safety concerns of consumers of this important fish.

Materials and Methods

Sample Collection

A total of eight *C. gariepinus* samples of average weight 800 $\pm$ 10g were collected from the reservoir of the University of Agriculture, Abeokuta (UNAAB), Ogun State in September 2008. They were transported live in plastic kegs to the laboratory of the Department of Microbiology, (UNAAB).

Preparation of Sample

Known weights of gill, mouth, gut, flesh and skin samples from experimental fish were aseptically dropped into 10ml sterile distilled water in order to release microorganisms on them. An aliquot of fish sample solution (1ml) was aseptically pipetted into 9ml sterile distilled water and mixed thoroughly to give 10ml of 10^2 dilution of the sample solution. The above procedure was repeated to obtain dilutions of 10^3 to 10^{12}.

Isolation of Microorganisms from Fish samples

Aliquots of 1ml fish samples were taken using a sterile syringe and cultured in Nutrient agar using the pour plate method. The plate was swirled to evenly distribute the agar and organisms; then allowed to stand for several minutes to cool. The plates were incubated at 37^{o}C for 18 – 24 hours. Further su b culturing was carried out to obtain pure colonies. The pure culture was of the organisms were inoculated on nutrient agar slants and incubation was at 37 ^{o} C for 24hours.

Total Viable Bacterial Count

Aliquots of 0.5ml of three dilution factors were introduced into three Petri dishes each. Molten nutrient agar at about 45 ^{o} C was added and then mixed thoroughly and allowed to set. The set agar was later incubated at 37^{o}C for 24hrs after which plates were examined and the number of colony forming units (CFU) per plate counted.

Identification of Microorganisms

Characterization of the organisms was based on colonial, morphological and biochemical characteristics of colonies (Table 2). Macroscopic examination of surface colonies on nutrient agar medium was used to determine the colour, edge, elevation, surface, shape and arrangement of microorganisms. Morphological characteristics were studied under the oil lens immersion microscope after Gram-staining.

Biochemical Tests

Biochemical tests carried out on the bacterial isolates were Catalase test, Coagulase test, Motility test, Indole production test, Citrate utilization test, Urease test and Sugar Fermentation tests (Table .3).

Results

Results of microbial load on the gills, gut, skin, mouth parts and flesh of freshly slaughtered *C. gariepinus* at 6-hour intervals (Figure 1) showed that microbial load on all the parts evaluated increased progressively from 0 – 42hrs post slaughter, though the rate of microbial proliferation varied within the period of study. Highest microbial load was found on the mouth, gut, gills, flesh and skin in that order. The microbial flora isolated 6-hourly from 0 – 42 hours and their significance revealed eleven bacterial species and they were identified (Table 1). Among the eleven isolates, dominant bacterial species were *Staphylococcus aureus* and *Klebsiella pneumoniae.*

Discussion

The trend of microbial proliferation was similar in all parts of *C. gariepinus* evaluated, with a distinct lag phase of little or no bacterial proliferation between 0 and 6hrs, followed by a more rapid bacterial proliferation period or log phase between 6 and 36hrs, then a stationary phase from 36 – 42hours post-slaughter in line with the findings of Al- Bulushi et. al. (2007). The relatively lower microbial load in fish flesh at 0, 6 and 12hrs of sampling confirmed the 'sterility' of the flesh during the lag phase as biochemical processes of rigor mortis within fish conferred some level of preservation on it until rigor was resolved with increased microbial proliferation encouraged by the build-up of nutrients post-rigor in line with the findings of Austin (2006). The high microbial population in the gills at these hours corroborates the findings of Al-Harbi and Uddin (2003). As time progressed post-slaughter, microbial load increased in all parts of the fish evaluated. This is probably due to the rapid

multiplication of microorganisms and their migration to all parts of fish that were initially 'sterile'. The fish were considered fit for consumption up till the 24hr post-slaughter based on their microbial load as suggested by the International Commission on Microbiological Specification for foods, ICMSF (1986) Organisms isolated included *Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae*, *Escherichia coli, Bacillus subtilis, Bacillus megaterium, Citrobacter freundii, Staphylococcus aureus, Pseudomonas lundensis, Proteus mirabilis* and *Acinetobacter species*. *Klebsiella pneumoniae* was found in all parts of fish evaluated. This confirms the ubiquitous nature and high medical importance of the *Klebsiella* group as reported by Obiamiwe and Berkowitz (2006). Organisms isolated comprised six spoilage organisms (*Bacillus subtilis, Proteus mirabilis, Citrobacter freundii, Acinetobacter species, Pseudomonas lundensis,* and *Bacillus megaterium*), three pathogenic organisms (*Staphylococcus aureus, Escherichia coli,* and *Enterobacter cloacae*) and two opportunistic pathogens (*Klebsiella pneumoniae* and *Klebsiella oxytoca*). Some of the organisms are significant as pathogens and also as spoilage agents. *Proteus mirabilis*, a spoilage agent which sometimes exist as an opportunistic pathogen under suitable conditions. *Pseudomonas species* are pathogens; however some of them are opportunistic pathogens, while four of the species (*Pseudomonas mudicolens, P. taetrolens, P. fragi,* and *P. lundensis*) are spoilage organisms. According to Gennari and Dragotto (1992), *Pseudomonas fragi* causes spoilage in dairy products, *P. mudicolens* and *P. taetrolens* cause mustiness in eggs, while *P. lundensis* causes spoilage in fish, cheese, meat and milk products.

Fish farmers should avoid culturing fish with water contaminated with fecal matter of animal origin including humans, by monitoring water source for pond aquaculture and the application of proper filtration when using other culture enclosures e.g. concrete and wooden tanks common in developing countries. This precaution will not only prevent the residence of bacteria in such waters but also protect the culture medium from the opportunistic activities of these microorganisms on fish under culture. Since most microorganisms, particularly pathogenic ones contaminate fish through improper handling and sanitation practices during processing, fish processors need to be thoroughly educated on the need for the maintenance of complete hygienic conditions during fish handling and processing.

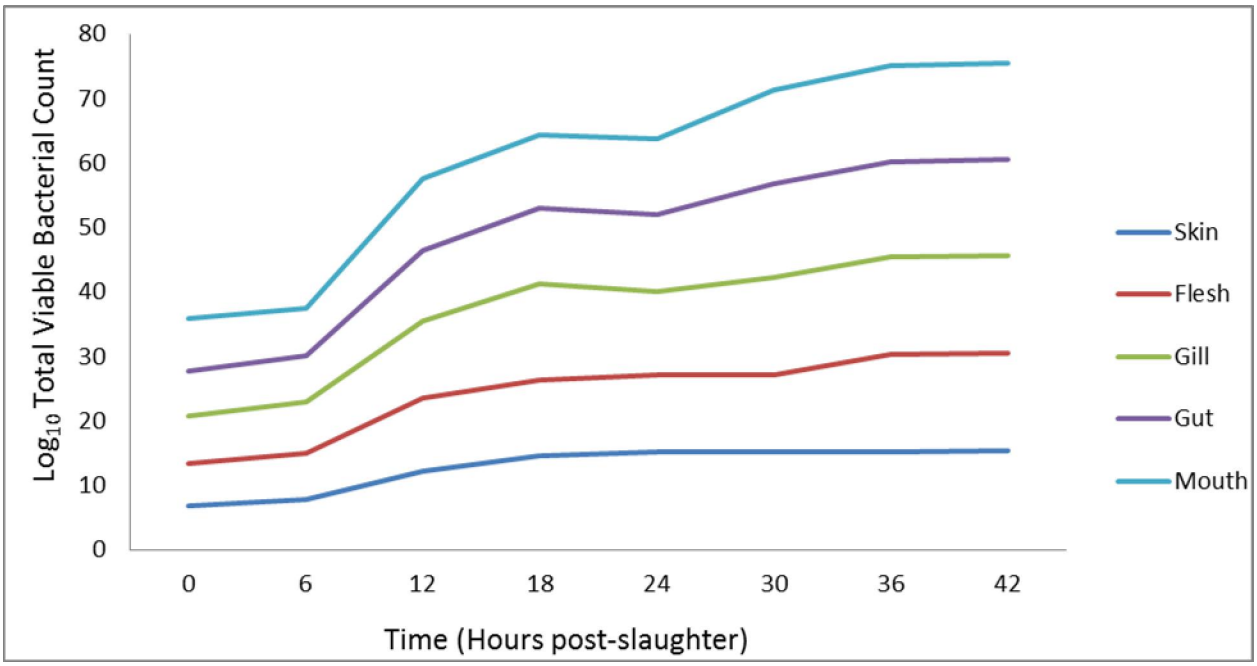

Figure 1: Bacterial load in different parts of *Clarias gariepinus* held at ambient temperatures

Table 1: Bacteria isolated six – hourly from different parts of *Clarias gariepinus* and their significance

Time (h)/Part of fish	Mouth	Gut	Gill	Flesh	Skin
0	*Staphylococcus aureus* **(P)** *Enterobacter cloacae* **(P)** *Klebsiella pneumoniae* **(OP)**	*Citrobacter freundii* **(S)** *Klebsiella pneumoniae* **(OP)** *Bacillus subtilis* **(S)** *Staphylococcus aureus* **(P)**	*Enterobacter cloacae* **(P)** *Bacillus subtilis* **(S)** *Klebsiella pneumoniae* **(OP)**	*Citrobacter freundii* **(S)** *Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Klebsiella oxytoc* *Bacillus megaterium* **(S)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Citrobacter freundii* **(S)**
6	*Enterobacter cloacae* **(P)** *Klebsiella pneumoniae* **OP)** *Staphylococcus aureus* **(P)** *Proteus mirabilis* **(S)**	*Staphylococcus aureus* **(P)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(P)**	*Klebsiella pneumoniae* **(OP)** *Staphylococcus aureus* **(P)**	*Staphylococcus aureus* **(P)**
12	*Staphylococcus aureus* **(P)** *Acinetobacter species* **(S)** *Enterobacter cloacae* **(P)** *Klebsiella pneumoniae* **(OP)**	*Klebsiella pneumoniae* **(OP)** *Enterobacter cloacae* **(P)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Klebsiella oxytoca* **(OP)**	*Staphylococcus aureus* **(P)** *Klebsiella oxytoca* **(OP)** *Citrobacter freundii* **(S)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Citrobacter freundii* **(S)** *Proteus mirabilis* **(S)**
18	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)**	*Klebsiella pneumoniae* **(OP)** *Staphylococcus aureus* **(P)** *Enterobacter cloacae* **(P)**	*Klebsiella oxytoca* **(OP)** *Klebsiella pneumoniae* **(OP)** *Enterobacter cloacae* **(P)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Klebsiella oxytoca* **(OP)** *Citrobacter freundii* **(S)**	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)** *Citrobacter freundii* **(S)**
24	*Staphylococcus aureus* **(P)** *Klebsiella pneumoniae* **(OP)**	*Citrobacter freundii* **(S)** *Staphylococcus aureus* **(P)** *Enterobacter cloacae* **(P)** *Escherichia coli* **(P)**	*Klebsiella pneumoniae* **(OP)** *Klebsiella oxytoca* **(OP)**	*Enterobacter cloacae* **(P)** *Klebsiella pneumoniae* **(OP)** *Staphylococcus aureus* **(P)** *Citrobacter freundii* **(S)**	*Staphylococcus aureus* **(P)** *Escherichia coli* **(P)** *Klebsiella pneumoniae* **(OP)** *Citrobacter freundii* **(S)**
30	*Staphylococcus aureus* **(P)**	*Klebsiella pneumoniae* **(OP)**	*Klebsiella pneumoniae* **(OP)** *Staphylococcus aureus* **(P)**	*Staphylococcus aureus* **(P)** *Citrobacter freundii* **(S)** *Klebsiella pneumoniae* **(OP)**	*Staphylococcus aureus* **(P)** *Citrobacter freundii* **(S)**

36	*Staphylococcus aureus*(P) *Enterobacter cloacae*(P) *Klebsiella pneumoniae*(OP) *Pseudomonas lundensis*(S)	*Klebsiella pneumoniae*(OP) *Citrobacter freundii*(S)	*Klebsiella pneumoniae*(OP) *Enterobacter cloacae*(P)	*Citrobacter freundii*(S) *Staphylococcus aureus*(P) *Bacillus subtilis*(S)	*Citrobacter freundii*(S) *Staphylococcus aureus*(P)	
42	*Staphylococcus aureus*(P) *Escherichia coli*(P)	*Klebsiella pneumoniae*(OP) *Klebsiella oxytoca*(OP)	*Klebsiella pneumoniae*(OP)	*Citrobacter freundii*(S) *Staphylococcus aureus*(P)	*Citrobacter freundii*(S) *Staphylococcus aureus*(P) *Klebsiella pneumoniae*(OP)	

Table 2: Colonial and Morphological characteristics of bacteria isolated from different parts of *Clarias gariepinus*

Colour	Edge	Elevation	Surface	Shape	Arrangement	Isolated Organism						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella pneumoniae*						
Cream	Undulated	Flat	Smooth	Irregular	Rod	*Citrobacter freundii*						
Translucent	Irregular	Raised	Mucoid	Irregular	Rod	*Pseudomonas species.*						
Cream	Undulated	Flat	Smooth	Irregular	Rod	*Proteus mirabilis*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella pneumoniae*						
Cream	Undulated	Flat	Smooth	Irregular	Cocco-bacillary	*Acinetobacter species*						
Cream	Undulated	Flat	Smooth	Irregular	Rod	*Proteus mirabilis*						
Cream to brown	Entire	Slightly raised	Wrinkled	Irregular	Rod in chains	*Bacillus subtilis*						
Yellow	Entire	Raised	Glossy	Round	Cocci in clusters	*Staphylococcus aureus*						
Cream	Serrated	Raised	Mucoid	Irregular	Rod	*Enterobacter cloacae*						
Cream	Serrated	Raised	Mucoid	Irregular	Rod	*Enterobacter cloacae*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella oxytoca*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella pneumoniae*						
Cream	Entire	Slightly raised	Wrinkled	Irregular	Strepto - bacillus rod	*Bacillus megaterium*						
Cream	Undulated	Raised	Smooth	Irregular	Short rod in singles	*Escherichia coli*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella pneumoniae*						
Yellow	Entire	Raised	Glossy	Round	Cocci in clusters	*Staphylococcus aureus*						
Cream	Undulated	Flat	Smooth	Irregular	Rod	*Citrobacter freundii*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella pneumoniae*						
Cream	Undulated	Slightly raised	Smooth	Irregular	Rod in pairs & singles	*Klebsiella. oxytoca*						
Cream	Serrated	Raised	Mucoid		Rod	*Enterobacter cloacae*						

Table 3: Biochemical characteristics of bacteria isolated from different parts of *Clarias gariepinus*

Gram Stain	Catalase	Coagulase	Motility	Indole	Urea	Citrate	Oxidase	Glucose	Mannitol	Doxytol	Capsulate	Isolated Organism			Isolated Organism
-			-	-	+	+	-	+	+	+		*Klebsiella pneumoniae*			*Klebsiella Pneumonia*
-			+	-	-	+	-	+	+	+		*Citrobacter freundii*			*Citrobacter Freundii*
-			+				+					*Pseudomonas species.*			*Pseudomonas species.*
-			+	-	+	+	-	+	-	-		*Proteus mirabilis*			*Proteus Mirabilis*
-			-	-	+	+	-	+	+	+		*Klebsiella pneumoniae*			*Klebsiella Pneumonia*
-			-				-					*Acinetobacter species*			*Acinetobacter Species*
-			+	-	+	+	-	+	-	-		*Proteus mirabilis*			*Proteus mirabilis*
+			+								-	*Bacillus subtilis*			*Bacillus subtilis*
+	+	+										*Staphylococcus aureus*			*Staphylococcus aureus*
-			+	-	+	+	-	+	+	-		*Enterobacter cloacae*			*Enterobacter cloacae*
-			+	-	+	+	-	+	+	-		*Enterobacter cloacae*			*Enterobacter cloacae*
-			-	+	+	+	-	+	+	+		*Klebsiella oxytoca*			*Klebsiella oxytoca*
-			-	-	+	+	-	+	+	+		*Klebsiella pneumoniae*			*Klebsiella pneumoniae*
+			+								+	*Bacillus megaterium*			*Bacillus megaterium*
-			+	+	-	-	-	+	+	+		*Escherichia coli*			*Escherichia coli*
-			-	-	+	+	-	+	+	+		*Klebsiella pneumoniae*			*Klebsiella pneumoniae*
+	+	+										*Staphylococcus aureus*			*Staphylococcus aureus*
-			+	-	-	+	-	+	+	+		*Citrobacter freundii*			*Citrobacter freundii*
-			-	-	+	+	-	+	+	+		*Klebsiella pneumoniae*			*Klebsiella pneumoniae*
-			-	+	+	+	-	+	+	+		*Klebsiella. oxytoca*			*Klebsiella. oxytoca*
-			+	-	+	+	-	+	+	-		*Enterobacter cloacae*			*Enterobacter cloacae*

Practical Application

Microbial load and flora have implications on shelf-life and preservation of fish and its safety as food. Fish is a major source of food for large populations of people in most developing countries where they are often produced under unsanitary conditions thus exposing consumers to risks of poisoning with microbial toxins and also pathogens. Evaluation of microbial load and flora will improve knowledge of the microbiology of fish, risks involved in fish consumption and target organisms for preservation strategies.

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