

Diversity of Bacteria Contaminants in Tissues of Fish Cultured in Organic Waste-Fertilized Ponds: Health Implications

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Abstract: Bacterial populations contaminating various tissues of fish cultured in fish ponds fertilized with different organic wastes have been studied. Twenty-five genera of bacteria were identified as associated with the fish tissues. The identified bacteria included one genus of spiral and curved bacteria, one genus of Gram-negative aerobic rod, sixteen genera of Gram-negative facultative anaerobic rods, one Gram-negative anaerobic bacterium, three Gram-positive cocci, two endospore-forming rods, and one Actinomycete. The bacterial contaminants were isolated from all tissues of the cultured fish. Higher bacterial contamination was associated with the gills, gut and skin than the blood and muscle. Although *Pseudomonas* sp. was most abundant species in the organic waste fertilized ponds and was present in considerable quantities in the five fish tissues, *Salmonella* sp. was the most important contaminant of the gills, muscle and skin. The presence of high diversity of bacteria in the fish tissues presents health hazards to both consumers and fish farm workers. Handling and cleaning of the fish can result in contamination of hands of farm workers and through them to their family members and others. The digestive tract and intraperitoneal fluid of fish in this study showed high concentrations of pathogens, such as *Salmonella* in fish cultured in the organic waste fertilized ponds, but lower concentrations in fish cultured in non-fertilized pond. Public health must therefore be of prime concern when dealing with fish farming and its products in countries, such as Ghana, with less restriction on release of waste into water bodies, and the use of untreated wastewater for aquaculture.

Keywords: Organic-waste fertilized ponds, bacterial contaminants tissues.

INTRODUCTION

Application of fertilizers to ponds substantially increases fish yield but the increase in cost of commercial fertilizers makes the practice unprofitable. Interest has, therefore, been diverted to other sources of enrichment of the water, such as use of animal manure which causes algal blooms, provides feed for the fish, and also serves as a source of nutrients for microorganisms present in the water.

In Ghana poultry waste, blood waste, sewage, cow dung and pig dung are mostly used to fertilize fishponds and are considered superior to inorganic fertilizers in producing and maintaining desirable species of planktonic and benthic organisms in fresh and brackish water ponds [1]. Some farmers make regular application at three or four months intervals. Others depend on visual observation and add the waste according to the colour of the pond.

Many studies have shown that bacteria belonging mostly to the genera *Aeromonas*, *Corynebacterium*, *Myxobacterium*, *Pseudomonas* and *Vibrio* cause infectious diseases in fish [2,3]. Most infections begin on the mucus membranes. To initiate infection the bacteria must reach susceptible organ or adhere to the epithelial cells [2]. In certain diseases they remain localized at the mucosal surface and cause damage by liberating toxins. In most cases, however, infection is caused

by the pathogen penetrating the epithelium and then growing in the sub-mucosa or spreading even further [3]. Successful invasion depends on the ability of the pathogen to out-compete the normal microflora for nutrients. Small breaks or lesions in the mucosal membrane also facilitate spread of pathogens [3].

The health of fish is dependent on the quality of the water. The presence of some obligate pathogens such as *Aeromonas salmonicida* or *Renibacterium salmonarum* in water is an indication of the presence of fish disease on a farm [4]. However the bacterial load in water *per se* does not relate to possible health hazard. Indeed majority are beneficial saprobes involved in the numerous re-cycling processes. Of much concern in fisheries is the contamination of fishes by faecal coliforms in polluted waters [4].

In every country where fish inspection programme exists, the load of faecal coliforms in farmed, feral or processed fish is evaluated to verify whether the harvest or product presents a health hazard or not [5,6]. Their presence in fish intended for human consumption may constitute a potential danger not only in causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human infecting bacteria from non-aquatic sources [7]. *Escherichia coli*, the predominant species of the faecal coliforms, has been found in the intestinal tract of fish [8], on the gills, in the muscles and on the skin [9], when sewage water has been used to rear fish. Salle [10] reported that the most heavily contaminated parts are the intestines and the skin. Presence of *E. coli* in water or food indicates the possible

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presence of causative organisms of many gastro-intestinal diseases [11, 12]. This study was therefore undertaken to investigate the diversity of bacteria pathogens in tissues of fish that are grown in ponds that receive fertilizers from various organic sources in Ghana and their health implications to the fish and human.

MATERIALS AND METHOD

Fish farms used in this study were ponds being used for commercial purposes, and which retained water throughout the year. They were drainable and had well conditioned dykes and each was fertilized with only one type of fertilizer. They included cow manure-fertilized pond, poultry manure-fertilized pond, pig manure-fertilized pond, and, blood manure-fertilized pond. Each pond was stocked with tilapia. Fish from River Volta, a non-fertilized pond was used as control.

Cast net was used to collect fish samples from the various ponds. Five live tilapias were randomly selected from the catch at each sampling time.

Sterile cotton bud was used to take a swap from the surface of the fish immediately upon collection from the pond. The cotton bud was placed immediately in 5ml sterile peptone water in sterile plastic applicator (Rayon), labeled and kept at 4°C and transported to the laboratory.

In the laboratory each fish was rinsed with de-ionized water and the surface of the fish decontaminated by dipping it in ethyl alcohol and lightly flames. The fish was aseptically dissected and parts of the gills, gut and muscle were taken for analysis. Blood sample was also collected using a sterile syringe. Each tissue was homogenized separately in a blender in sterile phosphate buffered saline PBS of pH 7.2 to achieve a 10% w/v suspension of fish.

Tests were carried out on each isolate following the procedures described by Bailey and Scott [13], Prescott *et al.* [14] and Cheesbrough [15] to enable identification to the generic and species levels with the aid of the Bergey's Manual of Determinative Bacteriology [16].

SELECTIVE CULTURE OF BACTERIAL ISOLATES

Forty isolates were randomly selected from plates of each culture. Isolation and subsequent identification of bacteria were done using Trypticase Soy Agar (TSA), standard biochemical tests [14, 15] and the API 20E Kit [17]. Strains of *E. coli* (1) 0 sera (Denka Seiken No 24506, Tokyo, Japan) were used to type for enterotoxigenic, enteropathogenic, enteroinvasive, anetroaggregative and enetrohaemorrhagic *E. coli*. Strains of *Salmonella* and *Shigella* were serologically typed with specific *Salmonella* and *Shigella* antisera (O grouping) (Remel Inc, USA). Each colony was identified to the generic level and the frequency of occurrence of each genus in the selected 40 colonies determined. In cases where tissue parts could not produce a total of 40 colonies, all isolates were identified and the frequency based on the total number of colonies.

STATISTICAL ANALYSIS

The Statgraphics Plus for Windows 4.0 [18] was employed to test for significant differences between the various

means of bacteria genera from the various tissues of fish. The data for number of isolated bacterial genera were subjected to analysis of variance (ANOVA), where $P < 0.05$ was judged indicative of a significant difference. Where the ANOVA revealed significant differences, Duncan's multiple range test [19] was applied in order to characterize and quantify the differences between tissue bacterial flora populations.

RESULTS AND DISCUSSION

Tilapia caught from the various culture systems fertilized with different sources of organic wastes and those caught from non-fertilized pond were found to harbor bacteria belonging to twenty-five genera of bacteria, in various tissues of the fish, but at different magnitudes (Table 1). The bacteria isolated from the various tissues of the fish from both the fertilized and unfertilized ponds included *Actinobacillus* sp., *Aeromonas* sp., *Bacillus* sp., *Bacteroides* sp., *Campylobacter* sp., *Citrobacter* sp., *i* sp., *Corynebacterium* sp., *Edwardsiella* sp., *Enterobacter* sp., *Escherichia* sp., *Flavobacterium* sp., *Hafnia* sp., *Klebsiella* sp., *Micrococcus* sp., *Pasteurella* sp., *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., *Serratia* sp., *Shigella* sp., *Staphylococcus* sp., *Streptococcus* sp., *Vibrio* sp. and *Yersinia* sp.

Bacterial genera isolated from the blood were between 12 – 16, and 17 – 21 genera in the muscle of fish. Between 24 – 25 genera were isolated from the skin, 25 genera from the gills and 10 – 23 genera from the gut.

The study indicated that certain species seemed to be associated more with certain tissues than with others. For example *Edwardsiella* sp., *Pasteurella* sp. and *Salmonella* sp. were abundant in the muscle of fish, while the blood contained higher presence of *Corynebacterium* sp., *Flavobacterium* sp. and *Pseudomonas* sp. For the gills, gut and skin, the respective dominant genera were *Bacillus* sp., *Pseudomonas* sp., and, *Streptococcus* sp. and *Vibrio* sp., respectively.

Analysis of variance determination showed significant difference at 95.0% confidence level between the means for the muscle, blood, gut, gill and skin of the fish. Duncan's multiple comparison determination showed homogeneity between the means for the blood and muscles, and, among the means for the gill, gut and skin.

With fish cultured in poultry manure-fertilized ponds the data showed the presence of 17 - 21 genera in the muscles, 11 - 20 in the blood flora, 24 - 25 in the gills and in the skin flora and 19 - 23 genera in the gut.

Fish cultured in pig manure-fertilized ponds contained between 17 - 24 genera isolated from the muscle, while 11 – 15 genera were isolated from the blood. Between 24 – 25 genera were isolated from the gills, 11 – 13 genera from the gut and between 24 to 25 genera from the skin.

Fish cultured in blood waste-fertilized pond had 15, 14 and 22 genera isolated from the muscle, blood and gut, respectively.

The dominant species of the muscle flora were *Pasteurella* sp., *Escherichia* sp. and *Salmonella* sp. The dominant species in the blood flora was *Micrococcus* sp., followed by *Campylobacter* sp. and then by *Aeromonas* sp. *Salmonella* sp. was the predominant species in the gill flora

Table 1. Bacteria Species Diversity of Fish Cultured in Organic Fertilized Ponds

Fish Part/Tissue	Bacteria Pathogens Isolated From			
	Type of Fertilizer Applied to Pond			Open System / No Fertilization
	Poultry Manure	Pig Manure	Blood Meal	
Blood	<i>Corynebacterium</i> <i>Flavobacterium</i> <i>Pseudomonas</i> <i>Micrococcus</i>	<i>Corynebacterium</i> <i>Pseudomonas</i> <i>Micrococcus</i>	<i>Aeromonas</i> <i>Campylobacter</i> <i>Flavobacterium</i> <i>Micrococcus</i>	<i>Hafnia</i> <i>Pseudomonas</i> <i>Micrococcus</i> <i>Yersinia</i>
Gills	<i>Actinobacillus</i> <i>Bacillus</i> <i>Pseudomonas</i> <i>Salmonella</i> <i>Shigella</i>	<i>Bacillus</i> <i>Clostridium</i> <i>Klebsiella</i> <i>Proteus</i> <i>Salmonella</i> <i>Staphylococcus</i>	<i>Bacillus</i> <i>Salmonella</i> <i>Serratia</i> <i>Vibrio</i>	<i>Bacillus</i> <i>Enterobacter</i> <i>Escherichia</i> <i>Flavobacterium</i> <i>Pseudomonas</i>
Gut	<i>Enterobacter</i> <i>Escherichia</i> <i>Pseudomonas</i> <i>Micrococcus</i> <i>Salmonella</i> <i>Shigella</i>	<i>Citrobacter</i> <i>Clostridium</i> <i>Klebsiella</i> <i>Streptococcus</i>	<i>Bacteroides</i> <i>Micrococcus</i> <i>Proteus</i> <i>Staphylococcus</i>	<i>Bacillus</i> <i>Edwardsiella</i> <i>Escherichia</i> <i>Micrococcus</i> <i>Pseudomonas</i>
Muscle	<i>Bacillus</i> <i>Edwardsiella</i> <i>Pasteurella</i> <i>Pseudomonas</i> <i>Salmonella</i>	<i>Edwardsiella</i> <i>Pasteurella</i> <i>Pseudomonas</i> <i>Salmonella</i>	<i>Edwardsiella</i> <i>Escherichia</i> <i>Pasteurella</i> <i>Salmonella</i>	<i>Edwardsiella</i> <i>Pasteurella</i> <i>Salmonella</i>
Skin	<i>Bacillus</i> <i>Pasteurella</i> <i>Salmonella</i> <i>Shigella</i> <i>Streptococcus</i> <i>Yersinia</i>	<i>Bacillus</i> <i>Proteus</i> <i>Pseudomonas</i> <i>Streptococcus</i>	<i>Bacillus</i> <i>Flavobacterium</i> <i>Salmonella</i> <i>Shigella</i> <i>Staphylococcus</i> <i>Streptococcus</i>	<i>Bacillus</i> <i>Citrobacter</i> <i>Edwardsiella</i> <i>Enterobacter</i> <i>Escherichia</i> <i>Pseudomonas</i> <i>Salmonella</i> <i>Streptococcus</i>

followed by *Bacillus* sp. and by *Vibrio* sp. *Micrococcus* sp. was found to be the predominant species in the gut. *Salmonella* sp. dominated the flora of the skin, followed by *Bacillus* sp., and then *Flavobacterium* sp.

Analysis of variance determination showed significant difference at 95.0% confidence level between the means of the values for the blood, gill, gut, muscle and skin flora. Duncan's multiple comparison determination showed homogeneity between the means of the blood and muscles, and between the gill, gut and skin.

This study has indicated that fish cultured in various types of organic waste fertilized ponds are susceptible to infection with pathogenic bacteria. Fish from the non-fertilized pond had bacteria detected in the different tissues, but at relatively low numbers. *Salmonella* spp. was most commonly identified with most of the tissues, and was de-

tected in the muscles, on the gills and skin. The occurrence of disease is a complex interaction between host species, pathogens and the environment [20]. Most aquacultural practices that favour disease occurrence include high stocking densities which increase stress in the stocks, intensive feeding which provides abundant substrate for microbial growth and sub-optimal environment of inadequate water exchange [7, 9]. Disease outbreaks would thus be relatively less common in the non-fertilized pond as it has less stressful environments, even though the pathogens and host species may be present.

Frazier [12] stated that species of *Clostridium*, *Flavobacterium*, *Micrococcus*, *Proteus* and *Pseudomonas* are the major spoilage bacteria at near freezing temperatures. Raj and Liston [20] found that some pathogenic and potentially pathogenic microorganisms including *E. coli*, *Staphylococ-*

cus and some anaerobes survived when uncooked and pre-cooked fish foods were stored at freezing temperatures. Studies by Roberts [2] showed that bacteria belonging mostly to the genera *Aeromonas*, *Corynebacterium*, *Pseudomonas* and *Vibrio* cause infectious diseases in fish. The presence of these twenty-five genera in the fish is, therefore, a threat to the fish industry as fish, which do not succumb to the attack may still be subjected to spoilage.

The presence of the coliform group of bacteria, mainly *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella* in fish and fish products presents a health hazard to humans [4, 6, 22]. Allen and Hephner [23] have stated that most of the epidemics attributed to wastewater sources are from raw sewage gaining access to food eaten directly by man, or from contamination of water supply systems by untreated sewage. Olayemi *et al.* [7] have reported that the presence of faecal coliform in fish intended for human consumption may constitute a potential danger not only in causing disease, but also because of the possible transfer of antibiotic resistance from aquatic bacteria to human infecting bacteria from non-aquatic sources. Some human pathogens such as *Aeromonas*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella* and *Vibrio* have been found to survive and multiply in the gut, mucus and tissues of fish and thus render fish a potential vector of human disease over long periods [24]. All these pathogens have been identified to be present in the tissues of fish that were cultured in the organic waste-fertilized ponds in this study. Public health must therefore be of prime concern when dealing with fish farming and its products in countries with less restriction on release of waste into water bodies, and in use of untreated wastewater for aquaculture. The digestive tract and intraperitoneal fluid of fish in this study showed concentrations of pathogens. Handling and cleaning of such contaminated fish can result in contamination of the hands of farm workers and through them to their family members and others.

The similar pattern of variation in the water and fish tissues of the population of bacterial flora is a possible indication that bacterial biomass forms an important part of the diet in *Oreochromis niloticus* and that gut microflora [25], whether it be autochthonous or allochthonous or both, could possibly play an important role in the nutrition of the fish. A higher bacterial load in the gut of fish has been observed than in the surrounding waters [25-27]. This study thus confirms the suggestion that fish selectively feed on detrital particles with high numbers of bacterial biomass per unit weight [28, 29], thus concentrating bacteria in their foreguts at levels higher than those in the surrounding environment. Henebry *et al.* [25] observed increased bacterial population in the midgut of silver carp and suggested that bacterial populations may increase in the midgut before being ultimately digested, thereby providing high quality protein for the fish. Sera and Ishida [30] observed increased total heterotrophic bacteria count from 10^4 to 10^8 cells per gram in the intestine of red sea bream snapper (*Pargus major*) 16 hours after ingestion of food. Many species of bacteria that are normally considered saprophytic, including species of *Bacillus*, *Micrococcus* and *Proteus* have been isolated from infections of tropical fish [31]. However, since in the present study separate counts for the flora in the foregut, midgut and hindgut were not done, it is difficult to determine whether bacteria

serve directly as source of protein for *O. niloticus* or indirectly by synthesizing vitamins required by the fish. Most probably they perform both functions, and their relatively high density in the gut of fish is of important survival value.

It is suspected that the organic wastes improve considerably the nutrients levels in the ponds, a situation that will increase the population of bacteria present in the ponds [7, 9]. This is ideal for the growth of the fish, as food will be in abundance. However, with conditions where pathogenic bacteria are introduced into the ponds with the wastes, the risk of transfer of diseases to the fish and humans is high [20]. The use of the organic wastes to fertilize the ponds is cost-saving and recommended if some form of treatment can be given to the wastes to eliminate the bacterial pathogens. One form of such reduced cost of treatment can be by solar treatment.

The safety of products for consumption is prime concern from the point of view of managing of the fish culture systems, as well as ensuring public health [32]. Official regulatory bodies in many countries specify maximum permissible concentrations of toxic substances or the number of harmful bacteria that a product may contain, in order to ensure that unfit or unwholesome food does not reach the consumer. Even though not usually covered by regulations, unattractive appearance and tainting of products affect their marketability [33]. Water quality and culture practices play important roles in determining product quality. In Ghana most of the fish consumed is bought directly from the fishermen and do not pass through any health-safety checks. Instituting and ensuring fish inspection programmes to regulate the quality of fresh fish from the farm or in the market in Ghana would be a safeguard to protect the health of consumers.

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