PREVALENCE OF *SALMONELLA ENTERICA* AND *SHIGELLA FLEXNERI* IN FRESH FISH PRODUCTS SOLD AT INFORMAL MARKET ALONG THE SHORELINE OF SOME LAKES IN ETHIOPIA

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ABSTRACT

Food borne diseases are considered a major public health challenge worldwide due to their incidence, associated mortality, and negative economic repercussions. Fish and fish products are one of the most important sources of food borne disease and well known medium favoring growth of several bacterial pathogens. This cross-sectional study was conducted to assess the occurrence and prevalence of Salmonella enterica and Shigella flexneri in fresh fish products of two common fish species - Nile tilapia (Oreochromis niloticus) and common carp (Cyprinus carpio) sold along the shoreline of two Ethiopian rift-valley Lakes Hawassa and Ziway. Among the examined 60 samples collected from the study areas, 33(55%) were positive for S. enterica and S. flexneri. This has implications for the consumer health and adherence to simple hygienic steps is advisable. Further, establishing a practice of regular inspection of fish products and fish processing channels for bacterial pathogens and environmental sanitation is necessary.

KEYWORDS

Fresh fish products; common carp (Cyprinus carpio), Nile tilapias (Oreochromis niloticus), Salmonella enterica, Shigella flexneri

1. INTRODUCTION

Food borne diseases have been an issue for all societies since the beginning of humanity. Types, severity and impacts of these illnesses have changed through the ages and are still diverse across regions, countries and communities [1]. Only fractions of the people who become sick from food they have eaten seek medical care and reported to public health authorities, and recorded in official disease statistics. Certain chronic diseases, such as cancer, kidney or liver failure, that result from contaminated food appear long after the ingestion of food and the causal link is never made for each case[1, 2].

Microorganisms play a crucial and unique role in fish and fish products safety. The presences of human bacterial pathogens make the control of pathogenic microorganisms critical for fish products safety [2, 3]. Fish plays an important role in human diet; the production and consumption of fish and fish products have increased worldwide over the years. However, fish and fish products have been involved in various food borne outbreaks [3].Fish and fish products are one of important sources of food borne disease and they are well known medium that favors

growth of several bacterial pathogens such as *S. enterica* and *S. flexneri*. *Salmonella enteric* are responsible for non-typhoidal infection and the second leading cause of food borne illness which can result in the patient's death [2].

Foodstuff contamination may occur directly from infected food-producing animals or may result from poor hygiene during production processes, or the retail and storage of food, since may also harbor microorganisms [2, 4].Non-typhoidal *Salmonella enterica* spp. infection from contaminated food is an important cause of both sporadic gastroenteritis and outbreaks internationally. A wide variety of foods can be contaminated with *Shigella* including fish products. *Shigella flexneri* is the most prevalent species of *Shigella* associate with illness in developing countries [5]. *Salmonella enterica* and *S. flexneri* are an important cause of food borne illness, regularly causing high-profile outbreaks involving commercially-available foods [4].

Salmonella is a leading cause of bacterial food borne illness in the world [6]. Although *S. enterica* and *S. flexneri* are typically associated with foods of animal origin including Milk, Eggs and egg-containing foods, aquatic foods, such as fish and fish products, are quickly gaining recognition as an important vehicle of *Salmonella* and *Shigella* exposure. Reports on food borne bacterial pathogens in fish products particularly *S. enterica* and *S. flexneri* are scant in Ethiopia. Hence, this study was aimed to assess the occurrence and prevalence of *S. enterica* and *S. flexneri* in fish products of two common fish species - Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) sold at the informal market along the shoreline of two Ethiopian rift-valley Lakes Hawassa and Ziway.

2. METHODS

2.1. The Study Area

The study was on two Ethiopian rift-valley lakes, Lake Ziway and Lake Hawassa, which are well-known for their common fish catches and tourist destinations. These lakes are some 163and 275 km to the south Addis Ababa respectively (Fig. 1). The information sources were from World Lake Database [7].

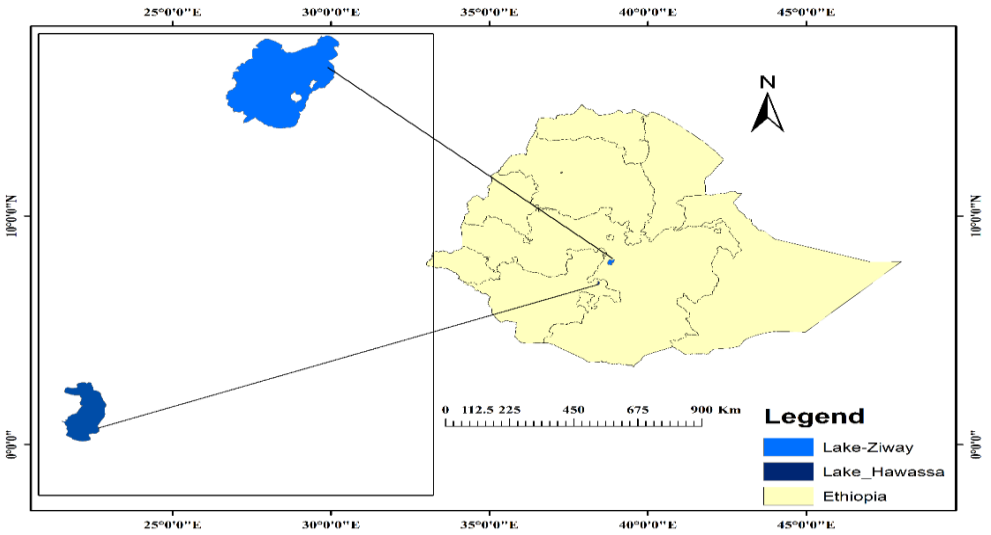

Fig. 1: The study area: (Lake Hawassa and Lake Ziway)

2.2. Study design and sampling strategy

The study design was cross-sectional and its period was from June to December 2022. For sampling, the two lakes were selected purposively that the lakes are known for their common fish catches from which fish are continuously harvested for subsistence and fresh fish products were sold informally along their shoreline for cash [8]. Fresh fish products samples caught from the two lakes was collected from the informal Market around the shorelines of each lake. They were packed in a sterile container with ice blocks to prevent sample deterioration on transit [9, 10] and transferred to the laboratory for identification and biological assays.

2.3. Sample collection

A total of 60(30 from each lake with 30 fresh Nile tilapias fish and 30 fresh common carps fish) samples of fresh fish products those caught from Lake Hawassa and Lake Ziway was bought from the informal markets around the shorelines of each lake, between 8.00 a.m. and 9.00 a.m. local time. They were separately packed in sterile plastic bags with ice blocks to prevent sample deterioration on transit and transferred to the Addis Ababa University, Institute of Biotechnology, Health Biotechnology laboratory for bacterial isolation and identification assays.

2.4. Bacterial Isolation and Identification

2.4.1. Sample preparation

All bacteriological experiments were performed following Society of American Bacteriologists *Manual of microbiological methods* [11], *Bergey's manual of determinative bacteriology* [12] and the respective manufacturer's instructions under complete aseptic conditions. Using sterile forceps, a piece of the body, muscles of the fishes were inoculated separately into McCartney bottles containing selenite-f-broth and Gram-negative broth (HIMEDIA, India) for the isolation of *Salmonella* spp. and *Shigella* spp. respectively, and incubated aerobically at 37ºC for 24 hours. A loop of the inoculated selenite-f-broth and Gram-negative broth was streaked on a plate of MacConkey agar (HIMEDIA, India) and incubated aerobically at 37 ºC for 24 hours. Suspected bacterial colonies were then further picked up with a sterile loop and spread on Desoxycholate citrate agar medium (HIMEDIA, India) and incubated aerobically at 37ºC for 48 hours. These colonies were further purified by repeated subculture on nutrient agar. Pure isolates were stored at -20°C in 50% glycerol (Fine Chemical, Ethiopia) using 1.8ml cryovial (IMEC, China) for biochemical identification.

2.5. Biochemical characterization

All the media used in the biochemical tests were from HIMEDIA, India. The tests began with inoculating respective media with 24-hour-old pure culture colonies. All incubations were at 37°C for 24 hours and expected color changes confirmed test positivity. Briefly, indole production was tested by inoculating 10ml of Dev tryptophan broth, incubating and adding 2-3 drops of indole reagent. Methyl red (MR) test was conducted by inoculating 10ml of MR Voges-Proskauer (MR-VP) medium, incubating, and adding 2-3 drops of 0.05% MR. Voges-Proskauer (VP) test was done by inoculating 10ml of MR-VP medium, incubating, and adding 2-3 drops of 5% α-nephtol followed by 40% of KOH and shaking and leaving it open for an hour.

For catalase enzyme test, a small amount of bacterial colony was transferred to a clean glass slide using a sterile loop and a drop of hydrogen peroxide was added, and the formation of bubbles was checked for. For citrate utilization test, Simmons citrate agar slant was inoculated and

incubated. Hydrogen sulfide or triple sugar iron (TSI) test was done by inoculating TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant, and incubating. Similarly, urea production was tested using Christensen's Urea Agar slant. Sugar fermentation test was conducted using sugar broth medium prepared by mixing 1g peptone, 0.3g meat extract, 0.5g table salt, 0.5g sugar and 0.008g phenol indictor in 100ml distilled water. Three tubes having three different sugars (glucose, sucrose, lactose) in the broth medium were inoculated, and incubated.

2.6. Molecular Characterization

The bacterial isolates that were biochemically characterized and identified as *S. enterica* and *S. flexneri* were to further identify and confirms using molecular techniques. These involve extracting their DNA using standards protocols, amplifying the DNA using PCR, and sequencing the gene of interest using Sanger sequencing at Leiden, The Netherlands [13].

2.6.1. DNA Extraction

DNA was extracted using phenol chloroform standard method as described by Biggin *et al.* [14]. Bacteria cells each of 200µl was added in a 1.5ml micro centrifuge tube, 400µlof lysis buffer and 10µl of proteinase K was added to the 1.5ml tube. The tube was vortexed and placed on heat block at 65℃ for 1 hour while vortexing at interval. Then 400µlof phenol was added and vortexed, briefly after which it was centrifuged for 10 minutes at 13,000rpm to separate the phases. The upper layer was carefully removed with a micro pipette. Chloroform (400µl) was again added and vortexed, centrifuged for 5 minutes at 13,000rpm, the upper layer was then carefully removed. Also 1000µl of 100% ethanol and 40µl of 3M sodium acetate was added and mixed by inverting the tube several times.

The micro centrifuge tubes containing pure DNA were incubated at 20℃ over night. The tubes were centrifuged for 5 minutes at 14,000rpm using centrifuge set at 4 ^{o}C; the upper layer was attentively discarded using micropipette. Then 400µl of 70% ethanol was added and the upper layer was again carefully removed using micro pipette, the tubes were centrifuged for 5 minutes at 14,000rpm using centrifuge set at 4^{o}C, the upper layer was carefully removed using micro pipette in order to remove all traces of ethanol, the DNA was then allowed to dry by leaving the tubes open for 20 minutes at room temperature and 50µlof sterile water was added to re-suspend the DNA.

2.6.2. PCR Amplification

It was performed in a DNA thermal cycler (Eppendorf, Hamburg, Germany). PCR reactions were performed in a final volume of 25µl containing 3µl of template DNA, 2µl of each primer (rD1 and fD1), 14µl of Double distilled water (ddH_2o), 2.5µl of Buffer solution, 2µl of MgCl2 and 1.5µl of Hot Star Taq DNA polymerase and deoxynucleotide triphosphate mix (dNTPs). PCR conditions were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. The PCR products (8 µl) were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide using 1 µM Tris-Acetate-EDTA buffer at 100v for 1 hour and were visualized by UV transillumination [13].

2.6.3. 16S Rrna Sequencing

Sequencing of the 16S rRNA was performed using the universal bacteria primers rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3') and fD1 (5'-CGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') by Sanger sequencing at

BaseClear DNA research laboratory, Leiden, The Netherlands. For sequencing of amplified 16S rRNA directly, four identical 100µl amplification reactions were performed on each sample, with the resultant material being pooled and purified. A 500 ng amount of template (16S rRNA) was combined with 10 ng of primer, 2µl of Sequence buffer, and 10µl water. This sample was held at 98°C for 7 min and cooled to room temperature for 1 min, and then the labeling reaction was performed at 37°C for 5 min. Chain elongation was terminated with sample loading buffer, and sequencing was performed on buffer-gradient gels [14].

2.6.4. Phylogenetic Analysis

The 16SrRNA gene sequences obtained were compared and aligned with sequences deposited in National Center for Biotechnology Information (NCBI) GenBanks database using BLAST for identification of bacteria [15]. Related sequences were obtained and aligned by Pairwise sequence alignments in MEGA6 program using ClustalW algorithm and phylogenetic tree was constructed by the neighbour-joining method program available online [16]. The 16S rRNA sequences of isolated bacteria were used for constructing phylogenetic tree. A total of 500 bootstrapped values were sampled to determine a measure of support for each node on the consensus tree.

2.7. Data Analysis

Bacterial infection status of the fresh fish products sample types of the two lakes was determined and the proportion of infected samples was compared between various categories using the Chi-squared test. Bacterial prevalence in the two different sample types was compared using one-way analysis of variance (ANOVA). Statistical analysis was performed using IBM SPSS software version 26 (IBM, Chicago, USA) and $p<0.05$ was considered the level of statistical significance.

3. RESULTS

3.1. Collected Fresh Fish Products

A total of 60 (30 from each) fresh fish products samples were bought and obtained from the informal market along the two lakes shoreline of which 33(55%) were positive for *S. enterica* and *S. flexneri* pathogens. Among the 55% positive samples, 28.33% (16.67% Nile tilapia fish and 11.67% common carp fish) and 26.67% (13.33% common carp fish and 13.33% Nile tilapia fish) of the fresh fish products were bought along the shoreline of Lake Hawassa and Lake Ziway respectively.

3.2. Colony Morphology of the Isolates

Colonies of the isolated isolates were found to be different in their form, elevation, margin, surface, colour and optical characteristics, and are Gram negative, rod-shaped, usually motile, non-spore forming bacteria.

3.3. Biochemical Identification of Isolates

To further identify the morphologically identified isolates, a range of biochemical tests (Indole, methyl red, Voges-Proskauer (VP), Citrate, H_2S gas, Urease, sugar fermentation and catalase test) were carried out. The results showed that thirty three (33) isolates of *S. enterica* (18) and *S. flexneri* (15) were identified.

3.4. Prevalence of Isolates Among the Examined Fresh Fish Products

Salmonella enterica (30%) was relatively more prevalent than *S. flexneri* (25%) in the examined fresh fish products samples. The highest numbers of *S. enterica* (33.33%) were recovered from the Lake Hawassa fresh fish products samples followed by Lake Ziway (21.2%).But, the highest numbers of *S. flexneri* (27.3%) were recovered from the Lake Ziway fresh fish products samples followed by Lake Hawassa (18.2%). However, even though highest numbers of both pathogens were detected from fresh fish products of Nile tilapia than common carp samples (Table 1), their prevalence showed statistically no significant difference in the samples source of the study lakes ($p<0.005$).

Table 1: Frequency of *S. enterica* and *S. flexneri* among the examined Nile tilapia and common carp fish products from Lakes Hawassa and Ziway (N = 33)

Bacteria isolates	**Lake Hawassa**		**Lake Ziway**		**Total**	**P-value**
	C. carp	Nile tilapia	C. carp	Nile tilapia		
S. enterica	5(15.15%)	6(18.2%)	3(9.1%)	4(12.12%)	18(54.54%)	0.062
S. flexneri	2(6.1%)	4(12.12%)	5(15.15%)	4(12.12%)	15(45.45%)	0.096
Sub-total	7(21.2%)	10(30.30%)	8(24.24%)	8(24.24%)	33(100)	0.056
Total	**17(51.5%)**		**16(48.5%)**		**33(100)**	0.192

Note: - C. carp=Common carp

3.5. Analysis of the 16S Rrna Gene

The results of the genomic DNA extraction and PCR amplifications of the samples on agarose gel electrophoresis shows the amplified 16S rRNA gene with band size of 1490bp obtained from fishes in two different informal markets location along the two lakes as depicted in Fig. 2.

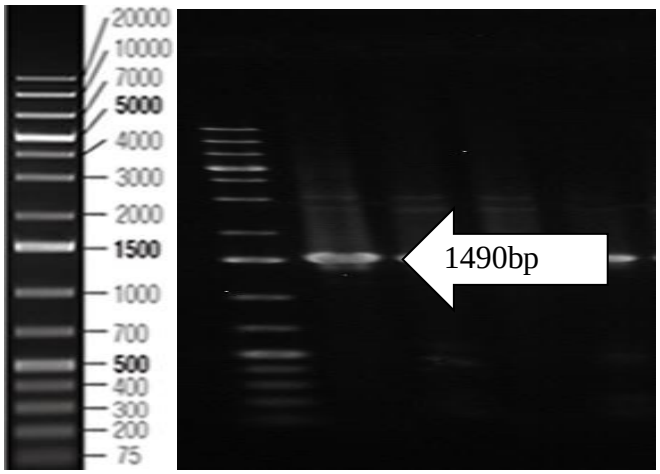

Fig. 2: Agarose gel electrophoresis pattern showing PCR amplified products of 16s rRNA from *S. enterica* and *S. flexneri*

3.6. Phylogenetic Analysis

This result represents the blast result of 16S rRNA gene sequences for isolated *S. enterica* and *S. flexneri*. The 16S rRNA ribosomal PCR amplified products sizes were 1490bp. Comparison of the *S. enterica* and *S. flexneri* isolates with known 16S rRNA sequences in the GenBank database using the BLAST program showed that the isolates had an identity rate of up to 99% to those of other members of *S. enterica* and *S. flexneri*. The phylogenetic relationship of this isolates was studied using the 16S rRNA gene with the respective *S. enterica* and *S. flexneri* strains that were downloaded from NCBI for phylogenetic analysis (Fig.3).

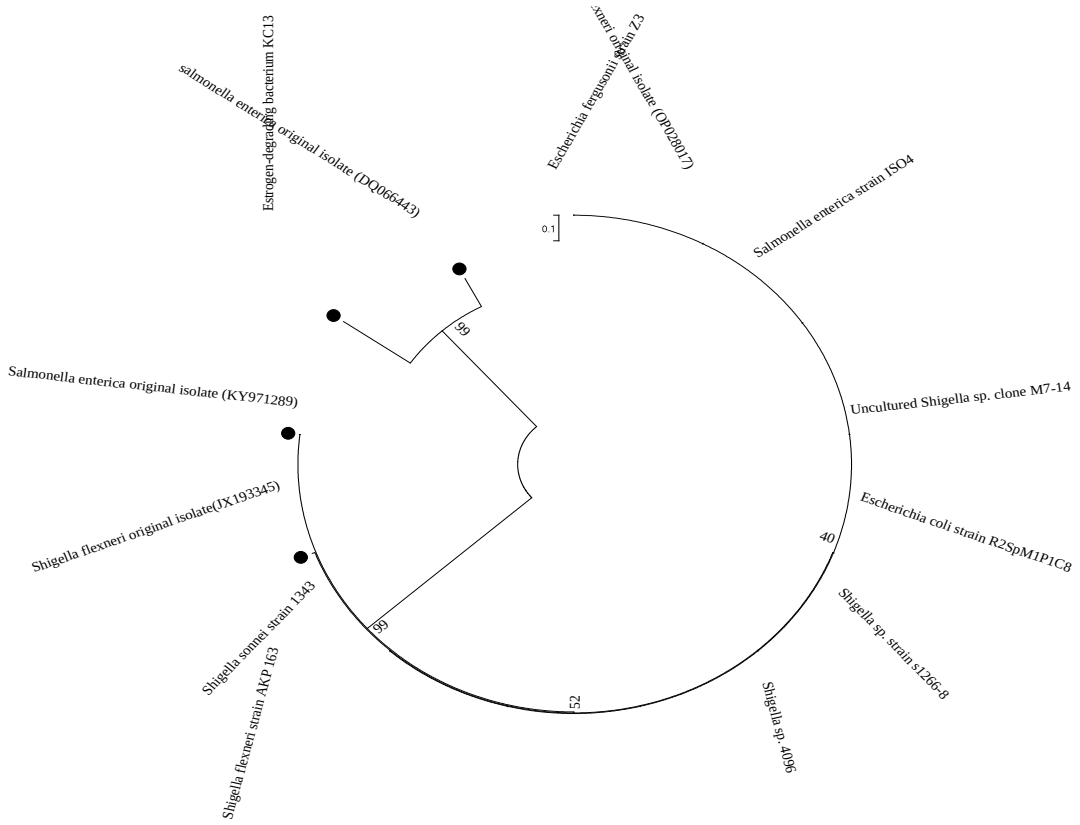

Fig. 3: The phylogenetic relationships based on 16S rRNA gene sequences of *S. enterica* and *S. flexneri* strains constructed by the neighbor-joining method showing homology with different sources of *S. enterica and S. flexneri.* Numerals at nodes indicate bootstrap percentages derived from 500 replications and original isolates were indicated by bold dot.

4. DISCUSSION

Assessing the occurrence of prevalent etiological agents in fish products is necessary to control food-borne diseases of fish origins. The result of this study confirms the contamination of fresh fish products by *S. enterica* and *S. flexneri* pathogens. Results obtained revealed the prevalence of *S. enterica* and *S. flexneri* in fresh fish products bought from the informal market along the shorelines of the two lakes. The results showed that 18 isolates of *S. enterica* and 15 isolates of *S. flexneri* were isolated and identified from the body, muscles of both common carp and Nile tilapia fish. Similarly, Coulombe and Tamber [17] isolated *S. enterica* from fish and fish products. The some authors indicated that, fish and fish products are an important vehicle for

food borne *S. enterica* and as this pathogen is the second leading food borne diseases in the world. Kousar *et al*. [18] and Onyango *et al*. [19] on the other hands reported the isolation of *S. flexneri* from different organs of apparently healthy and naturally infected fish of different species including Common carp and Nile tilapia.

Hidayah *et al*. [20] recorded *Salmonella* and *Shigella* from fresh fish product and smoked fish. The some authors suggested that fish products are among the foodstuffs that are prone to pathogenic microbial contamination which can cause food poisoning and disease in consumers. This result also agrees with the results of Ucak and Afreen [21] that showed the contamination of aquaculture products with *salmonella* spp., and *Shigella* spp., and as *salmonella* and *Shigella* are the main causing agents of infection in fish. Nisa *et al*. [22] also documented the isolation of *S. flexneri* from water, milk and fish products, and their association with an increased human risk to food-borne shigellosis.

Hazarika *et al*. [23] described as the consumption of food of animal origin including fish products are considered to be the primary source of human salmonellosis and shigellosis serving as the principal vehicles of human food borne *salmonella* and *Shigella*. The some authors suggested the prevalence of *salmonella* spp. and *Shigella* spp. in different sectors of the global food chain. Non-typhoidal *Salmonella* accounted for more than 60% of the food borne disease burden[24]. Dissasa *et al*. [25] also indicated the prevalence of *salmonella* spp., and *Shigella* spp. in fish, fish products and the natural source habitat.

Shigella spp. and *Salmonella* spp. are pathogenic bacteria found in animal, human or environmental reservoir. Although contamination of fish products with these bacteria is commonly from the environment, their incidence in ready-to-eat fish products due to unhygienic handling cannot be ruled out [26]. The presence of *Salmonella* and *Shigella* in fish could be due to microbiological pollution of rivers, including runoff and storm-water that contain deposits from wildlife, agriculture, urban, forestry and rural settlements which is a potential health hazard to human; another possibility includes contamination during a multistep handling process from harvest areas to the fish market resulting in deterioration of the quality of fish available for human consumption in the local fish markets [27].

Sources of *S. enterica* and *S. flexneri* infection can vary by environment and include animal and plant-based foods, as well as environmental reservoirs. Several studies have recognized the importance of increased ambient temperature and precipitation in the spread and persistence of *Salmonella* in soil and food [28]. Primary production of food animals remains the most important reservoir of *salmonellae* and *Shigella* entering the human food chain, since *salmonellae* and *Shigella*-free production system cannot be achieved in all animal species. Controls at slaughter and dressing are often not sufficient to prevent *salmonella* and *Shigella* entering the food chain. The prevalence of *Salmonella* in fish captured from aquaculture is relatively lower than in freshwater captured fish, due to possible exposure to untreated sewage, but overall, and in comparison with other food commodities, both remain low risk [29].

5. CONCLUSION

Fish are considered as a nutritious food that is widely produced, marketed, and consumed around the world. Nile tilapia and common carp are among the fish with the highest production, destined for human consumption. Fish are highly susceptible to contamination and deterioration along the food chain by different microorganisms, such as *Salmonella enterica* and *Shigella flexneri*, which is a causal agent of disease in fish, such as Nile tilapia and common carp, and humans negatively affecting primary production and food safety. The issue of *Salmonella* and *Shigella* in fish and fish products is one of the global concerns. The difficulty in eliminating *S. enterica* and *S.*

flexneri to safe levels in fish products underscores the importance of preventing contamination. For *S. enterica* and *S. flexneri*, which are common in natural water systems and is considered as part of the microbiota of freshwater fish such as Nile tilapia and common carp, the implementation of good hygiene practices in production, processing and preservation, and sanitary surveillance of regulatory authorities and the food industry, as well as the promotion of hygiene education in the handling and final preparation of these foods for consumption, can reduce the risk of contamination and related outbreaks of food borne diseases.

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