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Short communication

SCREENING OF INTERNAL AND EXTERNAL BACTERIA FOUND IN FARMED FISH IN RELATION TO FEED QUALITY

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ABSTRACT

Aquaculture in Kenya is progressively developing as an alternative means of human food production and subsistence. An enormous loss in aquaculture has been accelerated by the mortality of Nile Tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) fingerlings. This study aimed at screening the internal and external bacteria found in farmed Nile tilapia and African Catfish at the agro-science fish farm of the Egerton University. The microbiology laboratory collected and processed fifteen healthy fish and fish feeds (formulated and commercial). The skin, gills and intestines as well as the commercial and formulated fish feeds were processed and cultured in Salmonella Shigella and blood agar. The resulting bacteria colonies were subjected to morphological examination and biochemical tests for identification. The findings of this study demonstrated the existence of Enterobacteriaceae. *Escherichia*, *Salmonella* and *Bacillus* species were the most prevalent species identified on the skin, gills and intestines. Formulated feeds were more contaminated with bacteria than commercial feeds. The presence of the above microorganisms, some of which are pathogens (*Salmonella enteritidis*, *Enterobacter aerogenes*, *Klebsiella oxytoca*) to humans, is an indication that undercooked fish may pose health risks to susceptible human and an impediment to the rapid intensification of aquaculture because of mortality of fingerings.

Key words: aquaculture; pathogens; fish feeds; tilapia; catfish; health risks

INTRODUCTION

The aquaculture of Nile Tilapia *(Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) is expanding rapidly in Kenya (Kyule-Muendo *et al.*, 2022). The country is one of the sub-Saharan African countries that invests in aquaculture with tilapines dominating the sector. The fisheries and aquaculture sector account for about 0.7 % of the Gross Domestic Product (GDP) and offers over two million people indirect employment oppor-tunities in addition to more than 500,000 direct employment opportunities (Charo *et al.*, 2022; Afolabi *et al.*, 2020). However, due to various factors including the country's rapidly growing population, fish supply has been declining, while demand has increased significantly (Clols-Fuentes *et al.*, 2023). Studies on aqua-culture are being encouraged across the continent to reverse these trends. This has been marked by a record growth in fish farms over the past decade, ranging from widespread small-scale to intensive large-scale. One significant setback in aquaculture production is the risk of diseases (Jamabo *et al.*, 2019; Wamala *et al.*, 2018; Tesfaye *et al.*, 2018). It induces monetary losses and a dearth of fish and fish products due to fish mortalities and superfluous treatment expenditures (Ogbonne *et al.*, 2020; Mumbo *et al.*, 2023). The leading cause of death in aquaculture, particularly in the hatchery, is disease spurred on by bacterial presence (Njagi, 2019; Wanja *et al*., 2020). The types of feed and water sources are

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two elements that influence the diversity of bacteria in aquaculture (Ojwala *et al.*, 2018; Ahmed, 2019). Quality fish feeds are vital for the success of intensive fish farming since they influence fish development and, to some extent, bacterial growth in aquaculture (FAO, 2020).

"Fish health" refers to management strategies designed to mitigate fish diseases. To combat the risks and implications of aquatic diseases, a proactive and consistent biosecurity program is becoming increasingly vital (Kyule-Muendo *et al.*, 2022). Many cases of fish diseases reported in Kenya have focused on parasitic infections in aquaculture, which may have missed the bacteria of economic and health importance. The health of farmed fish should be closely monitored to produce fish that is suitable for human consumption. Limited data on the prevalence and potentially pathogenic bacteria in Kenya's aquaculture industry are available. While most published studies have concentrated on isolating single bacteria species, this study aims at screening the internal and external bacteria found in farmed fish and fish feeds at Egerton University agro-fish farm, Kenya. Studies on the bacterial flora will deliver insights that will aid in making informed decisions about the surveillance of disease control and prevention, the production of high-quality fish feed, analysis, and management.

MATERIAL AND METHODS

Study site description

The research was conducted at the agro-science fish farm of the Egerton University, Njoro, Kenya. The fish farm is about 20 km south-west of Nakuru city at an altitude of 1890 m, latitude 0.3725° S and longitude 35.9335° E. It is characterized by two seasons: prolonged rainfall from March to July, ranging from 760 mm to 1250 mm per annum, and short rain from September to November with less than 500 mm per annum. Due to these different seasons, temperature fluctuations range from 14.9 °C to 21.9 °C.

Sampling methodology

Fifteen of live mature tilapia and catfish, which appeared healthy, were randomly selected and aseptically collected from different locations at the fish farm and submitted to the bacteriological analysis. In the field of aquaculture, an extensive investigation was conducted across five distinct areas, with the collection of triplicate fish samples from each location to ensure thorough analysis and valuable insights.

Sample processing and isolation of fish bacteria

The fish were humanely sacrificed, and one square inch of fish skin was swabbed with sterile cotton wool and inoculated in nutrient broth for 24 hours at 37 °C. The fish were then dissected, and parts of the gills and intestines (1 g) were removed from each fish under aseptic conditions and were cultured in nutrient broth for 24 hours at 37 °C. Salmonella Shigella (SS) agar and blood agar (BA) were prepared and sterilized according to the manufacturer's instructions (The MacMillan Company, London (p. 226–229). The media were sterilized in an autoclave at 121 °C for 15 minutes. An inoculum from the 24-hour nutrient broth culture was sub-cultured on sterile prepared SS plate and BA using a sterile wire loop. The plates were inverted and incubated for 24 hours at 37 °C.

Isolation of bacteria from fish feeds

Five grams of the two fish feeds were weighed, put into separate test tubes containing 45 ml of distilled water and thoroughly homogenized by a vortex. One ml of the homogenate was transferred to a test tube containing 9 ml of distilled water to obtain a dilution of 10^{-1}. Similarly, 1 ml of the dilution homogenate was transferred to a test tube containing 9 ml diluents and the process was repeated until a dilution of 10^{-4} was obtained for both feeds (Njagi, 2019). This procedure was done in triplicates and the number of isolates was averaged. Aliquots from these dilutions were then spread onto the Salmonella Shigella and Blood Agar and incubated under optimal conditions for bacterial growth. Colonies that developed on the plates were then picked and streaked onto fresh plates to ensure purity. This process was repeated until single, isolated colonies were obtained. Theses isolated bacterial colonies were then subjected to biochemical tests to identify and characterize the bacteria present in the fish feeds.

Bacterial identification

To confirm the identity of bacterial isolates, referring to Bergey's Manual of Systematic (Bergey's Manual of Systematic Bacteriology, 2001–2005), the following biochemical tests were performed.

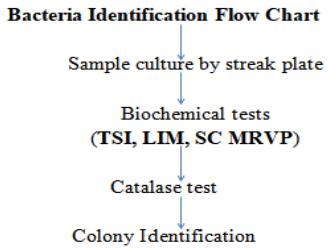

Figure 1. A simple flow chart of how the tests were done

Key: TSI – Triple Sugar Iron, LIM – Lysine Indole Motility, SC – Simmon's Citrate, MR – Methyl Red, VP – Vogues Proskauer, S – Slant, B – Butt, G – Gas, H_2S – Hydrogen Sulphide

Catalase test

This test was used to distinguish bacteria that produce the catalase enzyme. Here, a small colony of pure cultures of test organism with virtuous growth was smeared on a slide using a sterile wire loop. Then a drop of catalase reagent (3 % hydrogen peroxide) was added to the smear. The slide was checked for bubble formation. Rapid elaboration of bubbles within a few seconds indicated a positive result, while absence of bubble formation indicated a negative result.

The Triple Sugar Iron (TSI) test

The test was designed to differentiate among organisms based on the differences in carbohydrate fermentation patterns and Hydrogen sulphide production. Here, the top of a well-isolated colony was touched with a sterile straight inoculation needle. The TSI was inoculated by first stabbing through the centre of the butt and then streaking the surface of the agar slant. The cap was left on loosely and the tubes were incubated at 37 °C for 24 hours. The medium was then checked for reaction. A red slant/yellow butt indicated the fermentation of dextrose only. A yellow slant/yellow butt indicated the fermentation of dextrose, lactose and sucrose. A red slant/red butt showed the absence of carbohydrate fermentation. Production of hydrogen sulphide was indicated by the blackening of the medium.

Indole test

This test was used to demonstrate the ability of certain bacteria to decompose the amino acid tryptophan to indole, which accumulates to the medium. The bacterial culture was inoculated in the Lysine-Indole-Motility broth and incubated at 37 °C for twenty-four hours. When indole was combined with Kovac's reagent (which contained concentrated hydrochloric acid and p-Dimethylaminobenzyladehyde in amyl alcohol), a red coloration that formed an oily layer at the top of the broth indicated indole formation.

Citrate Utilization Test

This was used to test an organism's ability to utilize citrate as a source of energy. Following inoculation in the Lysine-Indole-Motility gel, the wire loop was used to stab the butt and streak the slant. Growth with colour change from intensive blue to green along the slant indicated a positive reaction. The slant with no growth and colour change remained blue indicating a negative reaction.

Methyl Red Test

The test was used to detect the production of sufficient acid during the fermentation of glucose. The test was conducted by inoculating a pure culture in sterile 2 ml glucose phosphate broth. After overnight incubation, a drop of methyl red solution was added. A red coloration was positive and indicated an acid pH resulting from glucose fermentation. A yellow coloration indicated negative result.

Voges-Proskauer Test

This test was used to differentiate between members of the *Enterobacteriaceae* family based on their pattern of glucose metabolism. Two ml of sterile glucose phosphate broth were inoculated with test organisms and incubated for 48 hours at 37 °C. Two drops of creatine were added and mixed. Three ml of sodium hydroxide (NaOH) were added and mixed well. The bottle cap was removed and left for an hour at room temperature. It was checked closely for development of pink colour for positive cases. In cases of negativity, they retained yellow colour.

Data Analysis

In the present experiment, statistical analysis was conducted using R software, employing both the Chi-square test and G-test of independence. These tests were utilized to examine the relationship between categorical variables and to determine if any significant associations existed. The Chi-square test was conducted with rescaled probabilities to assess if the observed prevalence in sample sources significantly differed from an expected uniform distribution. A G-test of independence was conducted to assess if there is a significant association between sample sources and prevalence of bacterial isolates.

RESULTS

Occurrence of bacterial isolates in fish organs and fish feeds

The agar plates used for isolation were categorized into two: Salmonella Shigella (Plate A) and Blood agar (Plate B).

Plate A: Colonies appeared small, pink to red, suggesting *Escherichia coli*. Other colonies appeared large and colourless, with black centres suggestive of *Salmonella* spp. On the same plate, other colonies appeared to be large, mucoid, opaque cream to pink, suggestive of *Enterobacter* spp. At the same time, others appeared to be pink to red without an area of precipitated bile suggestive of *Klebsiella* spp. A few more appeared to be colourless, suggestive of *Shigella* spp. They were further subjected to biochemical tests and identified using a standard biotyping chart.

Plate B: Colonies had a characteristic swarming following spot inoculation in the center of the Blood agar plate suggestive of *Proteus* spp. Other colonies appeared non-hemolytic, slightly convex, and glassy, suggesting *Bacillus* spp. They were further subjected to biochemical tests and identified using a standard biotyping chart.

Prevalence of bacterial isolates in skin, gills, intestines and fish feeds

Thirty-five bacterial isolates were recovered and characterized from 15 healthy appearing tilapia and catfish, formulated, and commercial fish feed samples. Bacteriological examination of the various organs showed seven bacterial isolates: *Escherichia coli*, *Salmonella* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Shigella* spp. and *Bacillus* spp.

The sample sources were sorted by prevalence in descending order. Gills (27.4 %), skin (27.1 %), and intestines (26.6 %) had the highest prevalence of organisms while formulated fish feeds (14.6 %) and commercial fish feeds (4.3 %) had the lowest. The bacterial isolates were sorted by prevalence in descending order; *Escherichia coli* (16.9 %), *Salmonella* spp. (10.2 %), *Enterobacter* spp. (8.3 %), *Bacillus* spp. (6.1 %).

DISCUSSION

Studies on bacteria flora in farmed fish aid in providing knowledge pertinent to developing robust biosecurity and sanitary measures (Kyule-Muendo *et al.*, 2022) since some of the organisms are crucial for public health and a substantial hindrance to the rapid intensification of fish farming (Opiyo *et al.*, 2018). This study isolated members of Enterobacteriaceae, *Escherichia coli*, *Salmonella* spp., *Enterobacter* spp., *Proteus* spp., and *Shigella* spp. from fish organs (Table 1). Bacterial species recovered from fish organ samples were also found in commercial and ormulated fish feeds (Table 2). This demonstrates that fish feeds influence the bacterial ecology of farmed fish.

Table 1. Fish Organ Sample Isolates Biochemical Test Characterization

TESTS			TSI					LIM			SC	MR	VP	Conclusion
Fish	Origin	Isolates	S	B	G	H_2S	L	I	M					
Tilapia	Skin	A2	+	+	+	-	-	+	+	-	+	-	*Escherichia* coli
	Skin	A1	-	+	+	+	-	-	+	+	+	-	*Salmonella* spp.
	Gills	A1	-	+	+	+	-	-	+	+	+	-	*Salmonella* spp.
	Intestines	A3	+	+	+	-	-	-	+	+	-	+	*Enterobacter* spp.
	Skin	A1	+	+	+	-	-	-	+	+	-	+	*Klebsiella* spp.
	Skin	A1	+	+	+	+	-	-	+	+	-	+	*Proteus* spp.
	Gills	A2	+	+	+	+	-	-	+	+	-	+	*Bacillus* spp.
Catfish	Intestines	B1	+	+	+	-	-	+	+	-	+	-	*Escherichia* coli
	Skin	B1	+	+	+	-	-	+	+	-	+	-	*Escherichia* coli
	Gills	B2	-	+	-	-	-	-	-	-	+	-	*Shigella* spp.

Key: TSI – Triple Sugar Iron, LIM – Lysine Indole Motility, SC – Simmon's citrate, MR – Methyl Red, VP – Vogues Proskauer, S – Slant, B – Butt, G-Gas, H_2S – Hydrogen Sulphide

Table 2. Fish Feeds Bacterial Isolates Biochemical Test Characterization

TESTS	TSI					LIM		SC	MR	VP	Conclusion
Origin	S	B	G	H_2S	L	I	M				
Commercial fish feeds	-	+	-	-	-	-	-	-	+	-	*Shigella* spp.
	+	+	+	-	-	+	+	-	+	-	*Escherichia* coli
	+	+	+	-	-	-	+	+	-	+	*Enterobacter* spp.
Formulated fish feeds	+	+	+	+	-	-	+	+	-	+	*Bacillus* spp.
	+	+	+	-	-	+	+	-	+	-	*Escherichia* coli
	-	+	+	+	-	-	+	+	+	-	*Salmonella* spp.
	+	+	+	-	-	-	+	-	+		*Enterobacter* spp.
	+	+	+	+	-	-	+	+	-	+	*Proteus* spp.

The Chi-square test yielded a significant result (X-squared = 21.219, d = 4, $p < 0.001$), indicating that the prevalence of bacterial species is not uniformly distributed among the sample sources. The G-test of independence yielded a significant result (X-squared = 21.219, $p < 0.001$), suggesting a non-random association between sample sources and prevalence.

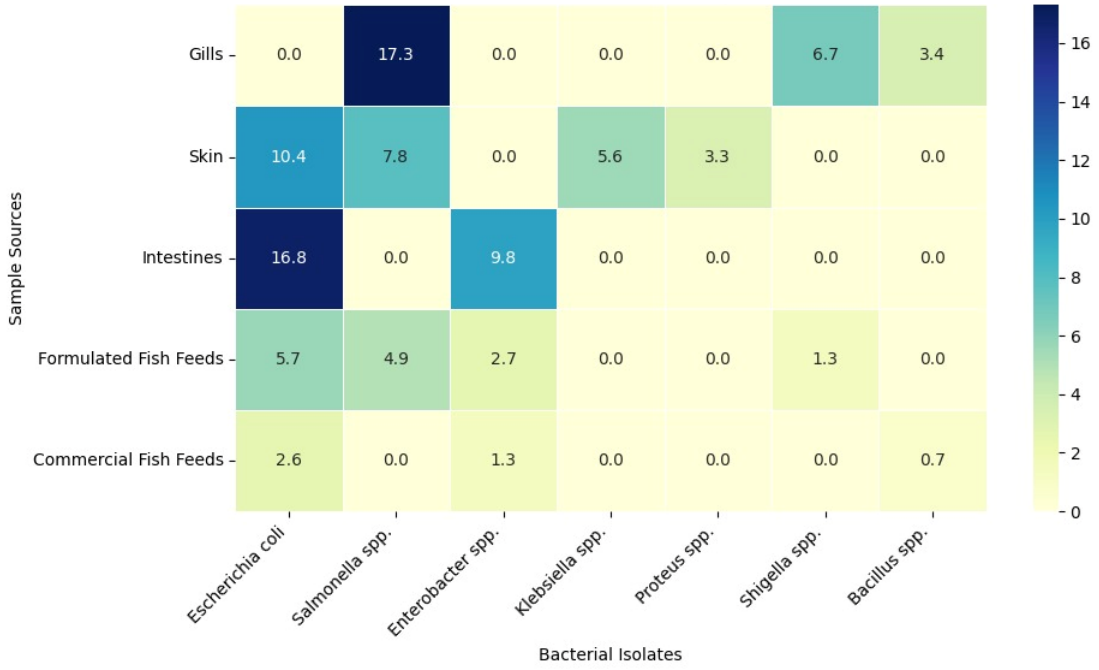

Figure 2. Cross-tabulation of bacterial isolates by sample sources

These results are in congruent with those reported by (Njagi, 2019) that there is a similarity between the microbial content of fish feeds and fish organs. The higher load of bacterial isolates in gills was contributed by the fact that gills perform a vital role in filtering microscopic organisms.

A significant number of the morphological and biochemical characteristics of the bacterial isolates identified in the study were consistent with previous researchers' results (Karimi *et al.*, 2022; Opiyo *et al.*, 2018). According to (Bekele *et al.*, 2019), Nile Tilapia's rapacious feeding behavior, the presence of organic debris, and the system's poor water quality were responsible for the higher prevalence of bacterial counts in the fish's gills and intestines. This suggests improper feeding habits may be related to the high bacterial count in the gills and intestines. Since gills are critically crucial in filtering tiny organisms, there were more bacterial isolates in gills.

Formulated fish feeds contain many bacteria of human origin, such as *Escherichia* species (Jamabo *et al.*, 2019). This might be caused by fish farm practices such as improper handling of fish feeds, which could introduce bacterial contaminants. Other studies discovered *Escherichia coli* contamination in fish and water (Wanja *et al.*, 2019). The tank water that may have contaminated the African Catfish raised in the tanks is in line with (Njagi, 2019), who reported that the most contaminated water source for African catfish ponds was borehole water, while river water was the least contaminated. Contamination of the ponds could be why enteric organisms were isolated from the African Catfish and tilapia water sources (Fakorede *et al.*, 2019; Zaky & Ibrahim, 2017). Salmonella species indicate formulated fish feeds prepared on the agro fish farm under poor hygienic conditions (Dissasa *et al.*, 2022). *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Proteus* contamination of fish may be caused by erroneous animal waste disposal and runoff from land surfaces during the rainy season into ponds and rivers that harbor fish, as well as by the rainy season's washing of land surfaces into water bodies (Ogbukagu *et al.*, 2021). The isolation of *Klebsiella* species from the collected tilapia organ samples as potential human pathogenic organisms and a measure of food quality offers a potential concern for public health since the organism may be pathogenic and result in pneumonia, urinary tract infections, and bacteremia in humans (Fakorede *et al.*, 2019; Chitambo *et al.*, 2023).

Consequently, the development of fish farming is threatened by the occurrence of these bacterial isolates in fish, which, depending on the environmental culture conditions, may result in low-to-high mortality. Some isolates, including *Enterobacter* species and genera *Bacillus*, have been reported to have beneficial health effects in fish and have been developed into fish probiotics. This may justify their occurrence in the gut of studied tilapia and Catfish. However, further studies are needed to assess their efficacy as probiotics (Debnath *et al.*, 2023; Ringø *et al.*, 2020).

CONCLUSION

This study has confirmed that farmed fish harbor bacterial flora, some of which could be a setback in fish farming output. Moreover, some of the bacteria have been documented to cause severe illness in humans; therefore, they may pose inherent dangers to consumers (in case the fish are improperly cooked) and occupational hazards to fish harvesters (improper handling).

RECOMMENDATION

There is a need to monitor fish feeds regularly to evaluate bacterial contamination and improve sanitary conditions under which the fish are reared following standard practices. The public should be enlightened on the inherent dangers that the consumption of improperly cooked fish may accompany. To evaluate the pathogenicity of each bacterium and the potential application of probiotics for management strategies, further studies on pathogenic bacteria and probiotics are recommended.

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Writing-review and editing: NGIGE, H., ABUBAKAR, M., LELMEN, E.

Project administration: NGIGE, H.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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