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Isolation and identification of pathogenic bacteria in edible fish: A case study of rural aquaculture projects feeding livestock manure to fish in Zimbabwe

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Keywords

Isolation, Faecal contaminatio, *Salmonella typhi*, *Pseudomonas aeruginosa.*

A B S T R A C T

Integrated fish farming combines livestock production with fish farming. Animal manure is shed directly into a fish pond as fertilizer and supports the growth of photosynthetic organisms. The use of different kinds of livestock manure in fish production may increase the level of pathogenic bacteria causing a public health risk to the rural community. Bacterial pathogens associated with fish can be transmitted to human beings from the fish used as food or by handling the fish, (biochemical and gram staining reactions). The following human pathogenic bacteria were isolated *Salmonella typhi, Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus* and *Enterococcus faecalis*. All the bacterial species which were isolated from the fish were also present in the initial water samples collected. The isolation of enteric bacteria in fish serves as indicator organisms of faecal contamination and or water pollution. Their presence also represents a potential hazard to humans. The mean bacterial load of the isolates was found to be markedly lower than the recommended public health and standard value of 5.0 x 10^5 CFU/ml which has been adopted by many countries.

Introduction

Fish is a vital source of food for people and contributes about 60% of the world's supply of protein. 60% of the developing countries derive 30% of their annual protein from fish (Abisoye *et al.*, 2011). It is man's most important source of high quality protein, providing approximately 16% of the animal protein consumed by the world's population (FAO, 1997). In Africa, fish supplies 17% of protein and it is one of the cheapest sources of protein in Africa (Claucas and Ward, 1996). The advantage of fish as food is as a result of its easy digestibility and high nutritional value. Fish should be viewed not only as food, but also as a ready source of

income in the smallholder farming sector (Smith and Yoshida, 2000). Fish production in earth dams or ponds can revive the once abandoned lands and make them productive. Small scale fish production also improves the livelihoods of the communal people and reduces the number of people who always depend on government for economic assistance.

However fish are susceptible to a wide variety of bacterial pathogens, most of which are capable of causing disease and are considered by some to be saprophytic in nature (Lipp and Ross, 1997). The microbiological diversity of fresh fish muscle depends on the fishing grounds and environmental factors around it (Cahill, 1990). It has been suggested that the type of micro-organisms that are found associated with particular fish depends on its habitat (Claucas and Ward, 1996). The bacterial pathogens associated with fish have been classified as indigenous and non-indigenous (Kvenberg, 1991). The non-indigenous contaminate the fish or the habitat one way or the other and examples include *Escherichia coli, Clostridium botulinum, Shigella dysenteriae, Staphylococcus aureus, Listeria monocytogens* and *Salmonella*. The indigenous bacterial pathogens are found naturally living in the fish's habitat for example *Vibrio* species and *Aeromonas* species (Rodricks, 1991). The bacteria from fish only become pathogens when fish are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to prevail (Austin, 2011). Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteium, Streptococcus spp., Vibrio spp., Aeromonas spp., Salmonella spp.* and others (Lipp and Ross, 1997).

Integrated fish farming combines livestock production with fish farming. Animal manure is shed directly into a fish pond as fertilizer and supports the growth of photosynthetic organisms. While supplemental feeding affects fish growth directly, fertilization contributes to growth via the planktonic natural food. In addition to acting as a food for fish, plankton perform other important functions in pond aquaculture: a net producer of dissolved oxygen, which is indispensable for fish growth and the most important sink of ammonia-nitrogen, which is excreted by fish (Green and Teichert-Coddington, 1993). The use of different kinds of livestock manure in fish production may increase the level of pathogenic bacteria causing a public health risk to the rural community (Musaiger and D' Souza, 2008). It has been highlighted that fish consumption can be an important avenue for human pathogenic bacteria and other food borne diseases exposure to man (Christopher *et al.,* 2009). Pathogens from fish can be transmitted to humans through both active and passive contact and may cause food borne diseases such as, dysentery, typhoid, fever, salmonellosis and cholera. The practice of livestock-fish farming needs to be placed in perspective with the likely health risks (FAO, 2003). One of the risks involved in livestock integrated fish farming is possible transfer of pathogens between livestock and humans. Previous research has shown that, different kinds of livestock manure are contaminated with pathogenic bacteria such as *Salmonella, Shigella, Pseudomonas, Vibrio, Streptococcus,* and *E. coli* species (Abdelhamid *et al.,* 2006). There is little research in Zimbabwe at the moment related to the effect of animal manure on bacterial contamination in rural aquaculture systems, especially the pathogenic species that may cause bacterial diseases in humans.

The transmission of these pathogens to people can be through improperly cooked food or the handling of the fish. There have been great economic losses reported due to food borne illness such as dysentery and diarrhea resulting from consumption of contaminated fish and such can be a problem to the immune compromised, children and elderly people.

The microbial association with fish compromises safety and the quality for human consumption; particularly critical is when the micro-organisms are opportunistic and / or pathogenic in nature (Mhango *et al.*, 2010). There may be a potential risk of infection from food borne diseases to the residents from the surrounding communities from consuming the fish from the earth dams. These circumstances prompted this research to investigate the occurrence of any human bacterial pathogens in the fish that was being caught from the ponds.

Overall Objective

To isolate and identify the bacterial species in the fish samples which are potentially pathogenic to humans

Specific Objectives

1. To isolate and quantify the human pathogenic bacteria associated with freshly caught edible fish
2. To observe the organ wise distribution of the human bacterial pathogens.

Materials and Methods

Study area: This study was conducted in the rural Chivi district where three functional pond sites, were selected. The area is situated South East of Zimbabwe 65km South West of Masvingo provincial capital which and approximately 360km from Harare. The area is in natural region IV respectively and 43% of the land is classified as arable. The area receives an average rainfall of about 500mm per year with temperatures ranging from 27–30°C from November to April and 22–27°C. Chivi district has different soil types ranging from course grained sand soil and a reddish brown sandy loam developed from granite and grasses.

Laboratory analysis

Fish samples: 150 fish samples of the Nile bream were collected from three different ponds chosen at random, at three locations namely Imbayago in ward 11, Nhengo in ward 19 and Nyamakwe in ward 21 of the district. Samples were collected using aseptic techniques early in the mornings (between 0500 and 0800 hours local time). The fish were collected aseptically and immediately transported in a thermal bag to the laboratory. They were processed within three hours of acquisition. The processed samples were stored in the refrigerator (4–8^{o}C). The fish samples were then analyzed at Midlands State University's Livestock and Wildlife Laboratory.

Sample preparation: Sample preparation was made using the method described previously (Obi and Krakowiaka, 1983). About 10 g of each of the fish samples 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 40 fish samples.

Sampling: The bacterial counts on the external surfaces, intestines and tissue were estimated as follows:

Skin surfaces: Sample from different locations of the skin of 40 raw fish was taken by rubbing the sterilized cotton swab over the skin and then inoculated into 9ml of Nutrient agar, MacConkey agar and Selenite F broth which are dispensed in separate tubes. 10 fold serial dilution of the bacterial suspension already inoculated in peptone water was prepared in duplicate and viable aerobic bacterial counts were enumerated using 0.1ml and 1ml inoculums in standard plate count agar as described by Slaby *et al.,* (1981) and then incubated at 37^{o}C for 48 hrs.

Intestines, Gills & Tissues: 1g of the fish sample was dissected out, blended and mixed properly in a mortar. It was aseptically transferred to a sample bottle containing 9mls of 0.1% sterile peptone water. The bottle was closed and shaken thoroughly for 10 minutes and allowed to stand for 20 minutes, after which a 10 fold serial dilution was carried out in duplicates and viable aerobic bacterial counts were enumerated in standard plate count agar after incubation at 37^{o}C for 48 hrs as described by (Slaby *et al.,* 1981). Coliform organisms and gram negative enteric bacteria counts were determined using pour plate method with MacConkey agar, EMB Agar, respectively.

Mueller-Hinton Agar for *Pseudomonas spp*. *Salmonella spp*. and *Shigella spp*. were enumerated using Salmonella Shigella Agar (SSA) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar for pathogenic *Vibrio spp*. The plates were incubated at 35^{o}C for 24 hrs. The observed colony growth were counted using Coulter™ Colony counter according to plate count method. Identification of the organisms was done using the phenotypic and biochemical characteristics as described previously (Slaby *et al.,* 1981) and (Cheesbrough, 1984).

Estimate 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 $\Sigma f\chi/\Sigma f$, where Σfx is the sum of the products of number of colonies and the colony forming unit per gram; while Σf is the summation of the number of colonies.

Results and Discussion

The human bacterial pathogens that were isolated and identified include *Escherichia coli, Pseudomonas aueriginosa, Shigella dynteriae, Staphylococcus aureus, Enterococcus faecalis and Salmonella typhi* as indicated in Table 1.

Staphylococcus, Escherichia coli, Pseudomonas, Shigella and *Salmonella*, were the common pathogenic bacteria found associated with fish from the ponds associated with integrated farming systems. Their presence was attributed to the contamination of the fish ponds by animal waste (Abdelhamid *et al.,* 2006).The isolation of *Salmonella, Shigella* and *E.coli* from the fish samples indicates faecal contamination of the ponds resulting from the livestock manure that they add to the fish ponds as feed.

Nyamakwe fish ponds had the highest number of bacteria compared to other ponds. The reason might be suggested that they are using more cattle manure (90kg/ha/week) compared to other livestock wastes (Abulreesh *et al.,* 2004). Nhengo earth dams use more chicken manure compared to other livestock waste, and it had the highest number of Salmonella compared to other sites. Chicken manure has been suggested to increase the percentage of positive water

samples for Salmonella (Pell, 1997). In general all the fish sites have coliform bacteria that range from 4 x 10^3 to 14 x 10^3 which are lower compared to the acceptable standard of 5 x 10^5.This might be attributed to the their design which allow water to flow out gradually and is not stagnant as it is used to irrigate their crop down the outlet pipe. The occurrence of different species of bacteria in the ponds is attributed to the type of animal waste the farmers use in their ponds as feed.

In this study, the total plate count (TPC) for all the fish samples ranged between 3.60 x 10^4 and 23.60 x 10^4 cfu/g as shown in Table 2. Out of the 150 fish samples analysed for TPC, the skin had the highest number of bacteria with 23.60 x 10^4 cfu/g at Nyamakwe. The gills had the lowest isolation with 3.60 x 10^4 cfu/ml at the Imbayago sites. The Coliform count was highest in Nhengo (19.66 x 10^4 cfu/g) as compared to other location. Table 2 revealed the isolation of *Pseudomonas spp*. with the skin having the highest number in *Oreochomis mossambicus* (26.60 x 10^3 cfu/g) at Nyamakwe. The intestines were notably some of the most colonized parts in the fish with having the highest count of 23.30 x 10^3 cfu/g. The gills likewise showed possible colonization but in the lowest count as compared to other parts. No isolation of *Vibrio spp*. on the gills of the fish. The intestine and gills were also heavily populated by *E. coli* with the highest exhibited in the gills of fish isolated from Nhengo (6.4 x 10^3 cfu/g). Likewise, the intestines exhibited the highest *Streptococcal* colonization rate of 17.64 x10^3 cfu/g. *Vibrio spp*. had the lowest counts which are largely insignificant

According to published microbiological guidelines as cited by Gilbert *et al.* (1996) the results suggest that the microbiological quality of the fish examined is within acceptable levels and does not yet pose a potential risk to public health. This could be attributed to the low quanties of manure (<50kg/ha/week) applied. In the rural areas studied there is a general high level of demand for livestock manure to fertilise cropping lands, and as such very little is spared for feeding fish in earth dams. The issue of drug residues in the manure used was not discussed as very little or no antibiotics are administered to the rural livestock under study, to elicit any response worth investigating. But however the diversity of potential pathogens from the samples of fish is of concern particularly at a time when many in our communities are immunologically compromised as a result of various illnesses.

Table.1 The occurrence of bacteria species in fish from the different fish ponds

Bacteria species	Imbayago	Nyamakwe	Nhengo
Staphylococcus	13%	40%	13%
Salmonella	6.7%	3%	20%
Shigella	6.7%	6.7%	6.7%
Escherichia coli	13%	20%	13%
Pseudomonas	6.7%	6.7%	6.7%

The high incidence of *Salmonella* in the fish from the earth dams is a major health concern. In addition to *Salmonella*, the presence of diverse enteric bacteria in fish indicates contamination representing a potential hazard to human health. Stringent regulations and monitoring activities coupled with food safety training of suppliers (fishermen and traders) and ultimately the consumers on various aspects of Good Hygiene Practice (GHP), Good Manufacturing Practice (GMP) and HACCP is strongly recommended.

Table.2 Mean count of the bacteria present at different parts of examined sampled fishes

Location	**Parts**	**TPC**	**Coliforms**	***P.aueruginosa***	***V. cholerae***	***E.coli***	***S.aureus***	***S. dysentariae***	***S.typhi***	***E.faecalis***
		cfu/g	**cfu/g**	**cfu/g**	**cfu/g**	**cfu/g**	**cfu/g**	**cfu/g**	**cfu/g**	**cfu/g**
		10^3	**10^3**	**10^3**	**10^3**	**10^3**	**10^3**	**10^3**	**10^3**	**10^3**
Imbayago	Intestines	4.6	5.70	19.49	0.07	6.2	4.18	3.86	-	6.68
	Gill	3.60	6.68	8.78	-	4.26	3.85	2.84	0.36	3.44
	Skin	19.6	7.1	18.89	-	15.72	4.47	1.96	0.94	4.20
	Mouth	13.6	4.2	15.3	-	4.34	2.34	2.44	1.10	4.70
Nyamakwe	Intestines	5.06	14.66	16.12	0.31	7.44	4.18	3.35	3.44	7.44
	Gill	5.60	9.07	10.65	-	6.12	3.58	2.48	2.22	5.12
	Skin	23.60	14.64	26.6	0.07	12.12	4.47	1.48	2.13	7.14
	Mouth	14.4	2.20	16.64	0.04	5.24	2.84	2.60	0.85	4.47
Nhengo	Intestines	8.03	9.66	23.12	0.21	6.44	4.18	3.35	3.44	9.44
	Gill	9.60	7.07	9.65	-	6.22	3.58	2.48	2.22	5.12
	Skin	21.60	12.64	26.6	0.07	6.12	4.47	1.48	2.13	7.14
	Mouth	14.4	4.20	16.64	0.20	5.24	2.84	2.60	0.85	4.47

The presence of coliforms in fish demonstrates the level of pollution of their environment because coliforms are not the normal bacterial flora in fish. Of the organisms that were isolated and identified that is *S. typhi, S. aureus, S. dysenteriae* and *E. coli* are non-indigenous pathogens that contaminate fish or fish habitats in one way or the other (Kvenberg, 1991).

The isolation of *Salmonella, Shigella*, and *E. coli* indicate faecal and environmental pollution (Yagoub, 2009). Coliforms such as *E. coli* are usually present where there has been faecal contamination from warm blooded animals (Chao *et al.,* 2003). The organism *E. coli* is recognized as the reliable indicator of faecal contamination in small numbers and in large numbers it is an indicator of mishandling (Eze *et al.,* 2011). *E. coli* is the only species in the coliform group that is found in the human intestinal tract and in the other warm blooded animals as a commensal and is subsequently excreted in large quantities in faeces (Geldreich, 1983).

The presence of *S. aureus* and *E. coli* was attributed to the contamination of the fish samples by the faecal material fed to the fish. Of concern is the fact that the high bacterial loads found in the raw fish at the source point are most likely to have a multiplier effect as the caught fish are poorly handled and stored until they are consumed. In similar studies, *Escherichia coli, Pseudomonas aeruginosa, Shigella dysenteriae, Staphylococcus aureus and Salmonella typhi were* isolated from the gills, intestines, muscle and skin of *Megalaspis cordyla* and muscles of *Priacanthus hamrur* from Royapuram waters in India (Sujatha *et al.,* 2011). This was attributed to the heavy load of sewage disposal into the seas which could act as a suitable environment for the growth and survival of the human pathogens. Members of the genus *Pseudomonas* are found in the soil and natural sources of water and are important phytopathogens and agents of human infections being considered opportunistic pathogens (Sujatha *et al.,* 2011).

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