Occurrence, Molecular Characterization and Antimicrobial Susceptibility of Sorbitol Non-Fermenting *E. coli* Isolates, and Assessment of Fish Handling Practices along the Supply Chain in Central Oromia, Ethiopia

PhD Dissertation

By

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Occurrence, Molecular Characterization and Antimicrobial Susceptibility of Sorbitol Non-Fermenting *E. coli* Isolates, and Assessment of Fish Handling Practices along the Supply Chain in Central Oromia, Ethiopia

A dissertation Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Veterinary Public Health

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We, the members of the Examining Board of the final PhD open defense have read and evaluated the dissertation prepared by Tesfaye Debelu Bedane, titled: Occurrence, Molecular Characterization and Antimicrobial Susceptibility of Sorbitol Non-Fermenting *E. coli* Isolates, and Assessment of Fish Handling Practices along the Supply Chain in Central Oromia, Ethiopia, and recommended that as a partial fulfilling of the dissertation requirement for the degree of Philosophy in Veterinary Public Health.

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DEDICATION

This dissertation manuscript is dedicated to my lovely father, Debelu Bedane Bati and my sweet mother, Toleshi Heyi Bedane, both of whom I lost during this PhD study. May God rest their sole in peace!

STATEMENT OF THE AUTHOR

This thesis is solely prepared during the accomplishment of the PhD degree where all sources of material used in this thesis have been duly acknowledged. As partial fulfillment of the PhD degree, this dissertation is submitted to Addis Ababa University, College of Veterinary Medicine and Agriculture, and is deposited in the college library to be made available to borrowers under the rules of the library. In this thesis, there is no part which has been submitted to obtain a degree, diploma, or certificate in my name in any institution.

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1. Bedane T.D., Agga G.E and Gutema F.D. (2022). Hygienic assessment of fish handling practices along production and supply chain and its public health implications in Central Oromia, Ethiopia. *Scientific Reports;* **12**:13910. https://doi.org/10.1038/s41598-022-17671-5.
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ABBERIVATIONS

BPW	Buffered Peptone Water
CAC	Codex Alimentarius Commission
CDC	Center for Disease Control
CFU	Colony Forming Units
CSA	Central Statistics Authority
CT-SMAC	Cefixime-potassium tellurite Sorbitol MacConkey Agar
EFNG	Ethiopian Federal Negarit Gazeta
EFSA	European Food Safety Authority
EMB	Eosin Methylene Blue Agar
ESZOLF	East Shewa zone Office of Livestock and Fisheries
FAO	Food and Agriculture Organization of the United Nations
FBD	Foodborne Disease
FDOSS	Foodborne Disease outbreak Surveillance System
MDR	Multidrug Resistance
mTSB	Modified Trypton Soya Broth
SN-F *E. coli*	Sorbitol Non-fermenting *Escherichia coli*
WHO	World Health Organization

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ABSTRACT

Fish-borne diseases are among the global public health concern. Contaminated water bodies and poor handling practices along the fish supply chain can lead to fish contamination. Raw fish consumption is a leading cause of fish-borne infection. Emerging sorbitol non-fermenting *Escherichia coli* strains including *Escherichia coli* O157:H7, may transmit to humans through contaminated water and fish. However, despite reports of contamination, there is limited information on the occurrence, molecular characteristics, and antimicrobial susceptibility of sorbitol non-fermenting *Escherichia coli* in Ethiopia. This study, attempts to determine these aspects in fresh water lakes, fish, and humans in central Oromia, Ethiopia. Data were collected using semi-structured questionnaire interviews and personal observations from a total of 200 purposively selected respondents comprising of 50 fishermen, 10 retailers, 20 food establishments serving fish, and 120 consumers. Moreover, a total of 750 samples including 150 fish meat, 150 fish skin swab, 150 fish feces, 150 fresh water of the lakes, 150 human stool samples were collected from five Lakes and three health facilities. Overall, 150 fish, 30 fish from each Lake, comprising of six fish species: *Oreochromis niloticus, Clarias gariepinus,* Tilapia Zilli, *Cyprinus carpio, Labeobarbus intermedius, Barbus ethiopicus*, which are commonly used for human consumption were included in the study. Fecal, meat and skin swab samples were taken from each fish. The samples were processed following the standard protocol recommended by European Food Safety Authority and Kirby–Bauer disc diffusion method for detection of the bacteria, and antimicrobial susceptibility tests, respectively. Molecular characterization of presumptive isolates was performed using Whole-Genome Sequencing for serotyping, determination of virulence factors, antimicrobial resistance traits, and genetic linkage of the isolates. The study revealed a wide range of hygienic breaches, including fish processing at unhygienic landing sites, washing filleted fish with contaminated water of the Lakes, use of a single knife for processing all fish with infrequent washing and with no disinfection in between. Majority (70%; n=10) of the retailers and all the food establishments transported fish in vehicles with no cold chain facilities. Over three-fourths (77%; n = 120) of the consumers preferred consuming raw fish; 80% of them lacked the knowledge of fishborne diseases. Few good hygienic practices appreciated include, the use of refrigerators for storage in all retailers and 70% (n =20) of the food establishments, and the use of vehicles with thermostat for the transportation of fish among 30% of retailers. From a total of 750 microbiological

samples analyzed, 3.9% (29/750) was found positive for sorbitol non-fermenting *Escherichia coli;* of which 6.7% (n=10), 1.8% (n=8) and 7.3% (n=11) were retrieved from water, fish, and diarrheic human patients, respectively. Whole-Genome Sequencing confirmed that all the isolates were sorbitol non-fermenting *Escherichia coli* strains; among which none of them were *Escherichia coli* O157:H7. Two novel *Escherichia coli* strains with unknown O-antigen were retrived from fish feces and human stool. All the strains have multiple virulence factors and one or more genes encoding for them. Genetic relatedness was observed among strains from the same sources (water, fish, and humans). Most isolates were resistant to ampicillin (100%), tetracycline (100%), cefotaxime (100%), ceftazidime (100%), meropenem (100%), nalidixic acid (93.1%) and sulfamethoxazole/trimethoprim (79.3%). Majority of the strains were resistant to chloramphenicol (58.6%) and ciprofloxacin (48.3%), while small fraction showed resistance to azithromycin (3.45%). Isolates have shown an overall multi-drug resistance profile of 87.5%. Majority, (62.1%; n=18) of the strains have acquired multi-drug resistance traits. Genes encoding for mutational resistance and extendedspectrum beta-lactamases were also detected. In conclusion, the study revealed a wide range of hygienic breaches along the fish production and supply chain; lack of infrastructure for post-harvest fish handling and processing, lack of appropriate transportation facilities and presence of knowledge gaps regarding fish borne diseases, and the occurrence of virulent and antimicrobial resistant sorbitol non-fermenting *Escherichia coli* strains in water, fish, and humans. Although no genetic relatedness was observed among strains from various sources, the genomic clustering among strains from the same sources strongly suggests the potential risk of transmission along the supply chain at the human–fish-environment interface if strict hygienic fish production is not in place. Hence, infrastructural development for hygienic fish production and processing, training for fish producers, consumer retailers and food establishments, and robust genetic study of the new strains with unknown O-antigens is required to improve fish safety and reduce the risk of public health.

Keywords: *Antimicrobial resistance, Contamination, Escherichia coli*, *Fish-borne diseases*, *Hygienic practices, Whole-genome sequencing*

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Background and Justification

Fish provide an affordable and nutritious source of protein, minerals, and other essential nutrients that are consumed by over 120 million individuals globally (FAO, 2020). The industry has recorded impressive growth, with global fish production reaching approximately 179 million tons annually and per capita consumption rising from 9 kg in 1961 to 20.5 kg in 2018 (FAO, 2020). Despite the aforementioned economic and dietary benefits, fish and the aquatic habitats are reservoirs of zoonotic pathogens that may impact human health in addition to the aquaculture industry. Recent studies have outlined the growing threat posed by the pathogens as they result in unprecedented health and economic effects (FAO, 2022; Ziarati *et al*., 2022; Sumanaa *et al*., 2023).

As the global growth of fisheries and aquaculture operations proceeds at an increased rate, the risk of fish-borne diseases has emerged as a growing concern. Amongst the bacterial pathogens are Mycobacterium species, *Clostridium botulinum*, *Vibrio vulnificus*, and *Streptococcus iniae.* Other pathogenic bacteria include *Salmonella typh*i, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus,* and *Enterococcus faecalis* (David, 2015; Irshath *et al*., 2023; Zhang *et al*., 2023). Furthermore, parasites like Anisakis, Diphyllobothrium, and Opisthorchis are common in fish, along with protozoan parasites such as Cryptosporidium. Fungal infections like Basidiobolomycosis and sporotrichosis are also on the risk list, reflecting the need for stringent safety measures and vigilant surveillance in the expanding aquaculture sector (Akbar *et al.*, 2023; Hegde *et al*., 2023).

As per estimates worldwide, fishborne disease infects around 1.5 million people each year, out of which *Escherichia coli* causes about 30% of them (WHO, 2023). In the United States, the issue is pertinent, with approximately 260,000 individuals falling ill annually from poisoned fish between 2017 and 2021alone (CDC, 2022). Similarly, fishborne illiness is equally a concern in Ethiopia, though specific data are less comprehensive. Recent reports indicate there have been fish-borne outbreaks in some regions of Ethiopia, resulting in a growing public health issue (Ayalew, 2019). This justifies the

necessity for enhanced fish safety measures and improved surveillance systems across the world, including Ethiopia, to reduce risks associated with consumption of fish.

Escherichia coli possesses a diverse range of pathogenic profiles, and seven distinct pathotypes have been classified on the basis of clinical, epidemiological, and pathogenic features. These include: Enteropathogenic *E. coli* (EPEC), which results in prolonged diarrhea in infants; Enterotoxigenic *E. coli* (ETEC), the primary cause of traveler's diarrhea; Enteroinvasive *E. coli* (EIEC), the invasive and destructive intestinal lining form; Enterohemorrhagic *E. coli* (EHEC), also referred to as Shiga toxin-producing *E. coli* (STEC), associated with acute foodborne illness and hemorrhagic colitis; Enteroaggregative *E. coli* (EAEC), which is adhesive to the intestinal mucosa and causes chronic diarrhea; Diffusely adherent *E. coli* (DAEC), which causes urinary tract infections and diarrhea; and Adherent invasive *E. coli* (AIEC), which is associated with Crohn's disease and intestinal inflammation (Nataro and Kaper, 1998; Croxen *et al*., 2013).

Sorbitol non-fermenting *E. coli* O157:H7 is a globally renowned foodborne pathogen, frequently causing severe outbreaks of gastroenteritis and hemolytic uremic syndrome (Paton and Paton, 2021). Recent studies also identified that enterotoxigenic and enteropathogenic *E. coli* strains are novel zoonotic pathogens in fish intended for human consumption and can have extreme health impacts (Ziarati *et al.*, 2022). Furthermore, it has been indicated that even non-pathogenic *E. coli* isolates carrying toxins can infect fish, which in turn cause foodborne disease in humans (Sok *et al*., 2023). The above facts highlight the need for continuous vigilance in detecting and preventing *E. coli* contamination in the fish supply chain.

Shiga toxin-producing *E. coli* is an emerging cause of foodborne illness with approximately 2,801,000 acute infections worldwide annually, including 10,200 African cases (Browne *et al.*, 2018; Lupindu, 2018). This pathogen also gives rise to 3,890 hemolytic uremic syndrome (HUS) infections and 230 fatalities each year. The sorbitol non-fermenting *E. coli* O157:H7, accounts for 10% of this disease burden (Majowicz *et al.*, 2014). Despite its lower prevalence as compared to other notifiable zoonoses (Majowicz *et al*., 2014), its impact of long-term kidney and brain damage in very young children is of significant public health concern (Rosales *et al.*, 2014). The agent is also harmful to the elderly, immunocompromised patients, and even a healthy individual following exposure to very high doses (Adley and Ryan, 2016;

CDC, 2020). While adults and immunocompetent individuals generally recover from bloody diarrhea, about 15% of children's cases can progress to HUS (Tarr *et al*., 2005). Moreover, about one-third of HUS cases can result in encephalopathy and brain injury, which is occasionally lethal (Siegler *et al*., 1994). Therefore, eating raw or under-cooked food, including fish and other aquatic food products, is greatly discouraged, especially among the high-risk populations (Jackie and Sarah, 2021).

Contamination of fish with pathogenic organisms like *E. coli* is a complex and not yet entirely clear process. Contamination from the environment plays a key role in facilitating the spread of *E. coli* from feces of animals into water bodies, and then to fish (Novoslavskij *et al*., 2016; Anyanwu *et al*., 2022; Bedane *et al.*, 2022). Pathogenic strains of *E. coli* are widespread among a variety of animal hosts, such as mammals, birds, amphibians, fish, and invertebrates (Espinosa *et al.*, 2018; Johnson and Russo, 2018). Infected hosts release these pathogens into the environment, mostly through fecal matter, at levels of 10^4–10^6 per gram in domesticated animals and 10^7–10^9 per gram in humans (Tenaillon *et al*., 2010). In the outside environment, *E. coli* possesses a remarkable ability to persist under different conditions such as soil, water, food, and sediments even in the absence of active growth (Leimbach *et al*., 2013). Fish may thus get infected at multiple points in the supply chain, from contaminated water sources and aquatic sediments to processing facilities, transportation, and storage channels (Yukgehnaish *et al*., 2020; Sheng and Wang, 2021). This calls for enhanced understanding of the ecology of pathogenic *E. coli* strains, including particular variants like SN-F *E. coli* for preventing infections of humans by maintaining the contamination under control and in check within the environment (Jang *et al*., 2017).

Even though *E. coli* is not a natural inhabitant within the microbial flora of fish (Guillen and Wrast, 2010), infection by certain strains of *E. coli* among various fish species would mean that fish could potentially act as a carrier for the bacterium within water environments (Hansen *et al*., 2008). *Escherichia coli* is most commonly recovered from the digestive tract of fish but also present in other tissues such as the gills, kidneys, muscles, and bladder in contaminated environments (Barbosa *et al*., 2014). Various factors, including seasonality, contact of humans with fish, environmental contamination, and immune status, influence these infections (Haile and Getahun, 2018).

Escherichia coli strains with significant virulence factors were documented in fish intended for human consumption in regions of poor hygienic conditions, e.g., the tropics (Thampuran *et al.*, 2005), Costa Rica (Marin *et al.*, 2009), South West Cameroon (Akoachere *et al*., 2009), Egypt (Mostafa *et al*., 2016), and India (Chandraval and Chandan, 2016), which would indicate the potential role of fish to disseminate *E. coli* to humans. The potential of *E. coli* infections, along with other water pathogens, is similarly high in developing nations, where weakened infrastructure and sanitation drive the transmission. Under such conditions, approximately 46% of these infections are caused by oral route, typically through consumption of infected raw fish or swallowing contaminated water while swimming because of negligence. In addition, 15% of infections occur through more than one route of infection, with 24% being acquired through contact with dirty water and 19% from direct skin contact when handling fish (Raissy, 2017).

Fish handling is compromised by inadequate sanitary practices, which in turn enhances the chances of contamination. The difficulty in ascertaining the true prevalence of such infection, especially of gastrointestinal disease, is compounded further by under-reporting due to their typically short and self-limiting nature (Novotny *et al*., 2004). In Ethiopia, limited availability of good quality health care facilities and sanitation puts more pressure on this public health threat, relaying the harshest reality for better infrastructures and intervention strategies to protect against the risk of aquatic pathogens.

The global emergence of antimicrobial resistant foodborne pathogens like *E. coli* strains with resistance to 3rd and 4th generation cephalosporins and reserve-line drugs like carbapenems is increasingly becoming a source of public health concern (Pfeifer *et al.,* 2010; Okwu *et al.,* 2019). Recent studies have shown a startling rise in antimicrobial resistance among *E. coli* O157:H7 strains (Haile *et al*., 2022; Abebe *et al.*, 2023), and increasingly among non-O157:H7 *E. coli* non-sorbitol fermenter strains (Anyanwu *et al.*, 2022; Wiriyaprom, *et al.*, 2022). This infers to a higher risk of environmental contamination; because feces from cattle that harbor these resistant pathogens can infiltrate water bodies, leading to their growth in aquatic environments and fish.

In Ethiopia, multi-drug resistant pathogens were identified in fish collected from Dambel and Hawasa Lakes (Guta *et al*., 2023). This represents a serious infection risk in humans, particularly by eating raw infected fish, a common practice around Lake Dambel

(Bedane *et al*., 2022). Thus, it is necessary to tackle the emergence of these non-sorbitol fermenting *E. coli* strains and their pattern of resistance to avert public health risks in such regions.

The abused use and overuse of antimicrobial drugs in terrestrial and aquatic food animals, including fish, have led to the emergence of antimicrobial resistant organisms (Guardabassi *et al.,* 2008). Such resistance can further be passed on to humans through the food chain (Caudell *et al*., 2018). This is more so a concern when zoonotic pathogens contaminate drinking water or food intended for use in humans, such as fish (Subbiah *et al.*, 2020). *Escherichia coli* isolates are among the main causes of this issue because they acquire and transfer resistance genes horizontally, leading to therapeutic failure in both animals and humans (Poirel *et al.*, 2018).

The aquatic ecosystem is highly prone to contamination by zoonotic pathogens such as *E. coli* and *Salmonella*, which can be transmitted from water to fish and ultimately to human beings, posing serious public health risks (Raissy, 2017; Wondimu, 2020). Contamination may occur due to contact with infected water, processing facilities, and equipment (Hastein *et al*., 2006; Chepkemoi *et al.*, 2015). Additionally, poor hygiene and unsupervised fish handling add to the risk, with sub-clinically ill handlers being able to contaminate fish throughout the supply chain (Svanevik and Lunestad, 2011; Liang *et al.*, 2021). This is especially a risk in nations like sub-Saharan Africa, where poor infrastructure adds to the risks of eating contaminated fish (Erasmus *et al*., 2022). Various studies have been demonstrated that virulent and multi-drug resistant strains of *E. coli* O157:H7 and *Salmonella* , and other antibiotic-resistant pathogenic bacteria like *Aeromonas* can spread across the fish supply chain (Novotny *et al.*, 2004; Maja *et al*., 2022). A recent study in Bahir Dar, Ethiopia, revealed serious hygiene breaches, where multi-drug resistant microorganisms were detected in fish produced for human consumption, highlighting the need for stronger food safety measures (Temesgen *et al.*, 2024).

In developing nations, the incidence of fishborne diseases is highly determined by unhygienic environments in fish handling and the absence of a proper cold chain at the time of transport and storage. These deficiencies, contrary to the FAO's code of international conduct for responsible fisheries (2011), have far-reaching implications for both public health and economic stability (Agyei and Maalekuu, 2014). Pathogens such as *E. coli* O157:H7 can be

added to the fish value chain at any stage in the process from aquaculture to consumption (Belina *et al.,* 2021). Consequently, the consumer is at risk of becoming ill from the consumption of contaminated or improperly processed fish (Mina *et al*., 2022).

In Ethiopia, while there is still limited systematic information regarding SN-F non-O157:H7 *E. coli* strains, previous studies documented the presence of *E. coli* O157:H7 in various contexts. The pathogen has been isolated in fish intended for human consumption (Ayalew, 2019; Tilahun and Engdawork, 2019), diarrheal patients (Gutema *et al.*, 2021), and children under five years (Adugna *et al*., 2015; Getaneh *et al*., 2021). Interestingly, Ayalew (2019) and Tilahun and Engdawork (2019) also reported the isolation of antimicrobial-resistant strains of *E. coli* O157:H7 from fish sampled from Lake Hyke and Tekrze Dam in northern Ethiopia and Lake Hawasa in the south, respectively, reporting an alarming antimicrobial resistance trend.

1.2 Statement of the Problem

Ethiopia possesses extensive water bodies and wetland ecosystems containing over 200 species of fish (Hirpo, 2017) with an estimated average annual fish production potential of 94,500 metric tons (Tesfaye and Wolff, 2014). Nevertheless, with this huge potential and overall demand for fish consumption, quality and safety control remains a crucial issue (Gazu *et al.*, 2023). Several bacterial pathogens have been identified to infect and kill fish in the nation, with a potential risk of transmission to humans (Sorsa *et al.,* 2019). Infection can be triggered by handling infected fish at fish farms, markets, or by consuming raw or under-cooked contaminated fish (Austin *et al*., 2006). The prevalence of such pathogens throughout the fish supply chain is often attributed to inadequate hygienic practices, with the result that fish meant for human consumption become contaminated by foodborne pathogens of significant public health concern (Sorsa *et al.*, 2019).

Among the key bacterial pathogens causing foodborne illness, including fishborne disease, are SN-F strains of *E. coli,* such as the Shiga toxin-producing *E. coli* O157:H7 (Fung *et al*., 2018). In Ethiopia, Central Oromia in particular, raw fish consumption is prevalent; however, there is limited data on levels of contamination of fish intended for human consumption with *E. coli* O157:H7 and other SN-F *E. coli* strains, and antimicrobial resistance profiles of the pathogens in water, fish and human. In addition, the virulence factors and antimicrobial

resistance determinants of such pathogens in water fish and humans is not documented. Furthermore, information on hygienic handling of fish in the production and supply chain is not available in the study areas.

1.3 Research Questions

1. Do SN-F *E. coli* O157:H7 and non-O157:H7 strains occur in water, fish and humans in central Oromia, Ethiopia?
2. Do SN-F *E. coli* strains prevailing in the area exhibit resistance to common antimicrobial agents used for the treatment of enteric infections?
3. What are the status of hygienic practices along the fish production and supply chain in central Oromia, Ethiopia?

1.4 Study Hypothesis

The study was initiated based on the following two key hypothesis;

Ho-1: Sorbitol non-fermenting *E. coli* strains including the potent Shiga toxin-producing *E. coli* O157: H7 do not occur in lake water and fish in central Oromia, Ethiopia; suggesting that consumption of raw fish has no risk of acquiring such pathogens in humans.

Ha-1: Genetically related antimicrobial resistant SN-F *E. coli* strains including the potent Shiga-toxin producing *E. coli* O157:H7 may occur in lake water, fish and humans suggesting human exposure via consumption of contaminated raw fish in central Oromia, Ethiopia.

Ho-2: The status of hygienic practices along the fish production and supply chain in central Oromia, Ethiopia may be good.

Ha-2: The status of hygienic practices along the fish production and supply chain in central Oromia, Ethiopia may not be good.

1.5 Research Objectives

1.5.1 General Objective

The overall objective of the study was to investigate the occurrence, molecular-linkage and antimicrobial susceptibility of SN-F *E. coli* strains occurring in water, fish and humans, and assess hygienic fish handling practices along the supply chain in central Oromia, Ethiopia.

1.5.2 Specific Objectives

- To investigate the occurrence of *E. coli* O157:H7 and other SN-F *E. coli* strains in water and fish sampled from Lakes Babogaya, Hora-Arsedi, Koftu, Koka, and Dambel (Chapter 2);
- To identify the presence of *E. coli* O157:H7 and other SN-F *E. coli* strains in diarrheic patients with a history of fish consumption (Chapter 2);
- To assess the genetic relationships of the isolates from water, fish, and humans (Chapter 2);
- To evaluate the antimicrobial susceptibility profiles of the isolates to commonly used antimicrobial agents for the treatment of enteric infections (Chapter 2);
- To assess hygienic fish handling practices along the production and supply chain in the study areas (Chapter 3).

1.6 Significance of the Study

Pathogenic strains of SN-F *E. coli*, including *E. coli* O157:H7, constitute a serious public health hazard in areas with high raw fish consumption. However, no data is available on the occurrence of such pathogens in central Oromia, Ethiopia. With the application of contemporary molecular techniques, such as genomic sequencing, to delineate the epidemiological associations between *E. coli* O157:H7 and other SN-F *E. coli* strains in fresh water, the fish, and humans, there is a need to unveil possible transmission pathways from aquatic reservoirs to human consumers. Furthermore, *E .coli* strains serve as reservoirs for antimicrobial resistance genes that drive the emergent threat of treatment-resistant infection. These strains carry the ability to acquire, transfer, and spread resistance mechanisms and are of public of health concern. Thus, assessment of SN-F *E. coli* and its antimicrobial susceptibility patterns is critical not only for determining the prevalence of resistance in such populations but

also for the determination of epidemiology, and identification of most appropriate therapeutic strategies. This data is critical to identify control action point and maximize treatment regimens for enteric infections caused by *E. coli* strains since it will enable doctors to select the most appropriate antibiotics and reduce treatment failure, chronic disease, and transmission of resistant strains. Genomic analysis also show biodiversity of the strain in these ecology. Thus, in the rising context of antimicrobial resistance, monitoring these susceptibility profiles becomes an essential component of infection control and public health practice.

Second, monitoring hygienic practice across the fish supply chain and community patterns of raw fish consumption is pivotal to the establishment of risk factors of exposure to fishborne pathogens like *E. coli* O157:H7 and other MDR SN-F *E. coli* strains. Policymakers and other stakeholders need such data to design effective efforts towards minimizing human exposure to antimicrobial resistant microbials and other fishborne pathogens. It will help in designing community awareness creation on control through training. The study will investigate the occurrence of *E. coli* O157:H7 and other SN-F *E. coli* throughout the fish supply chain, in addition to present hygienic practices.The outcomes will be precious in shaping effective interventions to enhance fish safety and public health.

1.7 Scope of the Study

The study, conducted from December, 2020 to December, 2024 analyzed 750 samples such as water (n=150), fish meat (n=150), fish skin swab (n=150), fish feces (n=150) and human stool (n=150) to investigate the occurrence, molecular characteristics, and antimicrobial susceptibility of *E. coli* O157:H7 and other SN-F *E. coli* strains in central Oromia, Ethiopia. The samples were analyzed using standard culture techniques, *E. coli* O157:H7 latex agglutination test and whole genome sequencing (WGS) based on standard protocols. Molecular clustering of the isolates from the three sources was screened using EnteroBase for epidemiological links, and *insilico* identification of virulence and antimicrobial resistance genes was performed. In addition, the study established hygienic fish handling practices of fish handlers and processors, and raw fish consumption habits of fish consumers to examine the risks associated with fish production, processing, transportation, storage, and consumption using questionnaire and observation. The integrated approach endeavored to

achieve the objectives of the study in an all-encompassing manner and provide insights that can guide policymakers to develop intervention strategies to enhance fish safety and public health.

1.8 Limitations of the Study

The study was geographically limited to certain lakes in Central Oromia due to economic and logistical constraints. It also did not include stool samples from clinical cases of fishermen, who come into direct contact with fish and water bodies. The study also did not construct a phylogenetic tree for the isolates found compared to *E. coli* strains in the genomic database. Fish from retail outlets and food premises, and samples from fish-handling equipment and workers' hands were not taken. The study also did not include sample from aquatic birds which could act as source of fecal contamination of the water bodies. This exclusion restrained a full evaluation of the existence of the pathogens throughout the fish supply chain. Future studies should address these limitations to give a comprehensive overview of the prevalence and dissemination of SN-F *E. coli* strains, particularly *E. coli* O157:H7, throughout the fish supply chain.

1.9 Structure of the Dissertation

The dissertation adheres to the PhD dissertation writing guideline of Addis Ababa University and employed paper-based structure. It is composed of a general introduction, two published papers (Paper 1 and 2), and a general discussion, divided into four distinct chapters: 1, 2, 3, and 4, respectively. Paper 1 responds to specific objectives 1, 2, 3 and 4, presenting crucial information on the occurrence, molecular characteristics, antimicrobial resistance, and epidemiological relationships of SN-F *E. coli* strains isolated from water, fish, and humans in central Oromia, and assesses the overall risk of transmission of the pathogen from contaminated water to fish, and then to humans. Paper 2 responds to specific objective 5, which assessed hygienic handling of fish throughout the supply chain in the study areas. Both papers are published on a reputable journal (Scientific reports). Chapter 4 is the general discussion chapter, which gives the overall findings of the study, relating the findings to other global findings and corresponding national and international food safety standards and

regulations, and suggests future intervention actions and research directions. The section is followed by a list of references used in the general introduction and discussion chapters, and appendices, which is the concluding section of the dissertation.

CHAPTER 2: OCCURRENCE, MOLECULAR CHARACTERIZATION, AND ANTIMICROBIAL SUSCEPTIBILITY OF SORBITOL NON-FERMENTING *Escherichia coli* IN LAKE WATER, FISH AND HUMANS IN CENTRAL OROMIA, ETHIOPIA

Abstract

Contaminated lake water and fish can be sources of bacterial pathogens of public health concern, including *Escherichia coli*. In central Oromia, Ethiopia, raw fish consumption is a common practice. Although there are few reports on the occurrence of *Escherichia coli* O157:H7 in fish destined for human consumption and children under five years, information on the transmission pathways of *Escherichia coli* O157:H7 and other sorbitol non-fermenting *Escherichia coli* strains from water-to-fish-to-humans, and their virulence factors and antimicrobial resistant determinants along the fish supply chain is lacking. The study aimed to investigate the occurrence, molecular characteristics, and antimicrobial susceptibility of *Escherichia coli* O157:H7 and other sorbitol non-fermenting *Escherichia coli* strains in fish, lake water and humans in central Oromia, Ethiopia. A total of 750 samples (150 fish meat, 150 fish skin swab, 150 fish feces, 150 lake water, 150 human stool samples) were collected from five lakes and three health facilities. The samples were processed following the standard protocol recommended by European Food Safety Authority and Kirby–Bauer disc diffusion method for detection of the bacteria, and antimicrobial susceptibility tests, respectively. Molecular characterization of presumptive isolates was performed using Whole-Genome Sequencing for serotyping, determination of virulence factors, antimicrobial resistance traits, and genetic linkage of the isolates. Overall, 3.9% (29/750) of the samples had sorbitol non-fermenting *Escherichia coli;* of which 6.7% (n=10), 1.8% (n=8) and 7.3% (n=11) were retrieved from water, fish, and diarrheic human patients, respectively. Whole-Genome Sequencing confirmed that all the isolates were sorbitol non-fermenting non-O157:H7 *Escherichia coli* strains. Two novel *Escherichia coli* strains with unknown O-antigen were retrived from fish and human samples. All the strains have multiple virulence factors and one or more genes encoding for them. Genetic relatedness was observed among strains from the same sources (water, fish, and humans). Most isolates were resistant to ampicillin (100%), tetracycline (100%), cefotaxime (100%), ceftazidime (100%), meropenem (100%), nalidixic acid (93.1%) and sulfamethoxazole

/trimethoprim (79.3%). Majority of the strains were resistant to chloramphenicol (58.6%) and ciprofloxacin (48.3%), while small fraction showed resistance to azithromycin (3.45%). Isolates had an overall multi-drug resistance profile of 87.5%. Majority, (62.1%; n=18) of the strains had acquired multi-drug resistance traits. Genes encoding for mutational resistance and extended-spectrum beta-lactamases were also detected. In conclusion, our study revealed the occurrence of virulent and multi-drug resistant sorbitol non-fermenting *Escherichia coli* strains in water, fish, and humans. Although no genetic relatedness was observed among strains from various sources, the genomic clustering among strains from the same sources strongly suggests the potential risk of transmission along the supply chain at the human–fish-environment interface if strict hygienic fish production is not in place. Further robust genetic study of the new strains with unknown O-antigens, and the epidemiology of sorbitol non-fermenting *Escherichia coli* is required to elucidate the molecular profile and public health implications of the pathogens.

Keywords: *Contaminated fish, Lake water, Diarrheic human patients, Sorbitol non-fermenting Escherichia coli, Central Ethiopia*

2.1 Introduction

Foodborne diarrheal diseases, including those acquired through fish consumption, are among the leading cause of morbidity and mortality globally with a mortality rate of 22.4 deaths per 100, 000 person-years, and a substantial impairment to socioeconomic development worldwide[1,2]. The mortality rate is higher among children under 5 years, elderly people over 70 years, and in low-income countries[3].

Perishable food items like ground beef, raw milk, meat, fish, vegetables, unpasteurized fruit juices originated from contaminated sources or contaminated during production or processing may harbor potential foodborne pathogens, including *E. coli*[4–6]. For instance, cross-contamination, inadequate cooking and storage are the dominant food safety impediments responsible for 60–78% of the foodborne disease burden (FBD); while raw foods accounted for an estimated 23–41% of the disease burden in France[7].

Both wild and cultured fish are sources of a wide variety of bacterial pathogens of public health concern[8,9]. Human infections due to potential pathogens acquired from fish or the aquatic environment are also quite common depending on season, poor hygienic fish handling practices, raw fish consumption habits and the immune status of the exposed individuals[10]; implying that detection of the microbial quality of fish intended for human consumption is crucial[11].

Although most strains of *E. coli* are normal inhabitants of the intestinal tract of humans, animals and fish[12], some strains have acquired genetic determinants encoding for various virulence factors giving them the capacity to exert intestinal and extra-intestinal illness in humans with a characteristic watery or bloody diarrhea[13]. Based on their clinical, epidemiological, and pathogenic characteristics, there are seven pathotypes of diarrheagenic *E. coli* strains; including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) or Shiga toxin producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) [13], and a new pathotype, adherent invasive *E. coli* (AIEC)[14]. Each pathotype has a distinctive virulence factor responsible for colonization and subsequent pathogenic effects of the pathogen[15,16], among which EPEC, ETEC, and EAEC are the dominant causes of infantile diarrhea in developing countries with a relatively low standard of living[17].

Escherichia coli may contaminate fish destined for human consumption and become a potential fish safety concern[12]. For instance, from 2009–2015 in the United States and Puerto

Rico alone, the FBD Outbreak Surveillance System (FDOSS) section of CDC received reports of 344 outbreaks and 2,288 illnesses associated with consumption of contaminated food of aquatic origin including fish[18]. A report from the Zhejiang Province of China has also shown, from 2010–2020 aquatic products including fish were responsible for 109 outbreaks, 1073 cases, and 77 hospitalizations, though no death was recorded[19]. Previous reports have also shown that zoonotic pathogens including *E. coli* can be transmitted to humans via consumption of contaminated and improperly processed fish or fish products[20].

However, despite reports of *E. coli* strains harboring many genes encoding for virulence factors from human and animal feces, only a few studies have investigated the occurrence of pathogenic *E. coli* strains in environmental water including lake water[21], and fish[12]. Although Ethiopia has a huge potential of fish production and its consumption is common in the country, the fish quality and safety aspect is overlooked by regulatory bodies due to limited implementation of food safety regulations[22]. The report of Bedane et al. 2022 indicated lack of infrastructure for fish production and processing, unavailability of cold chain facility for transportation, unhygienic handling practices, and the habit of consuming raw fish in central Oromia, Ethiopia[23]. Besides, some studies reported the occurrence of *E. coli* O157:H7 in fish and humans from various parts of the country. A 1.6% and 1.3% occurrence of *E. coli* O157:H7 in fish in Lake Hayq and Tekeze dam, respectively, was reported from northern Ethiopia[24]. Similarly, Tilahun and Engdawork[25], have reported 2.3% prevalence of the pathogen from fish in Lake Hawassa, southern Ethiopia. In humans, 15.3% prevalence and 28.9% isolation rate of *E. coli* O157:H7 related diarrhea in children under five years was reported from Eastern Ethiopia[26], and Bahir Dar town of northern Ethiopia[27], respectively. Similarly, Gutema et al.[28], have detected *E. coli* O157:H7 in 2.8% of 216 diarrheic patients' stool samples investigated in Bishoftu town, central Ethiopia. Besides, *E. coli* is the dominant donor and recipient of resistance genes through horizontal gene transfer, and directly or indirectly, it is the prime cause of treatment failures in both animals and humans. Thus, at a global scale, AMR *E. coli* is a public health concern[29].

In Ethiopia, Central Oromia in particular, although raw fish consumption is a common practice, and there are few reports of *E. coli* O157:H7 from fish destined for human consumption and children under five years, information on the transmission pathways of *E. coli* O157:H7 and other SN-F *E. coli* along the fish supply chain and, the virulence factors and antimicrobial resistance determinants of the pathogens is lacking. Therefore, this study was aimed to

investigate the occurrence, molecular characteristics, and antimicrobial susceptibility of *E. coli* O157:H7 and other sorbitol non-fermenting *E. coli* strains in lake water, fish, and humans to examine potential transmission pathways along fish supply chain in central Oromia, Ethiopia.

2. 2 Materials and Methods

2.2.1 Study sites and settings

The study was conducted from December 2020 to June 2022 in East Shewa zone **(**Bishoftu, Koka and Batu**)**. East Shewa zone is located in the upper Rift-Valley region and endowed with, a number of crater lakes. The zone has a total population of 1,356,342 within an area of 8,370.90 square kilometers with a population density of 162.03. Three-fourths (74.9%) of the population are mixed crop-livestock farmers dwelling in rural settings, while 25.1% are urban inhabitants[30]. The Rift-Valley lakes including lakes Dambel, Koka, Babogaya, Hora-Arsedi and Koftu are important source of fish for consumers in the adjacent towns (Bishoftu, Koka and Batu) and beyond[31].

2.2.2 Ethics statement

The study was reviewed and approved by Addis Ababa University College of Veterinary Medicine and Agriculture Ethical Review Committee (Ref No.VM/ERC/14/05/13/2021) and Oromia Health Bureau Health Research Ethical Review Committee (Ref No. BEFO/4BTW/1-16/10393); and all methods were performed in accordance with ARRIVE guidelines (https://arriveguidelines. org). Moreover, after explaining the purpose of the study, informed consent was obtained from all study subjects.

2.2.3 Study design and sampling

A cross-sectional study design was employed to collect data from five lakes that were conveniently selected based on their accessibility and fishing potential. These include Babogaya, Hora-Arsedi and Koftu Lakes in Bishoftu town; Koka Lake nearby Koka town, and Dambel Lake at Batu town. Stool samples were collected from diarrheic out-patients with a history of fish consumption at Bishoftu hospital, Koka health center and Batu health center (Fig. 1). On each sampling day, live fish were purchased at the lake shore from the fishermen who harvested them for commercial purposes, and placed in a bucket of water so that they feel as if they are in

their natural environment. To minimize pain during slaughter, the fish were first stunned by adding clove oil, which has anesthetic properties, to the water in which they had been placed a few minutes prior to severing with a sharp knife, as described by Davis et al.[32]. A total of 150 fish (30 fish from each Lake), comprising of six fish species (*Oreochromis niloticus, Clarias gariepinus,* Tilapia Zilli, *Cyprinus carpio, Labeobarbus intermedius, Barbus ethiopicus*), which are commonly used for human consumption were included in the study. Fecal, meat and skin swab samples were taken from each fish. A total of 750 samples, comprising of 150 water samples, 450 fish samples (150 fecal, 150 meat, 150 skin swab), and 150 stool samples were collected and processed. A maximum of ten fish were identified and sampled per sampling day from which 30 samples consisting of each ten fecal, meat and swab samples were collected on a visiting day.

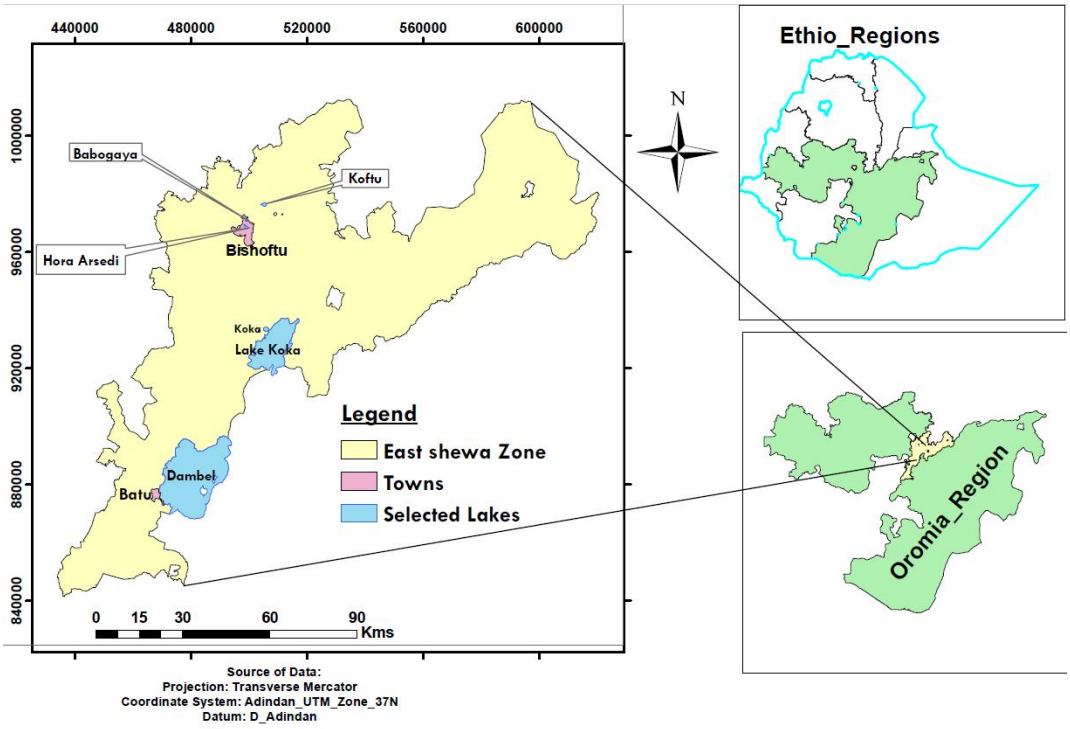

Figure 1: Map of the study areas and the Oromia regional state within the federal democratic republic of Ethiopia.

2.2.4 Sample collection

Skin swabs were collected using sterile cotton tipped swabs soaked in 10 ml of buffered peptone water (BPW) (Oxoid Ltd., Hampshire, England), as described by McEvoy et al.[33]. The skin was

swabbed several times first horizontally and then, vertically both in the left and right lateral commissars of the fish from the gill area towards the caudo-ventral region covering the entire dissection area of ~ 10 × 10 cm (an area with substantial risk of contact with a fillet during scaling). Then, the swabs were placed in a sterile universal bottle containing 10 ml of BPW by removing extra shafts and the bottles were screw capped.

After dissection, scaling, and gutting, ~ 10 g of meat (fillet) samples were taken from different parts of the fillet nearby the dissection area (the most suspicious part for external and fecal contamination) and pooled as a single sample; placed in a sterile universal bottle containing 10 ml of BPW.

After gutting, ~ 10 g of fecal samples were collected as described by Elder et al.[34]. Briefly, the whole intestinal organs were separated from the remaining parts of the dissected fish and placed in a sterile plastic bag. Then, the intestinal lumen was opened using a sterile surgical blade and the fecal samples were put into a sterile universal bottle containing 10 ml of BPW.

Water samples (~20 ml each) were collected by immersing sterile universal bottles containing 10 ml of BPW into a grossly visible dirty area of the water bodies near the Lake shore with an interval of about 2 m between each sampling points. Similarly, about 10 g of stool samples were collected from the stools submitted by the diarrheic patients having history of fish consumption for laboratory analysis, into a sterile universal bottle containing 10 ml of BPW in collaboration with the laboratory technicians using a sterile toothpick for each sampling. The procedures were done as aseptic as possible using a sterile disposable glove to avoid the risk of cross contamination during each sampling. Finally, all samples were carefully labeled using a permanent marker, kept in an ice box containing ice packs and transported to Addis Ababa University College of Veterinary Medicine and Agriculture Veterinary Public Health Laboratory for processing.

2.2.5 Detection of sorbitol non-fermenting E. coli and E. coli O157:H7

The samples were primarily processed targeting SN-F *E. coli,* and then Shiga toxin producing *E. coli* O157:H7 strain. Briefly, all the samples in the universal bottle containing 10 ml BPW were incubated at 41.5°c for 6 h to revive and increase the recovery rate of stressed bacterial cells[35]. Then, the meat samples were blended using stomacher (Seward Stomacher 400, Seward, London, UK) at a speed of 150 rpm for 60 s and, the aliquots from the meat, water, fecal, stool and swab

cultures were selectively enriched by measuring 10 ml of samples into 90 ml modified tryptone soya broth (mTSB) (Oxoid, Hampshire, England) using a sterile syringe, and the mixture was agitated using a vortex mixer for 60 seconds. After homogenization, the selectively enriched samples were incubated overnight at 41.5°c.

A loop-full of aliquots from selective enrichment was streaked on cefixime-potassium tellurite Sorbitol Mac-Conkey agar (CT-SMAC) and incubated at 37°c for 24 h. After incubation, sorbitol non-fermenting colonies (colorless colonies) were sub-cultured on CT-SMAC and incubated at 37°c for 24 h to obtain adequate number of pure colonies. To rule-out the growth of other Gram negative organisms with colorless colonies on CT-SMAC, including *Burkholderia*, *Vibrio, Proteus*, *Klebsiella*, *Aeromonads* and *Pseudomonas*[36], the pure colonies on CTSMAC were further sub-cultured onto eosin methylene blue (EMB) agar and incubated at 37°c for 24 h. Isolates with typical characteristic of green metallic sheen color of the generic *E. coli* on EMB agar were further tested for indole production. All indole positive presumptive colonies were preserved on tryptone soya agar slant after incubating at 37°c for 24 h and adding 80% glycerol for further analysis. The presumptive isolates were shipped to Belgian National Reference Centre, Brussels, Belgium for immunological testing and whole genome sequencing.

2.2.6 Escherichia coli O157 latex agglutination test

At the reference laboratory in Brussels, Belgium, the isolates were further sub-cultured on CT-SMAC to perform latex agglutination test. Then, each presumptive isolate was emulsified with normal saline on disposable reaction cards provided in the kit and mixed with a drop of test latex (a latex particle sensitized with specific rabbit antibody reactive with the O157 somatic antigen) and agitated for one minute. After one minute, the result was interpreted based on the standard protocol described by DeBoer and Heuvelink[37]. Due to lack of reference strains, known positive controls were not used. However, a suspension of inactivated *E. coli* O157 and *E. coli* O116 cells in buffer were used as a positive and negative controls, respectively.

2.2.7 Whole-genome sequencing (WGS)

Genomic DNA was extracted from pure cultures of SN-F *E. coli* isolates grown overnight on SMAC Agar; and its purity and quantity were measured with a Qubit double stranded DNA (dsDNA) BR assay kit. Then, fragmentation of 500 ng of genomic DNA was carried out using the NEBNextR Ultra™ II FS module. Sequencing libraries, with an insert size of on average 550

bp, were prepared using a KAPA Hyper Plus kit (Kapa Biosystems, Wilmington, USA) and a Pippin Prep (Sage Science, Beverly, MA, USA) size selection with a CDF1510 1.5% agarose dye-free cassette. Every sample was assigned an in-house truseq style adapter with a unique dual-indexed 8-bp barcode. PCR amplification (6 cycles) of the library was performed using the KAPA HiFi HotStart Libray Amplification kit (Kapa Biosystems, Wilmington, USA) according to the manufacturer's instructions. After equimolar pooling, libraries were sequenced on a Novaseq 6000 instrument (Illumina, San Diego, CA, USA) using a NovaSeq 6000 SP Reagent kit (500 cycles) generating 2×250 bp reads. For this, the library was denaturated and diluted according to the manufacturer's instructions. A 1% PhiX control library was included in each sequencing run. Lastly, the raw reads were uploaded, and de novo assembled, using SPAdes v.3.15.3, in BioNumerics v.8.1., and sequence quality was assessed using the quality metrics incorporated in BioNumerics v.8.1.

2.2.8 Comparison of SN-F E. coli strains retrieved from water, fish and humans in EnteroBase

The raw reads of 29 SN-F *E. coli* genomes were uploaded and automatically assembled in the public genome database, EnteroBase. All genome assemblies were subsequently compared to the available *E. coli* genomes using hierarchical clustering of cgMLST (HierCC) at different levels of resolution, ranging from HC0 (hierarchical clusters consisting of identical genomes with no AD) to HC200 (hierarchical clusters consisting of genomes with up to 200 ADs)[38]. However, up on quality check, the genomes of six isolates were poor and only 23 isolates were included in the cluster analysis.

2.2.9 Insilico identification of genes encoding for serotype, virulence factors, and antimicrobial resistance traits

The *E. coli* genotyping tool v.2.1, available in BioNumerics v.8.1 was used to predict *E. coli* serotypes, virulence gene profiles, acquired resistance genes, and point mutations starting from the genome assemblies. The presence of virulence and resistance genes was determined with a minimum % identity (ID) threshold of 85% and a minimum length for coverage of 60%.

2.2.10 Antimicrobial susceptibility test

Antimicrobial susceptibility test was conducted on a total of 29 SN-F *E. coli* strains retrieved in the present study following the standard protocol described by CLISI (2022)[39], using ten

essential antibiotics grouped under eight antimicrobial classes namely: class Penicillin (ampicillin 10µg), Tetracycline (tetracycline 30µg), Quinolone (ciprofloxacin 5µg and nalidixic acid 30µg), Macrolide (azithromycin 15µg), Chloramphenicol (chloramphenicol 30µg), Cephalosporine (ceftazidime 30µg and cefotaxime 30µg), Carbapenem (meropenem 10µg), and Sulphonamide (sulfamethoxazole /trimethoprim 25µg) obtained from commercial market (Thermo Scientific Fisher), using Kirby-Bauer disc diffusion method[39]. The antibiotics were selected based on their common usage in humans and animals and AMR reports. The discs were funded by Michigan State University through the 2019 faculty exchange program for one of our co-authors. To estimate the concentration of the isolates in the culture broth, 0.5 McFarland Standard (≈ 1.5×10^8 CFU/ml) was prepared by measuring 0.05 ml of 1% $BaCl_2$ and 9.95 ml of 1% H_2SO_4; and the turbidity of the culture broth was adjusted towards this standard using sterile saline solution. A sterile cotton swab was immersed into the broth culture and uniformly swabbed to Mueller–Hinton agar plates. The plates were allowed to dry for few minutes, and the antimicrobial discs were randomly placed on the surface of the agar plates with gentle pressure using sterile forceps. The plates were incubated at 37 °C for 18 h; and after incubation, the inhibition zones of each antimicrobial agent were carefully measured using a digital caliper. The test results were qualitatively interpreted as susceptible, intermediate, or resistant based on zone diameter interpretative standards established for *E. coli* and other enteric Gram negative rods[39]. Isolates resistant to at least one antibiotic from three or more distinct antibiotic classes were classified as MDR. Controls were accomplished with an *in-silico* identification of genes encoding for antimicrobial resistance traits using *E. coli* genotyping tool v.2.1, available in BioNumerics v.8.1 to predict acquired resistance genes, and point mutations following the standard procedure described by Feldgarden et al[40].

2.2.11 Data analysis

The data were entered into an excel spreadsheet (Microsoft office R excel 2013, Cengage Learning, version 15) and analyzed using Stata version 15.0 software (Stata Corp, College Station, TX). Descriptive statistics such as frequency and percentages were used to express the sociodemographic characteristics of the diarrheic patients and the antimicrobial susceptibility profiles of the isolates. The proportions of occurrence of sorbitol non-fermenting *E. coli* in diarrheic patients and, water and fish samples were calculated by dividing the number of culture

positive samples by the total number of samples tested from each sample source. Fisher's exact test was used to assess the difference in the proportion of sorbitol non-fermenting *E. coli* among the different sample sources.

The assembled sequencing data was analyzed using the *Escherichia* /*Shigella* cgMLST typing scheme in Bio- Numerics v.8.1 (core Enterobase). Both assembly algorithms, namely, the assembly-free k-mer-based approach using the raw reads and the assembly-based BLAST approach were used for allele calling. The default settings were used for both the assembly-free and assembly-based algorithms. The quality of the assembly-free and the assembly-based allele calls were verified using the quality statistics window in BioNumerics. The MLST profile of each isolate was determined using the three basic allele mapping experiments (Pub (Achtman) MLST, Pasteur MLST, Whittam MLST) incorporated in BioNumerics. Minimum spanning tree (MST) diagram of the cgMLST data was generated using the MSTree V2 algorithm and visualized by GrapeTree in EnteroBase.

2.3 Results

2.3.1 Demographic characteristics and proportion of SN-F E. coli strains per sample sources

Among the total of 150 diarrheic patients with a history of fish consumption who were participated in the study, 52.67% of them were males. The mean age of the patients was 30.7 years (range: 9 months to 70 years). From the patients investigated, 8.7% (13), 17.3% (26), 17.3% (26), and 56.7% (85) were observed with the clinical history of bloody, mucoid, mixed and watery diarrhea, respectively (Table 1).

Table 1: Demographic characteristics of the study participants

Variable	Health Facilities	Number of Participants	Gender		Average Age
			Male	***Female***	
Demographic Characteristics	Bishoftu Hospital	50	31	19	29.2
	Koka Health Center	50	29	21	32.6
	Batu Health Center	50	19	31	30.4

The overall culture-based positive proportion of SN-F *E. coli* strains in all samples was 3.9% (29/750), and this was also statistically significant (P=0.009). Specific sample level detection rate was 6.7% (10/150), 2.7% (4/150), 1.3% (2/150), 1.3% (2/150), and 7.3% (11/150) in water, fish meat, Fish feces, fish skin swab, and human stool samples, respectively (Table 2).

Table 2: Proportions of samples positive for SN-F *E. coli* strains

Sample taken	Quantity	Negative proportion, n (%)	Positive proportion, n (%)	Fisher's Exact Test (P-value)
Water	150	140 (93.3)	10 (6.7)	
Fish Skin Swab	150	148 (98.7)	2 (1.3)	
Fish Meat	150	146 (97.3)	4 (2.7)	0.009
Fish Feces	150	148 (98.7)	2 (1.3)	
Human Stool	150	139 (92.7)	11 (7.3)	
Total		**721 (96.1)**	**29 (3.9)**	

Of the six species of fish sampled, three species, namely: *Cyprinus carpio*, *Oreochromis niloticus* and *Tilapia zillii* were tested positive for SN-F *E. coli* strains from Lakes Hora Arsedi and Koka (Table 3).

Table 3: Species of fish and sample types from which SN-F E. coli Strains were detected

Species of Fish Sampled	Source Lakes	Species Positive for SN-F *E. coli*	Sample Positive for the Pathogen	Negative Proportion, n (%)	Positive Proportion, n (%)	Fisher's Exact Test (P-value)
Tilapia zillii	Hora Arsedi	*Tilapia zillii*	Skin swab	21 (4.7)	1 (0.2)	

Cyprinus carpio (Common carp)	Koka and Dambel	*Cyprinus carpio* (Common carp)	**Feces** (sampled from the fish in Lake Koka)	8 (1.8)	2 (0.4)	
		Cyprinus carpio (Common carp)	**Meat** (sampled from the fish in Lake Koka)	28 (6.3)	2 (0.5)	
Oreochromis niloticus (Nile Tilapia)	Babogaya, Hora Arsedi, Koftu	*Oreochromis niloticus* (Nile Tilapia)	**Meat** (sampled from fish in Lake Koka)	211 (47)	2 (0.4)	0.031
	Koka and Dambel	*Oreochromis niloticus* (Nile Tilapia)	**Skin swab** (sampled from fish in Lake Koka)	102 (22.5)	1 (0.3)	
*Clarias gariepinus(*Afri can cat fish*)*	Babogaya, Koka and Dambel	None	None	37 (8.2)	0 (0)	
Barbus ethiopicus	Dambel	None	None	11 (2.4)	0 (0)	
Barbus (Labeobarbus intermedius)	Koka and Dambel	None	None	24 (5.3)	0 (0)	
Total				**442 (98.2)**	**8 (1.8)**	

Among the total of 10 SN-F *E. coli* strains detected in water samples, higher proportion (40%, n=4) was detected in samples collected from lake Koftu, followed by lake Hora-Arsedi (30%; n=3). Majority, 87.5% (n=7), of the fish isolates were detected in fish sampled from lake Koka; 50% (n=4), 25% (n=2) and 12.5% (n=1) of which were recovered from meat, feces and

skin swab samples, respectively. Nevertheless, none of the fish samples collected from Babogaya, Koftu and Dambel Lakes were tested positive (Table 4).

In diarrheic patients, the overall detection rate of SN-F *E. coli* strains was 7.3% (95% CI: 3.7, 12.7%) with higher prevalence in younger age groups (5–17 years) and children under 5 years; at a detection rate of 10.5% and 8.3%, respectively (Table 4).

Table 4: Proportion of samples positive for sorbitol non-fermenting *E. coli* per sample types and sources.

Sample Type	**Source Lake**	**Number of samples**	**Positive n (%)**
Water	Hora-Arsedi	30	3 (30)
	Babogaya	30	1 (10)
	Koftu	30	4 (40)
	Koka	30	1 (10)
	Dambel	30	1 (10)
Fish Samples	Babogaya	90	1 (12.5)
	Hora-Arsedi	90	0 (0)
	Koftu	90	0 (0)
	Koka	90	7 (87.5)
	Dambel	90	0 (0)
	Age (Years)	**Number**	**Positive n (%)**
Human Stool	Children under 5	12	1 (8.3)
	Young (5-17)	19	2 (10.5)
	Youth (18-30)	49	4 (8.2)

	Adults (≥31)	70	4 (5.7)

Core genome multi locus sequence typing (cgMLST) confirmed that all the strains detected from water, fish and humans were SN-F *E. coli* strains. None of the *E. coli* strains were tested positive for *E. coli* O157:H7 using *E. coli* O157:H7 latex agglutination test, and whole genome sequencing (WGS). Core genome MLST also detected a new strain with unknown O-antigen from fecal sample of fish obtained from Lake Koka and stool sample of diarrheic patient presented to Bishoftu Hospital (Table 5).

Table 5: Antigenic characteristics and MLST of SN-F *E. coli* strains retrieved from water, fish, and humans.

Sample Type	Source	Antigenic Characteristics	MLST Pasteur ST	MLST Whittam ST	MLST PubMLST (Achtman) ST	Number of Isolates with the Same Antigen
Water	Lake Babogaya	O116: H49	publicST325	ST2001	publicST2520	1
	Lake Hora Arsedi	O20: H8	ST2121	ST611	publicST164	1
	Lake Hora Arsedi	O174: H43	ST2119	ST2001	publicST2244	1
	Lake Koftu	O116: H49	publicST325	ST2001	publicST2520	1
	Lake Koftu	O8: H10	publicST86	ST2608	N/A	1
	Lake Koka	O17/O44: H18	ST2118	ST2607	publicST69	1
	Lake Dambel	O17/O44: H18	ST2118	ST2607	publicST69	1
Fish skin swab	Lake Koftu	O176: H11	ST2120	ST1634	publicST48	2
	Lake Koka	O176: H11	ST2120	ST1634	publicST48	1

Fish	Lake Koka	O155: H21	publicST87	ST301	publicST58	1
Meat	Lake Koka	O10: H5	ST785	ST2605	publicST206	1
	Lake Babogaya	O18ac: H21	ST2123	ST2611	publicST223	1
Fish Feces	Lake Koka	O-Unknown: H28	ST2122	ST2609	publicST1633	1
	Koka Health Center	O168: H2	ST2117	ST2606	N/A	2
Human stool	Koka Health Center	O8: H25	publicST24	ST294	publicST58	1
	Bishoftu Hospital	O-Unknown: H40	publicST2	ST2610	publicST10	1
	Bishoftu Hospital	O155: H10	publicST21	ST284	publicST1049	1
	Batu Health Center	O155: H10	publicST21	ST284	publicST1049	4

2.3.2 Comparison of SN-F E. coli Genomes in EnteroBase

Comparison of the genetic linkage among the SN-F *E. coli* strains retrieved from the three sample sources (water, fish and humans) in EnteroBase showed lack of genetic relationships among the isolates. However, genetic relatedness was observed among strains from the same sample sources: 2 clusters of *E. coli* strains from humans (5 and 2 strains), water (2 strains) and Fish (2 strains) (Fig. 2).

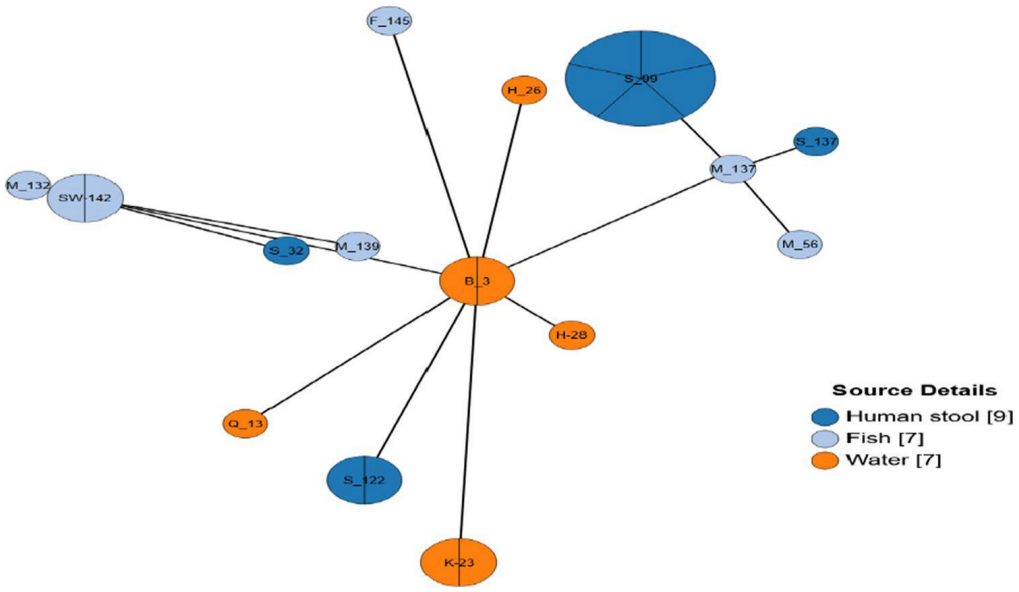

Figure 2: Grape-Tree diagram generated in EnteroBase showing lack of molecular clustering among water, fish and human isolates.

2.3.3 In silico identification of genes encoding for virulence factors and AMR traits

An in silico MLST showed that all the strains have multiple virulence factors and one or more genes encoding for them. The most repetitive virulence factors and respective encoding genes detected were survival (*iss*), adherence proteins (*papC, lpfA, irp2, k88ab*), invasion (*ompT, cia*), toxin production (*hlyE, hlyF, cvaC, mchF*), and metabolic regulation (*terC, eilA*). It also revealed genes encoding for acquired resistance of ampicillin, amoxicillin, cephalothin, piperacillin, and ticarcillin (*blaTEM-1B*), doxycycline and tetracycline (*tet(A)*), trimethoprim (*dfrA1*and *dfrA5*), sulfamethoxazole (*sul1*), fosfomycin (*fos7*), chloramphenicol (*catA1*), ciprofloxacin (*qnrS*1), and nalidixic acid (*gyrA*). Moreover, genes encoding for mutational resistance against ciprofloxacin and nalidixic acid ((*parC, gyrA*), and extended spectrum beta-lactamase (ESBL) against aztreonam, cefepime, ceftaxime, ceftazidime, and ceftriaxone (*blaCTX-M15*) were detected (Annex 1).

Overall, 18 (62.1%) of the isolates were virulent strains; of which, 7 (38.9%), 6 (33.3%), and 5 ((27.8%) were retrieved from water, fish, and human, respectively. The majority (55.6%; n = 10) of the virulent isolates have genes encoding for antimicrobial resistance. Among this, 5 (50%), 4 (40%) and 1 (10%) isolates were from humans, fish and water respectively (Annex 1).

2.3.4 Antimicrobial susceptibility

Of the 29 sorbitol non-fermenting *E. coli* strains tested, 96.6% (n = 28) of them were susceptible to azithromycin followed by ciprofloxacin (20.7%, n = 6), chloramphenicol (17.3%, n = 5) and sulfamethoxazole /trimethoprim (13.8%, n = 4). All of the isolates were resistant to ampicillin, cefotaxime, ceftazidime, meropenem and tetracycline (Table 6).

Table 6: Antimicrobial susceptibility profiles of sorbitol non-fermenting *E. coli* strains.

Antimicrobial Classes	**Antimicrobial Agents**	**Concentration (µg)**	**Susceptibility**		
			Susceptible n (%)	**Intermediate n (%)**	**Resistant n (%)**
Penicillins	ampicillin	10	0 (0.0)	0 (0.0)	29 (100)
Macrolides	Azithromycin	15	28 (96.6)	0 (0.0)	1 (3.45)
Cephalosporin	Cefotaxime	30	0 (0.0)	0 (0.0)	29 (100)
	Ceftazidime	30	0 (0.0)	0 (0.0)	29 (100)
Chloramphenicol	Chloramphenicol	30	5 (17.3)	7 (24.1)	17 (58.6)
Quinolones	Ciprofloxacin	5	6 (20.7)	9 (31)	14 (48.3)
	Nalidixic acid	30	0 (0.0)	2 (6.9)	27 (93.1)
Carbapenems	Meropenem	10	0 (0.0)	0 (0.0)	29 (100)
Tetracyclines	Tetracycline	30	0 (0.0)	0 (0.0)	29 (100)
Sulphonamides	Sulfamethoxazole /Trimethoprim	25	4 (13.8)	2 (6.9)	23 (79.3)

A multi-drug resistance (MDR) of 87.5% was observed; where the strains were resistant to seven antimicrobial classes from the eight antimicrobial classes tested. Five isolates and one

isolate were resistant to 90% (n=9) and 100% (n=10) of the antimicrobial agents used, respectively. An in silico MLST analysis also confirmed that the majority (62.1%; 18/29) of the strains have multiple acquired resistance against ampicillin, amoxicillin, cephalothin, piperacillin, ticarcillin, doxycycline, tetracycline, trimethoprim, sulfamethoxazole, and one or more genes encoding for them. Genes encoding for mutational resistance against ciprofloxacin and nalidixic acid, and extended spectrum beta-lactamase (ESBL) against aztreonam, cefepime, ceftaxime, ceftazidime, and ceftriaxone were also detected (Annex 1).

2.4. Discussion

In this study, we investigated the occurrence, molecular characteristics, and antimicrobial susceptibility of SN-F *E. coli* with particular emphasis to *E. coli* O157:H7 in Lake Water, fish and humans in central Oromia, Ethiopia. The study showed that none of the identified isolates are *E. coli* O157:H7. Interestingly, the study revealed antimicrobial resistant SN-F *E. coli* and new SN-F *E. coli* strains with unknown O-antigen, and genetic relatedness among strains from the same sources with no linkage among strains from different sample sources (water, fish and humans). The majority of the strains have multiple genes encoding for a single virulence factor and antimicrobial resistance traits; implying that the isolates are pathogenic and antimicrobial resistant *E. coli* strains.

The genetic relationships among the isolates from the same sources suggests the circulation and the potential risk of dissemination of virulent and antimicrobial resistant strains along the fish supply chain in the study area. Similar report from Southeastern Nigeria revealed the occurrence of MDR SN-F *E. coli* strains in other species of animals (broiler chicken, cattle and pig), and suggested that the pathogens may spread from animals to humans and the environment making it a public health threat[41]. Likewise, the recent report of Bedane et al.[23], on hygienic fish handling practices in the study area indicated that the Lakes are used as watering points for cattle, which are the principal carriers of *E. coli* O157:H7, and fully accessible to run-off water, which may carry many other pathogens of public health concern, including SN-F *E. coli*.

Reports on SN-F non- O157:H7 *E. coli* strains are limited and as a result, our findings are discussed in relation to previous reports on other *E. coli* strains and *E. coli* O157:H7 as appropriate to provide an overall insight. The observed 6.7% SN-F *E. coli* strains in lake water samples in the present study is lower than the finding of Mekonnen et al.[42], who reported 93.3%

E. coli in water samples collected from Lake Dambel, and nearly comparable with the report of Dissasa et al.[42], who retrieved 5.9% of generic *E. coli* from water samples of Lakes Dambel, Langano and Hawasa of Ethiopia. Similarly, Akoachere et al. , in Cameron and Ribeiro et al.[45], in Brazil found that bacteria of the family Enterobacteriaceae, including *E. coli*, are widely distributed in the aquatic ecosystem. Once the aquatic environment is contaminated with bacterial pathogens of public health concern, fish can easily acquire the pathogens naturally by direct physical contact[45]. This substantiates the higher odds of exposure of fish to pathogenic organisms and the subsequent higher odds of human exposure to the pathogens. The observed variations could be due to differences in the method of detection and targeted *E. coli* strains in this study. We primarily investigated the occurrence of SN-F *E.* coli as compared to other studies that reported the prevalence of the generic *E. coli* which yield higher detection rate. Moreover, the variations could be attributed to the temporo-spatial disparity of the studies, and related anthropogenic and natural elements which affect the magnitude of contamination of lake water.

Sorbitol non-fermenting *E. coli* strains were detected in 1.8% of fish samples analyzed during the present study. An important notable finding is that 50% of the fish isolates were detected in meat, showing a remarkable contamination of the filet either from the external environment including skin, or intestinal contents due to poor handling and processing practices. Similar study conducted in India reported 19.2% occurrence of SN-F *E. coli* in cattle feces[46], implicating the potential risk of contamination of Lake water and fish with SN-F *E. coli* strains of cattle fecal origin. The poor hygienic conditions along the fish supply chain in the study area might have contributed to the contamination of the lakes with animal feces during watering and through water run-off[23].

Compared to the previous studies by Haile and Getahun[46], Yohans et al.[48,] and Dissasa et al.[42], who reported the generic *E. coli* in 12%, 20%, and 5.7% of fish samples collected from Lakes Dambel, Tana, and three Rift-Valley Lakes (Dambel, Langano and Hawasa), respectively, our report is exceedingly lower. It is also extremely lower than the finding of Marijani[49], who recovered *E. coli* from 39% of fish sampled from marine and freshwater fish in Tanzania. Our detection of SN-F *E. coli* on the edible part of fish agrees with the findings of Akoachere et al.[44], from Cameron, and Ribeiro et al.[45], from Brazil who previously recovered *E. coli* from fish destined for human consumption. The report of Anyanwu et al.[41], from Nigeria have also shown

13.4% prevalence of SN-F *E. coli* in other food animals (broiler chicken, cattle and pigs), among which 0.51% of them were *E. coli* O157.

The 7.3% occurrence of SN-F *E. coli* strains in diarrheic patients is lower than the systematic review and metaanalysis findings of Zenebe et al.[50], who reported 25% pooled prevalence of a generic *E. coli* in diarrheic children under five years in Ethiopia, and 15.3% prevalence and 28.9% isolation rate of *E. coli* O157 related diarrhea in children under five years from Eastern Ethiopia[26], and Bahir Dar town of northern Ethiopia[27], respectively.

The occurrence of highly virulent MDR *E. coli* strains in humans may be related to unhygienic handling of animal products including fish or consumption of raw or undercooked products. Likewise, the reports of Haenen et al.[51], Santos and Vieira[52], and Mekonnen et al.[53], showed unhygienic handling and consumption of raw or undercooked infected fish may pose the risk of infection in susceptible individuals. Similarly, Wiriyaprom et al.[54], have reported 8.82% prevalence of SN-F Shiga toxin-producing *E. coli* (STEC) isolated from goats in Thailand; among which only 0.77% was *E. coli* O157:H7.

The majority, (62.1%) of the strains have virulence determinants encoding for adhesion, host invasion, toxin production, and promoting survival of the pathogen. In addition, genes encoding for deterioration of metabolic regulation and iron uptake of the susceptible hosts were detected in majority of the isolates retrieved from the three sample sources, implying that the strains are sharing many virulence characteristics. A similar study in Mozambique also showed the coexistence of different combinations of two or more virulence genes encoding for a single virulence factor in *E. coli* strains retrieved from ready to eat food items[55].

Sorbitol non-fermenting *E. coli* strains carrying virulence determinant traits were detected in all the study lakes, all health facilities and fish sampled from lakes Koka and Koftu. Among them, a higher proportion, (38.9%; n=7) was detected from water samples; suggesting that the pathogen is exceedingly disseminated across the aquatic ecosystem in the study areas. The higher prevalence of virulent strains in lake water implies that fish can be easily infected with such virulent strains from the aquatic environment, and the pathogens can be disseminated among the fish population in that ecosystem and become a potential public health threat, especially among raw fish consumers. Previous reports also showed that if the water bodies are contaminated with pathogenic organisms, fish can be easily infected from its immediate environment[56].

The new SN-F *E. coli* strains with unknown O-antigens, O-unknown: H28 and O-unknown: H40, from fish and human, respectively might be mutant progenies of known *E. coli* strain or newly emerging strains not yet characterized. Moreover, *in silico* MLST have shown that both the new strains with unknown O-antigen have multiple virulence factors, and O-unknown: H40 is MDR strain. Thus, further robust molecular study is required to determine the lineages of these strains relative to *E. coli* strains in the sequence database to elucidate fundamental information on the epidemiology and public health implications of the strains. Similar previous studies conducted on bacterial genomics have also shown that comparison of bacterial 16S rRNA gene (the most conserved genetic marker of a bacteria) with a known sequence of related bacteria in the database has emerged as a preferred genetic technique to identify new strains[57, 58].

Among the ten antimicrobial agents tested for their level of efficacy, azithromycin was effective for 96.6% (n=28) of the isolates and recognized as the most promising drug for the treatment of infections related to SN-F non-O157:H7 *E. coli* strains. Studies indicated that azithromycin is the most promising alternative and excellent drug for the treatment of diarrhoeagenic enteric infections caused by *E. coli*, Shigella, Salmonella, and Campylobacter species[59–61]. Reduced efficacy of ciprofloxacin, chloramphenicol and sulfamethoxazole /trimethoprim to SN-F non-O157:H7 *E. coli* strains was observed as compared to the efficacy of azithromycin.

The lower efficacy of ciprofloxacin and sulfamethoxazole /trimethoprim is in agreement with the current report of Yasmin et al.[62], from Pakistan, who reported 93% and 92% resistance of *E. coli* strains to the two drugs, respectively. Other study reported a better and moderate efficacy of ciprofloxacin and sulfonamides to *E. coli* strains recovered from neonates in China[63]. On the other hand, the low efficacy of chloramphenicol to SN-F non-O157 *E. coli* strains is lower than the finding of Ashenafi et al.[64], who reported 27.3% efficacy of the drug to *E. coli* O157:H7 retrieved from raw cow milk in central Ethiopia.

Conversely, in the present study, it was noted that all isolates are resistant to five antimicrobial agents, namely: ampicillin, cefotaxime, ceftazidime, meropenem and tetracycline; and besides all human isolates were resistant to nalidixic acid. These findings are comparable with the recent findings of Dejene et al.[65], from Ethiopia, who have reported an absolute resistance of *E. coli* O157:H7 to ampicillin, and Yasmin et al.[62], from Pakistan, who have shown 91% resistance of other *E. coli* strains to the same drug. Thus, based on the result of the present

study and previous findings, avoiding the use of ampicillin for the treatment of *E. coli* O157:H7 and other *E. coli* infections is recommended. The absolute resistance to cefotaxime, ceftazidime and meropenem observed in the present study is also comparable with the finding of Yasmin et al.[62], who reported a 98%, 86% and 71% resistance of *E. coli* strains to the same drugs, respectively.

The study revealed the widespread occurrence of MDR SN-F *E. coli* strains along the fish supply chain in central Oromia, Ethiopia, where the strains were resistant to 87.5% (seven-out of eight) antimicrobial classes. Based on in silico MLST, the majority (55.6%) of the virulent strains, have multiple acquired antimicrobial resistant traits and one or more genes encoding for them. This implies the higher AMR profile of virulent strains retrieved from humans as compared to fish and water isolates. Similar report from the city of Maputo, Mozambique have also shown that MDR *E. coli* strains was detected in drinking water[55]. Another study reported MDR Gram-negative rods, including *E. coli* as a public health threat globally[66]. Thus, as MDR Gram-negative bacteria, including *E. coli*, represent a global public health challenge[67], it is crucial to note that these resistant organisms may be disseminated along the fish supply chain, and become a potential public health threat. Moreover, genes encoding for mutational resistance against ciprofloxacin and nalidixic acid, and extended spectrum beta-lactamase (ESBL) against aztreonam, cefepime, ceftaxime, ceftazidime, and ceftriaxone were detected. Besides, previous reports have shown that *E. coli* strains may serve as reservoir of antimicrobial resistant genes which can be transferred to other pathogenic strains primarily through horizontal gene transfer[29], and become a serious public health concern[68]. Therefore, since they are highly prone to exchanging genetic material[69], the risk of horizontal gene transfer to other pathogenic or non-pathogenic *E. coli* strains and dissemination of mutant strains along the fish supply chain may be inevitable.

In general, although resistance of *E. coli* to older antibiotics such as tetracycline and ampicillin and those that acquire resistance through plasmid transfers may not be surprising, resistance to the last-resort drugs like meropenem which are used for the treatment of human infections caused by MDR enteric pathogens and classified by WHO as high priority critically important antimicrobials[70], is a potentially discerning public health threat; and rational use of drugs are critically important.

2.5 Conclusion

The study revealed the occurrence of considerable proportion of virulent and multidrug resistant SN-F *E. coli* strains in Lake Water, fish and humans in Central Oromia, Ethiopia. We also reported new SN-F *E. coli* strains with unknown O-antigen from fish and stool samples. However, the strains detected from the three sample sources are not genetically linked; and none of them were *E. coli* O157:H7. The observed occurrence of virulent and multi-drug resistant *E. coli* strains and the genetic linkage among strains originated from the same samples compounded with the poor fish hygienic production and raw fish consumption habit of the community suggest the potential risk of dissemination of the organisms along the supply chain and the transmission pathways from contaminated water-to-fish-to-humans. Further robust molecular study is required to characterize the new strains with unknown O-antigen to guide public health intervention programs to ensure fish safety in Ethiopia in general and in central Oromia in particular.

Data availability

The DNA Sequence datasets generated during the current study are available in the National Library of Medicine repository, with accession numbers: SUB13951691, SUB13951614, SUB13951611, SUB13951598, SUB13951583, SUB13951579, SUB13951565, SUB13951559, SUB13951552, SUB13951544, SUB13951396, SUB13951541, SUB13951294, SUB13951282, SUB13951298, SUB13951274, SUB13951308, SUB13951320, SUB13951382, SUB13951387, SUB13951393, SUB13942239, SUB13951735, SUB13951753, SUB13951569, SUB13949288, SUB13949315, SUB13949345, and SUB13949358. All other data were incorporated to the manuscript.

2.6 References

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CHAPTER 3: HYGIENIC ASSESSMENT OF FISH HANDLING PRACTICES ALONG PRODUCTION AND SUPPLY CHAIN IN CENTRAL OROMIA, ETHIOPIA

Abstract

Fishborne diseases are among the major causes of morbidity and mortality worldwide. Contamination of the aquatic ecosystem and unhygienic handling practices along the fish supply chain can lead to a contaminated fish. Consumption of raw or under cooked fish and fish products is a major source of fishborne infections in humans. Despite reports of fish contamination with foodborne pathogens in Ethiopia, information regarding the hygienic status of fish handling practices is limited. We assessed fish hygienic handling practices at production sites and along the fish supply chain in three towns in east Shewa zone of Oromia. Data were collected using a semi-structured questionnaire interviews and personal observations. The study consisted of purposively selected respondents comprising of 50 fishermen, 10 retailers, 20 food establishments serving fish, and 120 consumers. Descriptive statistics and Chi-square test were used to present the proportion of various actors along the fish production and supply chain and to compare the proportions of observations among the different categories respectively. We observed that the lakes were accessible to animals and exposed to chemical and microbial contaminations through rainwater run-off. Fish were processed under unhygienic practices like washing of filleted fish with lake water, indiscriminate processing at unhygienic landing sites, use of a single knife for processing all fish with infrequent washing and with no disinfection in between. Majority (70%; n = 10) of the retailers and all the food establishments transported fish in vehicles with no cold chain facilities. Good hygienic practices we observed were the use of refrigerators for storage in all retailers and 70% (n = 20) of the food establishments; 30% of retailers used vehicles with a cold chain facility for the transportation of fish. Over three-fourths (77%; n = 120) of the consumers preferred consuming raw fish; 80% of them lacked the knowledge of fishborne diseases. The study revealed a wide range of unhygienic handling practices along fish production and supply chain; lack of infrastructure for post-harvest fish handling and processing, lack of appropriate transportation facilities and presence of knowledge gaps regarding fish borne diseases.

Abbreviations

CAC: Codex Alimentarius Commission
CSA: Central Statistics Authority
ECHCPD: European Commission Health and Consumer Protection Directorate
EFNG: Ethiopian Federal Negarit Gazeta
ESZOLF: East Shewa zone Office of Livestock and Fisheries
FAO: Food and Agriculture Organization
HLPE: High Level Panel of Experts
WHO: World Health Organization

3.1 Introduction

Fish is a food of excellent nutritional value providing high quality protein and unique long-chain polyunsaturated fatty acids and highly bioavailable essential micronutrients, vitamins D and B, and minerals. These compounds, often not readily available elsewhere in diets, have beneficial effects for adult health and child cognitive development[1].

Production and supply of products or services involve various actors and networks with a wide range of activities that are required to bring a product or service from conception, through the different phases of production and supply chain to delivery to the final consumers[2]. In food industry, production and supply chain consist of actors that include producer, processor, wholesaler, exporter, importer, retailer, and consumer each with a unique role by adding a value to the products with an overall goal of supplying and accessing quality and safe food while generating maximum earnings from the business[3]. Within the context of fishery and aquaculture, fish production and supply chain start with collection of fish mostly from large water bodies such as lakes and oceans and end up delivering to consumer in markets far from thousands of miles. The major actors in the fish supply chain consists of a network of fishermen, retailers, distributors, transporters, storage facilities and suppliers that participate in the production, delivery, and sale of a product to the consumer[4, 5].

Long geographical distance that commonly exists between the fishermen and consumers makes the fish supply chain is a challenge in maintaining food quality and difficulty to tracing the pathways of the products when spoiled or poor-quality fish reaches the consumer[6]. In Ethiopia, fish production and distribution are operated both formally (e.g., organized

cooperatives) and informally by individuals[7]. Alike in most developing countries, the fish supply chain in Ethiopia predominantly follows informal market system with limited supply chain management for fish quality and lack of information for price determination along the chain[8, 9]. Under a predominantly informal marketing system which usually lack formal fish supply chain management and have no established system of traceability using either the traditional paper-based recording or the use of digital platforms such as blockchain technology[10, 11]. In the absence of traceability, marketing and consumption of fish raise concerns of trust and transparency on the quality and safety[12, 13] and the price of the products which ultimately affects both fish consumers and sellers[14].

Ethiopia has huge water bodies and wetland ecosystems that can support more than 200 fish species[15] with an estimated average annual production potential of 94,500 metric tons of fish[16]. As a land locked country, fish production in Ethiopia entirely depends on inland water bodies such as lakes, reservoirs, and rivers. Approximately 75% of the total annual catch originates from the major lakes Tana, Dambel, Langano, Hawassa, Abaya and Chamo which are situated in the northwest and southern parts of the country. The remaining 25% of the total annual catch is harvested from minor lakes such as Beseka, Lugo, Hashengie, and Small Abaya, reservoirs such as Koka, Fincha-Amerti, Denbi, Melka-Wakena, Alwero, Tekeze, Gilgel Gibe I and major rivers[17]. For example, the Baro-Akobo basin drained by Baro, Akobo, Gilo and Alwero rivers, found in the Gambella region has an estimated fish production potential of 3,720 tons per year[18].

Despite this huge fish production potential in Ethiopia, less attention has been given to the sector and the per capita fish consumption per year is very low (0.5 kg/year) due to poor integration of fish into the diet, lack of accessibility and limited supply[9]. If adequately utilized, fish production and marketing have different socioeconomic significance in improving the livelihood of the community by ensuring food security, generating income, and providing self-employment opportunities for low-income groups particularly for young labor forces and rural communities dwelling near large waterbodies[9, 19].

The fishery sector in Ethiopia is hampered by several factors such as lack of adequate infrastructures at the fishing sites, lack of appropriate transportation facilities (including vehicles with thermostat), lack of harvesting and processing technologies and established supply chain management systems[9, 20]. Consequently, various actors along fish production and supply chain can experience handling costs, financial loss due fish spoilage and more importantly consumers

can lose trust and lack fast traceability information on the product quality. Thus, consumers can be exposed to contaminated fish due to lack of information related to the sources and hygienic conditions along the supply chain[21]. Besides, fish loss and fish waste due to improper handling have significant negative impacts on food-security, economy, and the environment by decreasing fish availability in the market, which may in turn increase fish prices and reduce the capacity of low-income consumers to access food. Moreover, if the quality of food deteriorates so badly that the fish must be sold at a lower price or even discarded to the environment as food waste, the livelihood of farmers and producers is adversely affected[22]. An investment at each step of the supply chain is required to improve fish handling by fully exploiting the resources using efficient solutions (e.g., the use of cold chain at each step) and establishing interlinkages among the actors to maintain fish quality and safety along the entire supply chain.

Fish contamination with chemical and microbial hazards can occur due to fish exposure to contaminated water with hazards from environmental sources linked with poor waste management such as from industries and livestock farms that pollutes the water resources[23]. Microbial contamination can occur at any point along the upstream supply chain during collection and processing, distribution, storage, marketing, and preparation if proper hygienic handling practices are not maintained[24–26]. Exposure to the microbial pathogens is a serious threat for consumer safety, especially when raw or undercooked fish is consumed[27].

Bacterial diseases are considered the main cause of high mortalities and economic losses in fish industry worldwide[28]. *Mycobacterium* species, *Streptococcus iniae, Clostridium botulinum*, and *Vibrio vulnificus* are the major bacterial fish pathogens of public health concern. In addition, potential human pathogens such as *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Enterococcus faecalis* were isolated from edible fish and water samples[29]. The widespread distribution of these pathogens in the aquatic environments is mainly associated with potential contamination of the water bodies through run-off[30]. For instance, in Ethiopia the inland water bodies used as a natural source of fish receive many contaminant inflows from the nearby farmlands with fertilizers and untreated industrial wastes, municipal sewages, leaching from pit latrines and septic tanks[31].

In Ethiopia, few studies have reported the occurrence of common fishborne pathogens. A review by[32] showed that *Edwardsiella tarda, Salmonella*, *E. coli, S. aureus, Aeromonas* and *Vibrio* species are among the bacterial pathogens reported in fish in the country. The prevalence

of these pathogens in fish varies considerably in different lakes across the country. For instance, *Salmonella* was reported at prevalence of 5.2% from Hayike[33], 7.5% from Abaya and Chamo[34] and 30% from Tinike lakes[35]. Similarly, 1.5%[36] and 2.3%[37] prevalence of Shiga toxin producing *E. coli* O157:H7 were reported from fish samples tested at lake Hayike and Tekeze dam, and lake Hawassa, respectively.

Several bacterial pathogens cause infections and mortalities in fish and humans. People can be infected while handling infected fish on fish farms, at retail shops, or through ingestion of raw or inadequately cocked contaminated fish and/or fish products[38]. The widespread occurrence of these pathogens in fish along the fish supply chain could be due to lack of basic hygienic practices. Unhygienic handling practices lead to contamination of fish ready for human consumption with potentially foodborne pathogens; especially if fish is consumed raw or undercooked, it poses a significant public health risk[32]. Despite the availability of information on contamination of fish with common foodborne pathogens, data regarding fish hygienic handling practices along the production and supply chain is scarce in Ethiopia. The aim of this study was to assess the hygienic status of fish handling practices along production and supply chain in three towns of east Shewa zone of Oromia, Ethiopia that have natural lakes and potential reservoirs known to support fish production. Supply chain analysis is essential to closely examine the hygienic practices at each step along the production and supply chain to ensure fish quality and safety to increase consumer confidence. Information regarding the handling practices of fish at each step along the supply chain is critically needed to identify the critical control points and driving factors to devise preventive measures to ensure fish quality and safety. Moreover, it can pinpoint the extent of the problem to all stakeholders to initiate collective plans and actions to reduce associated costs and health risks and, optimize revenues to improve the livelihood of all actors relying on fishing business.

3.2 Materials and Methods

3.2.1 Study area and study design

A cross-sectional study was conducted from December 2020 to June 2021 in Bishoftu, Koka and Batu towns of east Shewa zone of Oromia, Ethiopia. East Shewa zone is found in Central Oromia, connecting the western and eastern parts of Oromia. The zone has a total population of 1,356,342 within an area of 8,370.90 square kilometers with a population density of 162.03.

While the majority (74.9%) of the population are mixed crop-livestock farmers or other rural dwellers, the remaining part of the population were urban inhabitants (25.1%) or few pastoralists (0.05%) [39]. Most of the fish population in Ethiopia is found in the rift-valley lakes, including a natural lake Dambel in Batu town, the crater lakes Babogaya and Hora-Arsedi in Bishoftu and reservoirs: Barbara or Koftu few kilo meters eastern to Bishoftu, and Koka reservoir near the town of Koka. Bishoftu, Koka and Batu towns are the three major towns in the east Shewa zone where fish is routinely harvested and consumed[40].

Lake Babogaya is among the crater lakes of Bishoftu. Three fish species, namely *Oreochromis niloticus* (Nile tilapia), *Clarias gariepinus* (African Catfish) and *Tilapia zilli* were introduced into the lake by the Ministry of Agriculture to enhance the fishery resource of the lake. The most dominant species are *Oreochromis niloticus*; followed by *Clarias gariepinus*[41]. Lake Hora-Arsedi consists of only *O. niloticus* which was introduced around[19, 42, 43]. Currently it supports a piscifauna, which is exclusively composed of Nile tilapia and *Tilapia zillii* although not much fishing is done except a few tons of fish caught for consumption by the town residents[43].

Barbara also known as (Koftu) is a man-made reservoir located few kilometers away from the eastern part of Bishoftu town. *Oreochromis niloticus* (Nile tilapia) is the only fish species inhabiting the reservoir. It was artificially introduced to the reservoir by the Bishoftu town office of livestock and fisheries with the objective of boosting fish production in the area[40]. Koka reservoir is the most important source of fish for small scale fisheries in general and riparian societies in particular[16]. It provides about 542 tons of fish annually. The currently existing commercially important species and their contribution to the total annual catch are common carp (*Cyprinus carpio* = 36%), catfish (*Clarias gariepinus* = 35%), tilapia (*Oreochromis niloticus* = 18%*)* and barbs (*Labeobarbus intermedius* = 11%) [44].

The six indigenous fish species at Lake Dambel include *Barbus ethiopicus, Barbus paludinosus, Labeobarbus intermedius, Garra makiensis, Garra dembecha* and *oreochromis niloticus*[45]. In addition, three more exotic fish species such as *Tilapia zillii, Carassius carassius* and C*arassius auratu* were introduced into the lake with the objective of enhancing fish production, while the 4th exotic species, namely, *Clarias gariepinus,* is believed to have been slipped into the lake accidentally. Among others, tilapia (*Oreochromis niloticus* and *Tilapia*

zillii), carp (*Carassius auratus* and *Carasius carrasius*) and catfish (*Clarias gariepinus*) are the most demanded and highly exploited type of fish species in the area[46].

3.2.2 Sample size and sampling

The sample size was estimated according to Shari[47] who suggested a minimum required sample size of 50 participants for qualitative survey study. We collected data from a total of 200 selected respondents comprising 50 fishermen, 20 food establishment workers, 10 fish retailers and 120 consumers. Except for the later, the participants with more than one year of experience handling fish were purposively included in the study. Consumers were selected as convenience sample in the study based on their availability at the time of data collection at the restaurants or lakes, and their willingness to participate in the study. Questionnaire survey and direct observation were used to collect the data (Annex 5 and 6). All methods were performed in accordance with accepted ethics and regulatory requirements.

3.2.3 Ethical clearance

The study was approved by Oromia Health Bureau Health Research Ethical Review Committee (Ref No. BEFO/4BTW/1-16/10393). Written informed consent was obtained from all study participants and privacy information are kept confidential to protect their business and identity.

3.2.4 Data analysis

The data obtained was entered into an excel spreadsheet (Microsoft office R excel 2007) and analyzed using Stata version 13.0 software (Stata Corp, College Station, TX) [48]. Descriptive statistics was used to calculate the frequency and proportion of various actors along the fish production supply chain involving handling, processing, transportation and storage practices, and consumption preferences. Chi-square test was used to compare the proportions of observations among the different categories. $P < 0.05$ was used for a statistical significance of an estimate.

3.3 Results

3.3.1 Demographic characteristics of the participants

Among a total of 200 respondents participated in the study, 90% of them were males. The mean age of the participants was 33.3 years (range: 17-56 years) (Table 7).

Table 7: Gender and age distribution of respondents along fish production and supply chain in three east Shewa zone of Oromia, Ethiopia

Respondents' Category	Gender		Age (Mean)	Number (%) of respondents by town		
	M	**F**		**Bishoftu**	**Koka**	**Batu**
Fishermen (n=50)	50	-	34.4	30 (60)	10 (20)	10 (20)
Fish retailers (n=10)	7	3	33.8	1 (10)	1 (10)	8 (80)
Food establishment workers (n=20)	14	6	39	7 (35)	6 (30)	7 (35)
Consumers (n=120)	109	11	31.8	40 (33.3)	40 (33.3)	40 (33.3)

3.3.2 Fish production and supply chain

In the study area, fish is primarily harvested from freshwater lakes and reservoirs. The main actors in the supply chain include fishermen, fish retailers, food establishments and consumers. The fishermen take the lion's share in harvesting and processing fish. All fishermen were males in this study (Table 5). Consumers can acquire fish through five different gateways each of which require their own standard of hygienic handling processes as shown in the fish production and supply chain conceptual framework depicted below (Figure 3).

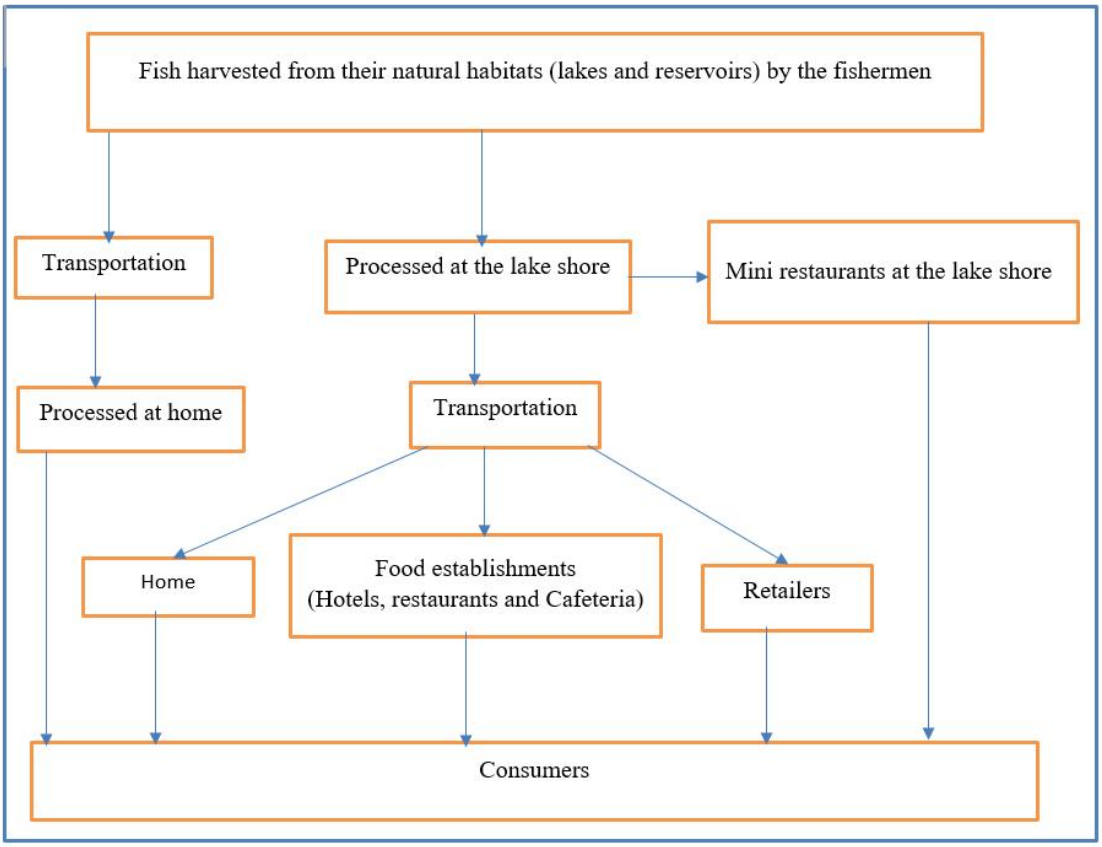

Figure 3: The observed conceptual flow diagram showing fish production and supply chain in east Shewa zone of Oromia, Ethiopia

3.3.3 Hygienic conditions at lakes and reservoirs

All the respondents reported that the lakes and reservoirs are accessible to animals, and most of them are watering points. They also indicated that an occasional open defecation by the people living and working around the lakes, and exposure of the water bodies to run-off water. We also observed animal feces, and cattle and cart horse roaming and grazing around the study lakes.

3.3.4 Post-harvest handling and processing practices

It was observed that all the fishermen handled and processed fish under unhygienic conditions at the lake shore. All of them were performing the activity under no processing facilities such as with no clean and impervious processing room/area, clean/potable water, equipment washing and disinfection facility, hand washing facility, product handling facility etc., using a single knife and the same cutting board for processing all fish with infrequent washing and with no disinfection in between processing. The fishermen visually inspect each fish for any external defects followed

by manual incision to separate the head, tail, scale, and skin, gut removal and filleting. Filleted fish were washed using unclean water directly obtained from the lakes.

Table 8 summarizes fish processing, transportation, and handling practices by the fishermen. Majority (88%) of the fishermen reported that their customers prefer processed fish at the shore compared to whole fish. Also, 84% of the fishermen reported selling the processed fish at the lake shore; and 64% of their customers were direct consumers. The most commonly used container by the fishermen for fish handling and transportation was sack, and in some instances a plastic bag. They also used the same container (either sack or plastic bag) to transport a filleted fish for home consumption using a public transport with no cold chain.

Only 30% of retailers transported their fish using vehicles equipped with cold chain facilities. Overwhelmingly, 70% of them used either ordinary trucks or a public transport with no cold chain using sacks and/or crate containers. All retailers stored fish in a refrigerator for a maximum duration of 7 days but they never checked the storage temperature.

Similarly, none of the food establishments used a cold chain facility for fish transportation; and 70% of them stored fish in a refrigerator for a maximum duration of 24 hours. However, none of them checked the storage temperature. The remaining 30% of the food establishments have reported that no fish will remain from the daily supply, and they did not store fish.

Table 8: Fish processing, transportation, and handling practices by the fishermen (n=50) in east Shewa zone, Oromia

Variables	Category	% (number of observations)
Fish preference of customers	Whole fish	16 (8)
	Processed fish	84 (42)
Catching frequency	Every day	74 (37)
	Once per week	2 (1)
	Three times per week	24 (12)
Where to sale	At the lake shore	82 (41)
	At restaurants	12 (6)
	Used for personal consumption	6 (3)
Where to process	At the lake shore	88 (44)
	At home	12 (6)
Who will process	Fishermen	88 (44)
	Other processors	12 (6)
Fish customers	Consumers	64 (32)
	Retailers	14 (7)
	Hotels/restaurants	16 (8)
	Used for personal consumption	6 (3)
Total		**100 (50)**

3.3.5 Fish consumption

Among the 120 consumers completed the questionnaires, 13.3% of them preferred raw fish only while the majority (63.3%) of them preferred both raw and heated/fried fish. Thus, about 77% of the consumers had the preference of consuming raw fish. There was statistically significant difference in the fish consumption preference based on gender and study sites ($P < 0.05$) (Table

9). The majority (72.7%) of female consumers preferred consumption of heat-treated fish. However, there was no difference in consumption preferences of fish among the consumers based on age, religion, educational status, occupation, or spatial location ($P > 0.05$).

Table 9: Fish Consumption Preferences of Different Categories of Respondents (n=120)

Variables	Category	% Consumption Preference (number of observations)			Fisher's Exact Test (P-value)
		Raw	**Heat Treated**	**Raw and Heat Treated**	
Study Site	Bishoftu (n=40)	15 (6)	40 (16)	45 (18)	0.015
	Koka (n=40)	10 (4)	10 (4)	80 (32)	
	Batu (n=40)	15 (6)	20 (8)	65 (26)	
Gender	Male (n=109)	14.68 (16)	18.35 (20)	66.97 (73)	0.001
	Female (n=11)	0.00 (0)	72.73 (8)	27.27 (3)	
Educational level	No formal education (n=6)	0.00 (0)	33.33 (2)	66.67 (4)	0.138
	Adult education (n=5)	20 (1)	40 (2)	40 (2)	
	Primary (n=63)	9.52 (6)	15.87 (10)	76.60 (47)	
	Secondary (n=41)	19.51 (8)	29.27 (12)	51.22 (21)	
	Higher (n=5)	20 (1)	40 (2)	40 (2)	
Religion	Orthodox (n=112)	13.39 (15)	25 (28)	61.61 (69)	0.284
	Protestant (n=8)	12.50 (1)	0.00 (0)	87.50 (7)	

Majority (83.3%) of the consumers had no knowledge about the significance of avoiding cross contamination between heat treated fish and raw fish including other raw foods.

Furthermore, 80% of them had no knowledge about fishborne zoonotic diseases. Consumers believed that the use of spices like red pepper, which are commonly consumed with a raw fish, followed by consumption of local alcoholic drink "Haraqee" avoids the risk of acquiring fishborne illnesses.

3.4 Discussion

In this study, we assessed the hygienic status of fish handling practices at the production sites and using a combination of questionnaire survey and direct observation approaches to identify the fish supply chain and to qualitatively measure the hygienic conditions along the supply chain. The approaches we used are essential in generating relevant information needed as inputs in food quality assessment model. However, we did not investigate the prevalence and concentration of pathogenic microorganisms and examine the effects of unhygienic handling practices on fish microbial quality and safety of fish along the supply. Further comprehensive food safety risk assessment that consists of hazard identification at each critical point, hazard characterization (dose response relationship), human exposure assessment to the hazards via consumption of contaminated fish and risk characterization (e.g., illness per exposed people) is required to quantify the probable risk and identify the risk mitigation measures to implement efficient and feasible fish safety management system along the supply chain[49, 50]. Nonetheless, our study revealed significant basic hygienic gaps in the fish handling practices that can be targeted for interventions. Considering the inherent differences in the scope and outcomes of the different food safety assessment approaches, we discussed the convergences and divergences of the findings from the perspectives of national and global sets of standards and regulatory requirements. Specifically, the major findings at each critical points in the fish supply chain were discussed in view of Ethiopian national proclamations (Ethiopian Public Health Proclamation No. 200/2000 and Ethiopian Food and Medicine Administration Proclamation No.1112/2019), and internationally recommended hygienic standards and regulatory requirements (FAO, WHO and Codex Alimentarius Commission, CAC) for hygienic handling of fish to prevent fish contamination to ensure fish safety and to reduce financial loss due to fish spoilage.

The free access of cattle to the freshwater lakes and reservoirs, the exposure of the water bodies to contaminated water run-off and open defecation by people in the area are potential factors that can lead to the contamination of the natural habitats for fish production. We also

observed the use of contaminated water for fillet washing which is contrary to FAO and WHO code of practice for fish and fishery products[51]. The code affirms the availability of adequate supply of clean sea water or potable water for washing fish prior to filleting or cutting, fillets after filleting, skinning, or trimming and filleting equipment and utensils. The codex general principle of food hygiene also declares the control of water quality to minimize the risk of potential biological, chemical, and physical hazards[52]. The Ethiopian public health proclamation no. 200/2000 prohibits the supply of any food which is unhygienic, contaminated, and unwholesome, or does not meet the standards of food safety and quality[53]. Therefore, the indiscriminate contamination of freshwater lakes and reservoirs from which fish is massively produced and consumed coupled with the use of potentially contaminated water for fillet washing at the production sites are important areas of concern for public health where interventions can be targeted.

Fecal contamination that may contain pathogens from animals and people particularly as the result of the observed open defecation can reach the lakes through run-off water. This contradicts the general principle of food hygiene[52], which states that the control of fecal contamination minimizes the potential for contamination with many foodborne pathogens such as *Salmonella*, *Campylobacter*, *Yersinia* and pathogenic strains of *E. coli*. Most importantly, contaminated water run-off may carry antimicrobial residues and resistant pathogens that can play an important role in the dissemination of antimicrobial resistance and transmission of such resistant pathogens among fish, animals and humans[50]. According to FAO and WHO[51], bilateral code of practice for fish and fishery products, contamination of fish depends on the environment and the bacteriological quality of the water from which it is harvested. Once the aquatic environment is contaminated with a zoonotic pathogen, it is highly probable for the aquatic fauna including fish to acquire the pathogen and become a potential threat for fish consumers[54]. Thus, limiting the access of animals to the water bodies, diverting the direction of water run-off against the natural habitat of fish, and circumventing the open defecation by providing access to public restrooms around the beaches and at the shores are the most likely intervention strategies. Our study found significant breaches in the basic hygienic practices where the environment is likely contaminated by animal and human feces.

It was observed that, fish was washed with lake water having a potential risk of contamination with pathogenic microbes. According to FAO[54], cross-contamination and contact

with contaminated water may lead to dissemination of microbial contaminants into other fresh or heat-treated fishery products. Thus, due to unhygienic landing sites and unrestricted cross contamination of the processed products, the situation worsens the safety of consumers.

Our study indicated that fish processing was practiced by all fishermen at unhygienic landing sites at the lake shores that can result in the contamination of the fish. This contrasts with the recommendations of FAO, which states unhygienic post-harvest handling and processing procedures deteriorate the safety of consumers[54]. Fish requiring gutting on arrival at the processing facility should be gutted efficiently, without undue delay and with care to avoid contamination[55]. A study by Huss et al.[56], also showed that contamination of fishery products is primarily due to unhygienic processing or poor water quality. Training of fishermen and infrastructural development at the landing sites are important interventions to promote hygienic fish processing in the area.

Our observation of the use of the same knife and one cutting board for processing all fish without disinfection of equipment shows risky practices that contribute to contamination and cross contamination of fish during production. The European commission health and consumer protection directorate requires that equipment and premises approved for slaughtering, carcass preparation and meat cutting to be carefully cleaned and disinfected several times during and at the end of the working day, and before being re-used, to prevent contamination by pathogenic microbes or other microbes associated with meat spoilage. In addition, the European Union meat hygiene directives require that during the production process the temperature of water used to decontaminate hand-held tools (knives, hooks, saws) should be maintained at 82 °C or higher[57]. However, these basic facilities were not present in the present study area and intensive training of the fishermen on the concepts of contamination, cross contamination and an overall hygienic production and processing of fish are significant tools to reduce fishborne illnesses and to increase the shelf life of the fish.

Fish transportation and storage practices are not in line with the required standards. All food establishments did not have refrigerated vehicles for transportation; and only 30% of the retailers shipped fresh filleted fish using vehicles equipped with cold chain facilities. The use of unhygienic sacks or plastic bags as a packaging material is also a common practice. However, according to FAO[54], fish is one of an exceptionally perishable product and post-harvest handling, processing and transportation channels should be performed under hygienic and cool temperature

conditions to prevent microbial contamination and fish loss due to spoilage. The transportation and storage methods of fish should also be environmentally friendly, and assure the safety of consumers[58]. The recent recommendations of FAO and WHO[59], also declares that fresh fish should be transported using clean and suitable vehicles at a temperature closer to 0 °C, preferably in containers with dry freezer bags instead of ice while frozen products should be maintained at –18°C or below. It also obliges the use of clean, sound, and durable packaging materials and maintaining adequate protection against contamination from dust, exposure to higher temperatures and the drying effects of the sun or wind[59]. The Ethiopian Food and Medicine Administration and Control Proclamation No. 1112/2019 also enforces the use of clean and suitable packaging material, adhering to the national and international quality and safety standards[60].

The Codex Code of practice for fish and fishery products requires that fish should be maintained at the chilling temperature of 4 °C for short time storage and freezing temperature of ≤ − 18 °C for long time storage with regular temperature monitoring[51]. However, all retailers and 70% of the food establishments in the study area stored fish in a refrigerator for a maximum duration of 24 h and 7 days respectively, but none of them had known the storage temperature at which the fish were kept. Interestingly, 30% of the food establishments did not store fish; they supply consumers with freshly produced fish though the way it has been harvested, processed, and transported is still under sub hygienic conditions. This entails that fish retailers and food establishments should make every effort to store fish at required temperature to avoid fish spoilage and to ensure consumer safety.

The present study showed that consumption of raw fish is popular in the study area where about 77% of the respondents had the preference of consuming raw fish. In addition, 80% of them did not know the potential risk of fishborne pathogens associated with the consumption of contaminated fish. FAO[54], indicated that food poisoning including consumption of contaminated fish, occurs because of eating raw or undercooked products or cooked products that have been cross-contaminated. Several studies elsewhere showed the linkage of the raw fish consumption with fishborne diseases. For instance, a study by Kalimuddin et al.[61], showed that 283 infections due to *Streptococcus agalactiae* was linked to the consumption of raw fish in Singapore. Austin et al.[38], also reported the presence of several bacterial pathogens of public health concern, causing serious morbidity and mortality in humans, through consumption of raw or inadequately

cocked contaminated fish and/or fishery products. As stated above Shiga toxin producing *E. coli* O157:H7 was reported from fish examined at various lakes in Ethiopia[36, 37], implying the potential risk of consuming raw or inadequately coked fish or fishery products. However, the Ethiopian food safety regulations almost exclusively target commercial markets while most people consume what they produce or buy from the local informal markets[62]. Therefore, adoption, establishment and maintenance of operational safety standard protocols will ensure the safety of fish and fishery products to protect consumer health[22, 58].

Although the number of female participants included in the questionnaire survey was small, the study identified a significant gender difference with respect to raw fish consumption. In addition, in the study area, most of the fish handling practices (processing, transportation and storage) are managed by the male counterparts under unhygienic conditions and without adhering to the national or international safety standards. This is contrary to the FAO and WHO, code of conduct for responsible fisheries[59], which declares a remarkable importance of naturally occurring food safety hazards in the environment from which fish are harvested. To that end, risks to consumer health associated with fish and fishery products obtained from unpolluted marine environments are low provided that the products are handled in line with the principles of good manufacturing practices. However, it would be increased when fish is mishandled along the value chain[59]. The report of Austin et al.[38], also showed the potential for public health risk of handling infected fish on fish farms or in retailer shops. It implies that in men the risk of occupational exposure to fishborne pathogens (hazards) is more likely. So, women are less likely to be exposed to fishborne pathogens as compared to men and also by avoiding raw fish consumption.

Taken all together, the study indicates that the identified unhygienic handling practices that could be due to less stringent food safety regulation practices in Ethiopia calls for an urgent timely intervention to establish and maintain effective national fish quality and safety plans to ensure public safety. More importantly, consumption of a heat-treated fish should be promoted as effective risk mitigation strategy through public education to prevent risk of acquiring fishborne zoonotic diseases.

Moreover, the informal fish marketing system that dominates in Ethiopia should be regulated for implementation of effective supply chain management system. To reduce the perceived risk associated with food consumption and to increase the consumers trust, many

countries have introduced Food Traceability System such as HACCP (Hazard Analysis and Critical Control Points), block-chain technology and other electronic systems; which can provide an information platform for all the supply chain members[12, 63–65]. Block-chain technology in particular is a digital ledger platform which is becoming an important tool in different industrial production and agribusiness in managing food supply chain by providing secure and immutable information[12, 14]. Block-chain is a digital, decentralized, and distributed ledger in which transactions are logged and added in chronological order with the goal of creating permanent and tamper-proof records[33, 66, 67]. One of the most critical aspects in the use of block-chain applications is related to monitoring social and environmental conditions to control and avoid the occurrence of health and safety problems[68]. It provides the ability to track food such as fish and fish products through all stages of production, processing, and distribution to signal quality in the food supply chain[12, 21].

Under the current Ethiopian context, it is more prudent to transform the prevailing informal fish production and marketing system by strengthening the existing fish producer and supplier cooperatives to a large-scale commercial enterprise, establishing well networked formal marketing system, and regulating the market dynamics and improving infrastructural development. These will be foundational to evaluate the application of the digital technologies such as block-chain and adopt the technology in future studies for sustainable management of fish production and supply chain by addressing perishability of fish, demand–supply mismatch, unfair prices, and quality related data to satisfy the demands of intervening actors across the supply chain to encourage transparency and accountability in the entire value chain in Ethiopia[5].

3.5 Conclusion

Our study revealed a wide range of unhygienic handling practices along fish production and supply chain such as the use of contaminated lake water for fillet washing; the use of a single knife and cutting board to process multiple fish with infrequent cleaning and without any disinfection between processing; and the use of unhygienic fish containers and packaging materials. Furthermore, the study identified lack of infrastructure for post-harvest fish handling and processing, evidence of conspicuous cross-contamination during processing, lack of appropriate transportation facility and lack of adequate knowledge about fish borne diseases linked with the consumption of raw fish. The unhygienic handling practices and the raw fish

consumption preference of the consumer imply the potential risk of fishborne diseases in the area necessitating timely interventions. Infrastructural development, establishing standard fish processing plant/house with basic facilities, provision of food safety training to all actors along the production supply chain and the use of easy to clean containers and cold chain facilities for transportation of fish are critically needed to ensure fish safety. Further studies should aim at investigating the role of the unhygienic handling practices, microbial quality of fish and other risk factors for the occurrence and contamination level of fish with foodborne pathogens to identify critical control points along the production and supply chain at which future interventions will be targeted. In addition to investigation into the intrinsic fish qualities such as changes in colour, aroma, flavor and texture of fish due to spoilage, examining the extrinsic factors such as method of production and processing, price determination, duration of storage, context and appropriateness (purchase/consumption occasion), origin and geographic distance between production and market, and consumption patterns are required for evidence based evaluation and decision for fish quality to enhance consumers' confidence and acceptance and promote willingness to choose and pay for better quality. Moreover, to curb the immense economic losses and implications of public concerns on fish quality and safety emanating from lack of information on how the fish is produced and marketed along the value chain, evaluation, and adoption of various food supply management systems such as block-chain technology are critically required.

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CHAPTER 4: GENERAL DISCUSSION

The goal of the present study was to investigate the occurrence and public health implications of SN-F *E. coli ,* with particular emphasis to *E. coli* O157:H7, in central Oromia, Ethiopia. The study employed standard conventional and molecular techniques to isolate SN-F *E. coli* strains and confirm their occurrence, molecular characteristics and antimicrobial susceptibility profiles. It also assessed the hygienic practices in fish handling along the supply chain in the study areas based on combined questionnaire survey and direct observation to get credible data. It provided evidence of virulent and antimicrobial resistant SN-F *E. coli* strains, including novel strains with previously undetected O-antigens from both fish and human sources (Chapter 2). Genetic analysis revealed some relatedness between isolates of the same source, but no molecular relationship existed between strains of diverse sources, i.e., water, fish, and humans. Most of the strains examined had more than one gene related to individual virulence factors and antimicrobial resistance characters, thereby emphasizing their pathogenicity and inherent resistance potential (Table 8). Here, it is interesting to note that none of the isolates were classified as *E. coli* O157:H7, reflecting widespread distribution of non-O157:H7 SN-F *E. coli* strains throughout the fish supply chain in the study areas. Apart from this, the study found critical failures in hygienic practices throughout the fish supply chain (Chapter 3). This practice is critical for the transmission of virulent and antimicrobial resistant (AMR) SN-F *E. coli* strains from aquatic environments to fish and subsequently to humans. Together, these results highlight the urgent need for technical interventions to improve fish safety and public health against the new hazards presented by AMR SN-F *E. coli* strains. Considering few existing reports on SN-F non-O157:H7 *E. coli* strains, the present study attempted to compare its findings with studies conducted on generic *E. coli* and, *E. coli* O157:H7 to provide better insights.

4.1. Occurrence in Water, Fish, and Humans

The study revealed the occurrence of virulent and antimicrobial resistant SN-F *E. coli* strains in water, fish, and humans with the following proportions: 6.7%, 1.8%, and 7.3%, respectively (Chapter 2). The occurrence of SN-F *E. coli* in lake water at 6.7% is much lower than the finding of Mekonnen *et al*. (2014) and Marijani (2022, who reported 93.3% of generic *E.*

coli from Lake Dambel; but agrees with Dissasa *et al.* (2022), who recovered 5.9% in Lakes Dambel, Langano, and Hawasa. Such variations can be emanated from variations in detection protocols, *E. coli* strains targeted, and environmental factors such as human activities influencing lake water contamination levels.

Likewise, SN-F *E. coli* strains were isolated from 1.8% of fish samples; in which 50% of them were detected in fish meat (Chapter 2), indicating widespread contamination from the external environment, skin or intestinal contents (Chapter 3). Similar study in India has also shown that 19.2% of cattle feces were positive for SN-F *E. coli* (Mana *et al*., 2010), whereby it was suggested that contaminated animal feces may contaminate lake water and fish. However, the relatively low percentage of SN-F *E. coli* in fish recovered in the present study does not coincide with greater reports from other countries, like the report of Marijani (2022), who detected generic *E. coli* in 39% of Tanzania's marine and freshwater fish.

The present study also detected SN-F *E. coli* strains in 7.3% of diarrheic human patients with a history of fish consumption (Chapter 2). This finding is proportionally lower than the 25% pooled prevalence of generic *E. coli* reported by Zenebe *et al*. (2020), and 15.3% prevalence of *E. coli* O157:H7 among diarrheal cases in children from eastern Ethiopia (Getaneh *et al.,* 2021) and Bahir Dar city of northern Ethiopia (Adugna *et al.*, 2015). These findings emphasize the public health risks associated with poor hygiene and raw fish consumption (Chapter 3), as echoed by concerns raised in other studies (Haenen *et al.*, 2013; Santos and Vieira, 2013; Mekonnen *et al*., 2019; Wiriyaprom *et al*., 2022) regarding the danger of human infection due to contaminated food sources.

Although strains from the same sample sources were genetically related, neither of the isolates from different sources were molecularly clustered (Chapter 2). Genetically related strains having occurred either in water, fish or humans (Chapter 2), combined with a wide range of hygiene failures in the fish supply chain (Chapter 3), raises the risk of circulation and transmission of virulent strains from water to fish to humans (Chapter 2). A study from Turkey also revealed the occurrence of SN-F *E. coli* O157:H7 in fish samples, pointing to the public health risk posed by fish as a transmission vehicle to the consumer (Onmaz *et al*., 2020).

4.2 Antimicrobial Resistance Profile

In the present study, SN-F *E. coli* strains that were detected in water, fish, and humans have shown mono and multi-drug resistance profiles (Chapter 2). This also raises public health concern since they are resistant to the majority of antimicrobial agents used for the treatment of enteric infections. Previous findings have indicated that these strains are frequently resistant to several classes of antibiotics, including beta-lactams, tetracyclines, and fluoroquinolones. This complicates treatment and undermines infection control in clinical and community settings (Zhu *et al*., 2020). Besides, some of them harbor multiple virulence factors, such as adhesins, toxins, and invasins, along with antimicrobial drug resistance genes. Not only do they enhance their pathogenicity, but also the ability to thrive in the host and environmental populations (Nüesch-Inderbinen and Stephan, 2016).

Unhygienic handling along the fish supply chain, particularly in areas where raw fish is consumed on a regular basis (chapter 3), offers direct linage for the transfer of such resistant strains from water to fish and then to humans (WHO, 2021). The transmission channel points towards the significant position that environmental pollution and unhygienic fish handling occupy as factors in transmitting antimicrobial resistance in areas with such handling practices. The 87.5% proportion of multi-drug resistant SN-F *E. coli* strains recovered in this study (Chapter 2) is again evidence of the ubiquity of such resistant strains along the fish supply chain, and proof of the need for targeted interventions.

Reports have intermittently described the worldwide establishment of multi-drug resistant *E. coli* strains, highlighting the significant health and economic impacts they have and the increasing difficulties of control and management (Ejaz *et al*., 2021; Mdegela *et al.*, 2021; FAO, 2022; Salamandane *et al*., 2022; Ziarati *et al.*, 2022; Sumanaa *et al*., 2023). The present finding is also consistent with the finding of Anyanwu *et al.* (2022), who recorded the occurrence of multi-drug resistant SN-F *E. coli* in animal hosts like broiler chickens, cattle, and pigs in Southeastern Nigeria. This study highlights the potential of these resistant strains to spread to other animal populations and habitats, thereby increasing their ecological reservoir of resistance genes to influence both aquatic and terrestrial environments, further adding public health significance. In addition, *E. coli* acts as a reservoir for antimicrobial resistance

genes that are transferred to other bacterial species by horizontal gene transfer (Poirel *et al*., 2018). This gene-exchange ability amplifies the public health risk posed by multi-drug resistant *E. coli* strains, emphasizing the need for appropriate fish handling practices and public health efforts to reduce the risk of spreading antimicrobial resistant strains of SN-F *E. coli* along the fish supply chain.

4.3. Novel Strains with Unknown O-Antigen

The study noticed the occurrence of SN-F *E. coli* with unidentified O-antigens in fish sampled from lake Koka and diarrheic human patient admitted to Bishoftu Hspital. These novel strains may be mutant progenies of known *E. coli* strains or newly emerging variants not yet characterized. The finding underlines the urgent need for robust molecular studies to deepen the insight into the phylogenetic positioning of these strains among *E. coli* lineages documented in the sequence database. Previous research has demonstrated the effectiveness of cross-comparison of 16S rRNA gene sequences with available bacterial taxa sequences in databases as a desirable and efficient technique for new strain identification (FAO, 2009; FAO and WHO, 2020). These molecular tests will be crucial to establish the evolutionary dynamics of the isolates and their potential public health relevance.

4.4. Public Health Repercussions of Unhygienic Fish Handling Practices

The present study identified severe hygienic gaps throughout the fish supply chain in central Oromia of Ethiopia. These deficiencies include washing fish with potentially contaminated water, processing at unsanitary lakeshore landing points, cutting different fish with the same knife and on the same cutting board without proper washing and disinfection, and lack of proper cold chain facilities for fish transportation among majority, (70%) of fish retailers and all food establishments. Besides, the usual practice of packing with unhygienic sacks or plastic bags is of concern. However, Such hygienic compromises are a significant threat to fish safety and public health. Earlier reports emphasized the spread of pathogenic microbes to other fresh or heat-treated foods through cross-contamination and exposure to contaminated water (Huss *et al.*, 2003; FAO, 2009). In addition, Fresh fish must be carried in clean, appropriate vehicles at near to 0°C temperatures, preferably in dry freezer bags containing containers rather than ice, and frozen products must be stored at –18°C or lower, as per FAO and WHO (2020) recommendations.

Consistent with earlier findings, Manna *et al.* (2010) identified SN-F *E. coli* strains from cattle feces and demonstrated a potential risk of environmental contamination and its impact on aquatic ecosystems. The Food and Agriculture Organization (FAO, 2009) further highlighted that contaminated water is a direct infection source for fish which in-turn exposes humans to zoonotic pathogens. Moreover, the CDC's Foodborne Disease Outbreak Surveillance System (FDOSS) recorded 857 foodborne disease outbreaks due to fishborne pathogens with 4,815 illnesses, 359 hospitalizations, and 22 deaths (Barrett *et al*., 2017). This indicates how highly susceptible fish are to zoonotic pathogens like SN-F *E. coli*, which is a serious public health concern with the consumption of contaminated fish.

Fish is extensively produced and consumed in Central Oromia, with majority of the consumers having a preference of raw fish. The present study revealed, 77% of the interviewees prefer raw fish and 80% of them were unaware of the potential public health risks associated with consumption of raw contaminated fish. Previous reports have shown that consumption of contaminated raw products, like fish, or cross-contaminated cooked food is significantly responsible for causing foodborne illnesses (Austin *et al*., 2005; FAO, 2009; Kalimuddin *et al*., 2017). However, food safety legislation in Ethiopia primarily aims at commercial markets; while most of the population consumes food from neighborhood or informal markets (Dinede *et al.*, 2021). This highlights an urgent need for the country to revise its food safety legislation to effectively counter the risks of foods produced and consumed locally, including fish.

In this study, 50% of the fish isolates were from fish meat that was meant for human consumption, again highlighting the risk of infection from consuming raw fish. The same was indicated by Lassen *et al.* (2016) and Kalimuddin *et al*. (2017), who isolated fishborne pathogens from smoked and raw freshwater fish, respectively. A recent study conducted by Zorriehzahra and Talebi (2021) estimated that a total of 260,000 people in the USA get infected each year by the consumption of contaminated fish, with the most frequently implicated food being fish meat. The consumption of raw fish significantly increases the incidence of fishborne diseases, thus the need to have public awareness programs on the risk posed by the consumption of raw or undercooked fish in central Oromia, Ethiopia.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The study investigated the occurrence, molecular characteristics, and antimicrobial susceptibility of SN-F *E. coli*, with major emphasis to *E. coli* O157:H7, in lake water, fish, and humans in central Oromia, Ethiopia. In addition, it attempted to assess the status of hygienic fish handling practices in the area to obtain crucial information related to the public health risks associated with fish handling and consumption among the study communities. The primary aim was to elucidate the transmission pathways of this pathogens along the fish supply chain. It revealed genetically linked, antimicrobial-resistant SN-F *E. coli* strains obtained from the same sources, and lack of phylogenic relationship among strains obtained from different sample sources (water, fish and humans). A widespread hygienic deficiencies was also evidenced throughout the fish supply chain in the areas. Of specific concern is, the detection of virulent and multidrug-resistant SN-F *E. coli* strains, and novel strains with unidentified O-antigens and MDR profiles, which are of serious public health concern.

Although molecular clustering among the water, fish, and human isolates was not detected, the recovery of virulent and MDR strains with increased frequencies among these sources, coupled with unhygienic handling practices and raw fish consumption habits of the community, indicate a high risk of transmission of the pathogens from water to fish to humans. These findings highlight the necessity of improved hygienic practices in consuming and handling fish to minimize the risk of fishborne disease and the spread of antimicrobial resistant pathogens.

5.2. Recommendations

Based on the aforementioned conclusive remarks, the following points are suggested:

1) Urgent technical interventions, such as public training on safe handling and consumption of fish, strict regulation on antimicrobial use, enforcement of polices , need to be initiated to tackle the spread of virulent and MDR SN-F *E. coli* strains across the fish supply chain.

2) There should be infrastructural development for hygienic fish production and processing; including, well established processing units, easily cleanable containers and cold-chain facilities for fish transport.

3) To improve the subsistence fishery situation in Ethiopia, where small-scale fish catchment and informal marketing dominate, modern food supply management systems, such as block-chain technology, need to be adopted. While this will not be appropriate for the short-term situation, improving fish handling, processing, and marketing practices will allow evidence-based evaluation and decision-making, thereby boosting consumer confidence and enhancing product traceability in the long-run.

4) Robust molecular study should be conducted to identify the lineages of new strains with unproved O-antigens relative to established *E. coli* strains in the sequence libraries and assess their public health issue.

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CHAPTER 7: ANNEXES

Annex 1: Supplementary File

Table 10: Whole Genome Sequencing (WGS) Result Showing Serotypes, Common Virulence Factors and AMR Traits of SN-F *E. coli* Strains Retrieved from Water, Fish and Humans in Central Oromia, Ethiopia

Sample Taken	Source	*E. coli* Serotype Detected	Number of Isolates with the Same Antigen	Virulence Traits	Locus	AMR Traits	Locus	AMR Identity Scores (%)
Water	Lake Babogaya	O116: H49	1	Adherence Protease Regulation Toxin	gad, IpfA k88ab terC hlyE	No Results	No Results	
	Lake Hora-Arsedi	O20: H8	1	Toxin Protease Adherence Regulation Complement Protease Survival Invasion	hlyE gad lpfA terC traT iss ompT	No Results	No Results	
		O174: H43	1	Toxin Protease Adherence Regulation Survival Invasion	hlyE gad lpfA terC iss ompT, cib	Doxycycline Trimethoprim Sulfamethoxazole Tetracycline	tet(A) dfrA1 sul1 tet(A)	100 99.79 100 100
		O8: H10	1	Toxin Protease Adherence Regulation	hlyE gad lpfA terC	No Results	No Results	
		O116: H49	1	Toxin	hlyE	No Results	No	

	Lake Koftu			Protease Adherence Regulation	gad k88ab, lpfA terC		Results	
	Lake Koka	O17/O44: H18	1	Toxin Protease Adherence Regulation Survival Invasion Iron uptake Complement protease Secretion system [not specified]	cma, hlyE, hlyF gad, vat lpfA, air terC, eilA iss cia chuA, sitA, iroN traT kpsE, kpsMll _k52 etsC	No Results	No Results	
	Lake Dambel	O17/O44: H18	1	Toxin Invasion Protease Adherence Secretion system Regulation Iron uptake Survival Complement Protease [not specified]	cma cia vat, gad air kpsmll -k52 terC, eilA sitA, chuA iss traT etsC	No Results	No Results	
Fish skin	Lake Koka	O176:H11	1	Toxin Adherence	hlyE, hlyF,	ampicillin amoxicillin	blaTE M-1B	100 100

swab				Protease Regulation Iron uptake Survival Compleme nt protease Invasion	cma, cvaC lpfA gad terC sitA, iroN iss traT ompT	Doxycycline piperacillin ticarcillin cephalothin Tetracycline	blaTE M-1B tet(A) blaTE M-1B blaTE M-1B blaTE M-1B tet(A)	100 100 100 100 100 100 100
Fish Meat	Lake Koftu	O18ac: H21	1	Toxin Protease Adherence Regulation Survival Invasion	hlyE gad lpfA terC iss cia	ampicillin amoxicillin cephalothin Doxycycline piperacillin ticarcillin Tetracycline Minocycline	blaTE M-1B blaTE M-1B blaTE M-1B tet(A) blaTE M-1B blaTE M-1B tet(B) cat (B)	100 100 100 100 100 100 100 100
	Lake Koka	O176: H11	2	Toxin Protease Regulation Survival	hlyE gad terC iss	ampicillin amoxicillin cephalothin Doxycycline piperacillin ticarcillin Tetracycline	blaTE M-1B blaTE M-1B blaTE M-1B tet(A) blaTE M-1B blaTE M-1B tet(B)	100 100 100 100 100 100 100
	Lake	O155: H21	1	Toxin Protease Adherence Regulation	hlyE gad lpfA terC	No Results	No Results	

	Koka	O10: H5	1	Toxin Protease Regulation	hlyE gad terC	Doxycycline Tetracycline Ciprofloxacin (mutational) Nalidixic acid (mutational)	tet(A) tet(A) parC parC	100 100 56 (position) 56 (position)
Fish feces	Lake Koka	O-unknown: H28	1	Toxin Invasion Protease Regulation Iron uptake Survival	hlyE ompT gad eilA, terC chuA iss	No Results	No Results	
Human stool	Koka Health Center	O168: H2	2	Toxin Protease Adherence Regulation Complement protease	hlyE gad lpfA terC traT	Fosfomycin	fosA7	94.09
		O8: H25	1	Toxin Protease Adherence Regulation Complement protease Iron uptake Invasion Survival [Not specified]	hlyE, hlyF, cvaC, mchF gad lpfA, irp2 terC traT fyuA, sitA, iroN, iutA ompT, cia iss etsC	ampicillin amoxicillin cephalothin Doxycycline piperacillin Trimethoprim Sulfamethoxazole Ticarcillin Tetracycline Nalidixic acid (mutational) Ciprofloxacin (mutational)	blaTEM-1B blaTEM-1B blaTEM-1B blaTEM-1B blaTEM-1B tet(A) blaTEM-1B blaTEM-1B dfrA5 sul1 blaTEM-1B tet(A) gyrA gyrA	100 100 100 100 100 100 100 100 86 86
		O155: H10	1	Toxin Adherence	hlyE, hlyF,	ampicillin amoxicillin	blaTEM-1B	100 100

	Bishoftu Hospital			Protease Regulation Iron uptake Survival Complement protease Invasion	cma, cvaC lpfA gad terC sitA, iroN iss traT ompT	Doxycycline piperacillin Trimethoprim Sulfamethoxazole ticarcillin cephalothin Tetracycline Ciprofloxacin Chloramphenicol	blaTE M-1B tet(A) blaTE M-1B dfrA12 sul3 blaTE M-1B blaTE M-1B tet(A) qnrS11 cmlA1	100 100 100 99.87 100 100 100 100 99.84
		O-unknown: H40	1	Toxin Adherence Protease Regulation Iron uptake Survival	hlyE irp2 gad terC fyuA iss	ampicillin amoxicillin cephalothin Doxycycline piperacillin Trimethoprim Sulfamethoxazole ticarcillin Tetracycline Nalidixic acid Ciprofloxacin Aztreonam Cefepime Ceftaxime Ceftazidime Ceftriaxone	blaTE M-1B blaTE M-1B blaTE M-1B tet(A) blaTE M-1B dfrA40 sul2 blaTE M-1B tet(A) gyrA qnrS1 blaCTX-M15 blaCTX-M15 blaCTX-M15 blaCTX-M15 blaCTX-M15	100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100 100

							(ESBL)	
	Batu Health Center	O155: H10	4	Toxin Adherence Protease Regulation Iron uptake Survival Complement protease Invasion	hlyE, hlyF, cma, cvaC lpfA gad terC sitA, iroN iss traT ompT	ampicillin amoxicillin Doxycycline piperacillin Trimethoprim Sulfamethoxazole ticarcillin cephalothin Tetracycline Ciprofloxacin Chloramphenicol	blaTEM-1B blaTEM-1B blaTEM-1B tet(A) blaTEM-1B dfrA12 sul3 blaTEM-1B blaTEM-1B tet(A) qnrS11 cmlA1	100 100 100 100 99.87 100 100 100 99.84

Annex 2: Ethical Clearance Certificates

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/14/05/13/2021

Name of Applicant: **Tesfaye Debelu (DVM, MVSc in Epidemiology, PhD fellow)**

Address: Department of Microbiology, Immunology & Vet. Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *An investigation into the occurrence, molecular characteristics and antimicrobial susceptibility of E.coli O157:H7 in fish and humans in different parts of Ethiopia*

Date of application:	**February, 2021**
Nature of the project:	**Mildly invasive**
Target animal species:	**Fish**
Number of animals involved:	**150**
Study area:	**Different parts of Ethiopia**

Minutes No. and date of review: **VM/ERC/05/13/021, 21/03/2021**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary
3. Separate clearance is required from relevant authorized body for works involving human subjects

Getachew Terefe (DVM, PhD)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

መልሱን በሚጽፉልን ጊዜ እባክዎን የኛን ደብዳቤ ቁጥር ይጥቀሱልን
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Biiroo Eegumsa Fayyaa Oromiyaa
Oromia Health Bureau
Saarbet (Calcalii) - Finfinnee

የኦሮሚያ ጤና ጥበቃ ቢሮ
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Lakk/Ref.NO BEFO/ሀBTኪ/1-16/10393
Guyyaa/Date 23/9/20—

W/E/F/Godina Shawaa Bahaa tiif **Hospitaala Bishooftuu tiif**
W/E/F/Go/Shawaa Kaabaa tiif **Hospitaala Baatuu tiif**
B/J

Dhimmi: **Xalavva Deggersa Kennuu Ilaala.**

Akkuma beekamu Biiroon Keenya Ogeyyii, dhabbile akkasumas namoota qorannoo fi Gamaggama jalqabaa fi Xumuraa geggessuuf propozaala dhiyeffatan propoozaala isaanii madaaluun akkasumas iddoo biratti ilaalchisani fudhatama argatan (approved) dhiyeffatan, propoozaala isaanii ilaaludhaan waraqa deggersa ni kenna. Haaluma kanaan Dhabbanni ***"Addis Ababa University"*** *jeedhu qorannoo mata duree* ***"An Investigation into the Occurrence, Molecular Characteristics and Antimicrobial Susceptibility of E. coli O157: H7 in Fish and Humans in Central Ethiopia*** "jedhuu irratti Magalaa fi Godina keessan keessaatti qorannoo gaggessudhaaf propoozaala isaani koree **"Health Research Ethical Review Committee"** Biiroo keenyatti dhiyeffatani jiru. Haaluma kanaan koreen **"Health Research Ethical Review Committee"** Biiroo keenya piropoozaalaa kana ilaaluun mirkanesse qorannoon Kun akka geggeeffamuu murtesse jira. Kanaafuu, hojii qorannoo kana irratti deggersa barbaachisa akka gootaniif isin gaafachaa, **dhabbata kana bakka bu'uun qorannoo kana kan gaggessuu Dr Tesfaayee Debeluu** qorannoon Kun qacceffamee eerga xumuramee booda firii isaa koppii tokko **BEFO** tiif akka galii godhaan galagalcha xalayaa kanaan isaan beeksifna.

Anis, **Dr Tesfaayee Debeluu** wayitti qorannoon Kun qaaceffame xumuramu firii isaa koppii tokko **BEFO** tiif galii gochuuf mallattoo kootiin ni mirkanessa.

Nagaa Wajjin

Maqaa: **Dr Tesfaayee Debeluu (PI)**
Mallattoo______
Bilbila:_________

G/G
Dr Tesfaayee Debeluu

B/J

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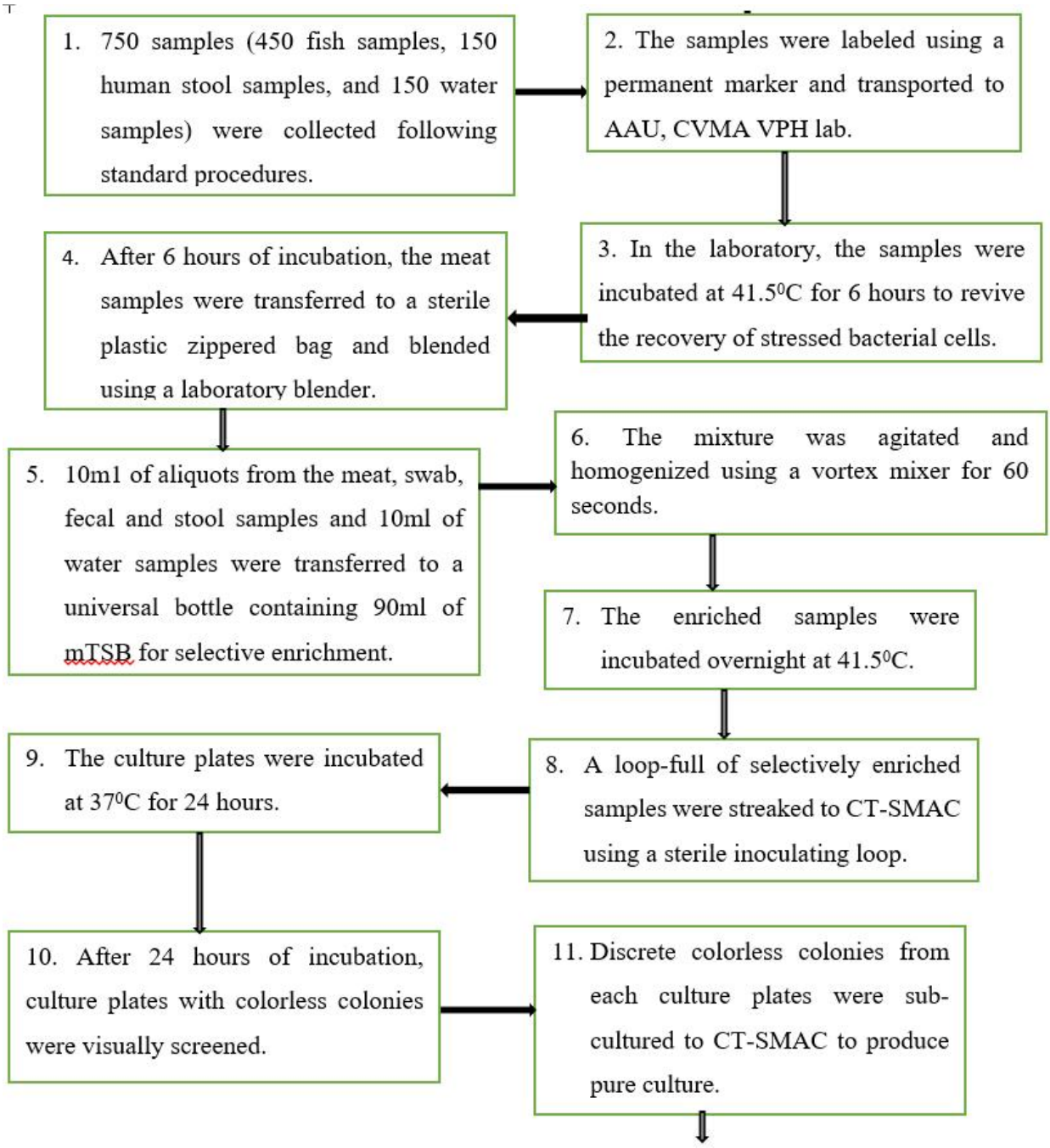
Annex 3: Conceptual Flow Diagram of Core Field and Laboratory Activities Performed to Isolate S-NF *E. coli* Strains Retrieved During the Present Study



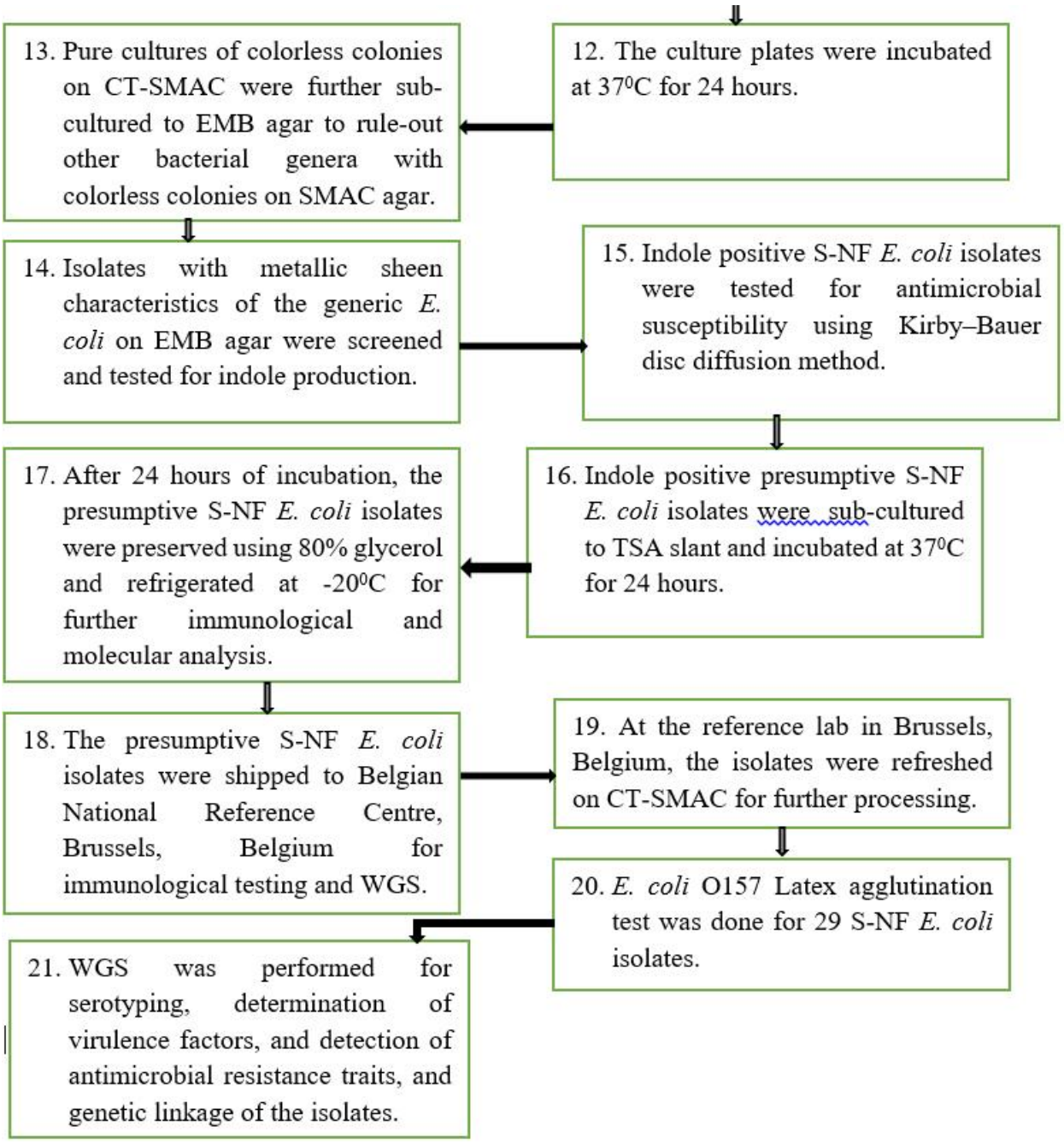


Figure 4: Conceptual Flow Diagram of Core Field and Laboratory Activities Performed to Isolate S-NF *E. coli* Strains Retrieved During the Present Study

Annex 4: Sample Information Sheets

Table 11: Fish sample information recording sheet

S/N	Source Lake	Species of Fish Sampled	Sample Taken	Sample Code

Table 12: Water sample information recording sheet

S/N	Source Lake	Sample Taken	Sample Code

Table 13: Stool sample information recording sheet

S/N	Source /Health Facility	Sample Taken	Type of Diarrhea	Sample Code

Annex 5 : Summary of Core Laboratory Activities and Results

Figure 5: Visual screening of the culture plates and sub-culturing made to produce pure cultures of SN-F *E. coli* on CT-SMAC

Figure 6: Indole test positive result

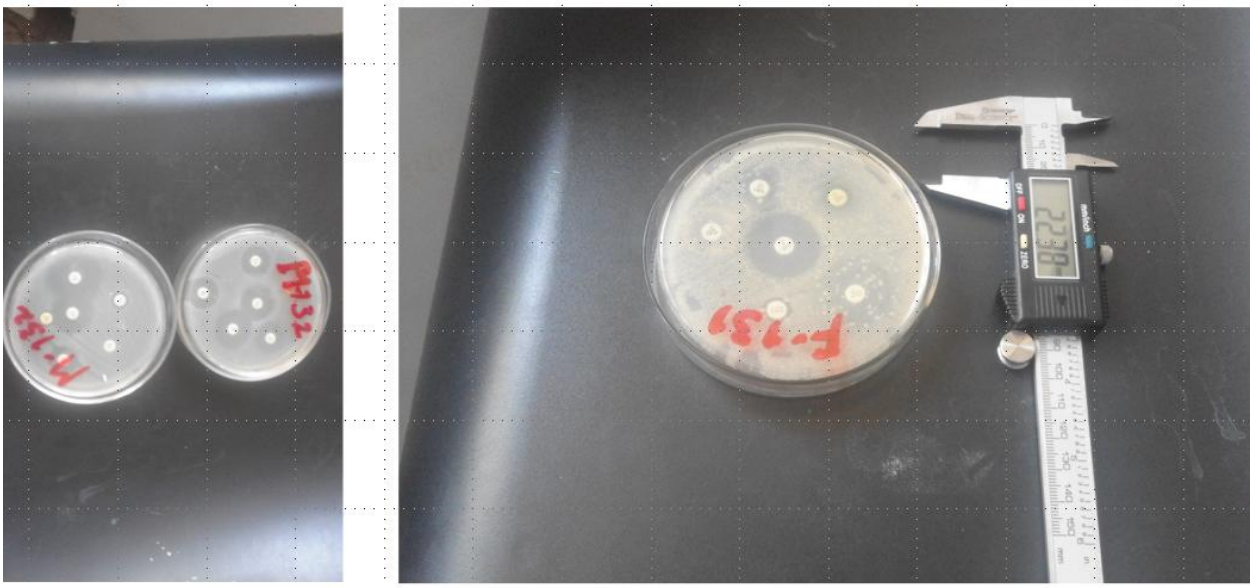


Figure 7: Antimicrobial susceptibility test and zone of inhibition measured using a digital caliper

Annex 6: Potential Contaminants of the study Lakes and Fish Handling, Processing and Raw Fish Consumption Practices in the Study Area

Figure 8: Cattle and Cart Horse roaming around Lake Dambel, Batu, Ethiopia: as observed during the study period.

Figure 9: Cattle drinking Lake Babogaya, Bishoftu, Ethiopia: as observed during the study period

Figure 10: Young fish processors processing fish at unhygienic landing site at the shore of Lake Dambel, Ethiopia

Figure 11: An interview with raw fish meat consumers at the shore of Lake Dambel during the study period.

Annex 7 : Questionnaire for Information Appraisal along the Fish Supply Chain and in Diarrheic Patients

A Questionnaire prepared to assess fish hygienic handling practices of fishermen that potentially leads to contamination of fish with *E. coli* O157: H7

Basic Information

Date: _____

Study Site: _____

Respondent ID: _____

Part 1: Socio-demographic characteristics

1. **Sex:**

   A. Male B. Female

2. **Age**: _____ (yrs)

3.**Educational level:**

   A. Illiterate D. 2^0 education

   B. Adult education E. 3^0 education

   C. 1^0 education

4.1^0 sources of income _____?

Part-2: Hygienic handling practices of fish meat

1. For how long have you been engaged in fishing? _____ (yrs)
2. How often do you catch fish per week?

   A. Once C. Three times

   B. Twice D. Every day

3. How many fishes do you catch per fishing day? _____
4. For what purpose do you catch a fish?

   A. For personal consumption B. For income generation

5. If you catch a fish for income generation, where do you sell your fishes?

   A. At the lake shore C. In the open market

   B. At retailer shops D. Hotel

6. Who are your major customers?

A. Consumers
C. Hotels
B. Retailer shop owners
D. Fish traders

7. What is the price of a kilo of raw fish meat in your town /village?

A. During fasting _____ (ETB)

B. During non-fasting _____ (ETB)

8. What is the preference of your customers on forms of fish (whole fish or processed)?

A. Whole fish
B. Processed fish

9. If your customers prefer a processed fish, who will process the fishes?

A. I will process by myself
B. I will give to other people who will process

10. If you process the fish by yourself, where does the processing takes place?

A. At home
B. At the lake shore

11. What about if others process?

A. At home
B. At the lake shore

12. How do you clean equipment like cutting board, knife and other related materials used for fish processing?

A. Using water of the lake from which the fishes are harvested with no detergent

B. Using potable water and detergents

13. Do you clean the equipment before and in between each processing (using sterile equipment for each processed fish)?

A. Yes
B. No

14. Do other processors thoroughly clean the equipment before and in between processing fishes using potable water and detergents?

A. Yes
B. No

15. If you process the fishes at home or supply to the market or retailer shops, how do you transport them?

A. In a sack
C. Hanging on hand without container
B. In a crate
D. On a cart or Bajaj without container

16. From the three fish processing stages (pre-processing, 1^0 processing, 2^0 processing), at which stage do you supply the fish for your customers?

A. Pre-processed fish
C. 2^0 processed fish
B. 1^0 processed fish
D. Pre and 1^0 processed fish

17. Do you use an ice box or any other cold chain facility for fish transportation?

A. Yes
B. No

18. Do cattle graze around the lake from where you are catching fish?

A. Yes
B. No

19. Do people living or working around the lake have the trend of open defecation?

A. Yes
B. No

20. Do the lake directly accessible to run-off water?

A. Yes
B. No

Thank you for your time and critical attention!

Questionnaire to assess the consumption preference and hygienic practices of fish meat consumers that potentially leads to contamination of fish meat with *E. coli* O157: H7

Basic Information

Date: _____

Study Site: _____

Respondent ID: **_____**

Part I: Socio-demographic characteristics

1. **Sex:**
   A. Male
   B. Female
2. **Age**: _____ (yrs)

3.Educational level:

A. Illiterate
B. Adult education
C. 1^0 education
D. 2^0 education
E. 3^0 education

4. **1^0 sources of income _____?**
5. **Religion_____?**
6. **Occupation_____?**

Part-2: Fish meat consumption and hygienic handling practices

1. Do you like a fish meat?
   A. Yes
   B. No
2. If yes, from where do you get the meat?
   A. From the surrounding fishermen
   B. From a retailer shop
   C. From restaurants
3. How do you transport the meat to your home?
   A. In a sack, using a public transport
   B. In a sack, using an Isuzu vehicle
   C. In a crate, using a public transport
   D. In a crate, using an Isuzu vehicle
   E. In a vehicle having a cold chain facility
4. Do you store the meat at your home?
   A. Yes
   B. No
5. If yes, where do you store?

A. In a crate, on the ground
B. In a sack, on the ground
C. In a refrigerator
D. Without a sack or crate, on the ground
E. Other, Specify_____

6. If you use a refrigerator, what is your optimum storage temperature? _____ ^{0}C
7. For how long do you store the meat? _____(days)
8. How do you prefer to consume?
   A. Raw
   B. Heat treated
   C. Both raw and heat treated
9. If you prefer a heat treated meat, do you have a separate room for fish meal preparation at your kitchen?
   A. Yes
   B. No
10. Do you have a facility to avoid a contact between a heat treated fish meat and raw fish or other raw food materials?
    A. Yes
    B. No
11. Do you know why the contact between a heat treated fish meat and raw fish or other raw food materials will be avoided?
    A. Yes
    B. No
12. If yes, why? _____
13. How often do you consume a fish meat?
    A. Every day
    B. Every 3 days
    C. Weekly
    D. Every 2 weeks
    E. Every 3 weeks
    F. Monthly
14. Do you know any disease of fish which can be transmitted from fish to humans?
    A. Yes
    B. No
15. If yes, what are the major means of transmission?
    A. Unhygienic handling of fish /fish meat
    B. Consumption of raw and /or under cooked fish meat
    C. Poor storage condition of fish /fish meat
    D. Poor personal and environmental hygiene
    E. Other (specify)_____
16. Have you ever encountered any diarrheic disease related to fish meat consumption?
    A. Yes
    B. No

17. Is there any one from your family who has such history?

A. Yes B. No

18. If yes, which group of your family had been encountered the case?

A. Child C. Pregnant woman

B. Adult D. Elder

19. Have you or your family member visited a health center or hospital due to such a case?

A. Yes B. No

20. If yes, what was its response to treatment?

A. Rewarding B. Refractory

Thank you for your time and critical attention!

Questionnaire to assess hygienic handling practices of fish retailers that potentially leads to contamination of fish meat with *E. coli* O157: H7

Basic Information

Date: _____

Study Site: _____

Respondent ID: _____

Part-1: Socio-demographic characteristics

Sex:

A. Male B. Female

Age: _____ (yrs)

Educational level:

A. Illiterate

B. Adult education

C. 1^0 education

D. 2^0 education

E. 3^0 education

Part-2: Hygienic handling practices of fish meat

1. Who are the major fish or fish meat suppliers for your retailer shop?
   A. Fishermen B. Fish traders
2. Which lakes are the major sources of your fish?
   A. Bishoftu lakes
   B. Koka reservoir
   C. Lake Ziway
   D. Arbaminch lakes
   E. Both Arbaminch lakes and lake Ziway
   F. Other, Specify_____
3. How do you or your suppliers transport a whole fish or fish meat from source lakes to your retailer shop?
   A. In a sack, using a public transport
   B. In a sack, using an Isuzu vehicle
   C. In a crate, using a public transport
   D. In a crate, using an Isuzu vehicle
   E. In a vehicle having a cold chain facility
4. At your retailer shop, where do you store your fish or the fish meat until it will be sold?

A. In a crate, on the ground
B. In a sack, on the ground
C. In a refrigerator
D. Without a sack or crate, on the ground
E. Other, Specify_____

5. If you use a refrigerator, what is your optimum storage temperature? _____ ^{0}C
6. For how long do you store? _____(days)
7. Who are your major customers?

A. Consumers
B. Hotels
C. Fish traders
D. Other, specify_____

8. What is the price of a whole raw fish in your retailer shop?
   a) During fasting _____(ETB)
   b) During non-fasting _____(ETB)
9. What about the price of a kilo of raw fish meat?
   a) During fasting _____(ETB)
   b) During non-fasting _____(ETB)
10. How many ETB does a roasted whole fish costs?
   a) During fasting _____(ETB)
   b) During non-fasting _____(ETB)

Thank you for your time and critical attention!

Questionnaire to assess clinical information of diarrheic patients and their exposure to *E. coli* O157: H7 via consumption of fish meat

Part -1: Basic Information

Date: _____

Residence: _____ A. Urban B. Rural

Respondent ID: _____

Sex:

A. Male B. Female

Age: _____ (yrs)

Educational level:

A. Illiterate
B. Adult education
C. 1^0 education
D. 2^0 education
E. 3^0 education

Occupation: _____

2. Clinical Information (*Taking the incubation period of E. coli O157: H7 into account***)**

2.1 Duration of diarrhea since onset _____ (days)

2.2 Consistency of diarrhea: A. Watery B. Bloody C. Mucoid D. Mixed

2.3 Maximum number of episode of diarrhea during the last 24hrs_____

2.4 Number of episode of diarrhea during the last one year_____

3. Exposure assessment

3.1 Are you a fisherman /woman or fish processor? A. Yes B. No

3.2 If yes, have you practiced fishing /fish processing in the last two weeks before illness?

A. Yes B. No

3.3 Do you have a retailer shop /restaurant? A. Yes B. No

3.4 If yes, have you made a contact with whole raw fish /raw fish meat in the last two weeks before illness? A. Yes B. No

3.5 Have you had a visit to a swimming pool (lake) in the last two weeks before illness?

A. Yes B. No

3.6 If yes, have you practiced swimming during the visit? A. Yes B. No

3.7 Have you consumed raw fish meat in the last two weeks before illness? A. Yes B. No

3.8 What is your fish meat consumption preference?

A. Raw C. Both raw and heat treated

B. Heat treated

3.9 Did you attend a large gathering like wedding ceremony the week before your illness?

A. Yes B. No

3.10 If yes, did others develop similar illness?

A. Yes B. No

Thank you for your time and critical attention!

Annex 8: Author's Biography

The author, was born on December 4, 1980, in Ilamu Arjo Salale Kebele, G/Jarso District, North Shewa Zone, Oromia, Ethiopia, from his father Debelu Bedane and his mother Toleshi Heyi. Raised in a supportive and nurturing family environment, he received strong encouragement throughout his educational and personal development.

He began his formal education in 1987 at Tinkoch Elementary School, where he completed grades 1-6. He then attended Abdisa Aga Junior Secondary School (grades 7-8) and Fiche Comprehensive Secondary School (grades 9-12). After completing his secondary education, he pursued higher studies at Addis Ababa Universit, CVMA. He earned Diploma in Animal Health in 2001, followed by DVM degree in 2010, and MSc degree in Veterinary Epidemiology in 2017. In 2019, he returned to AAU to pursue his PhD in Veterinary Public Health, further advancing his academic career.

Professionally, he has built a diverse career in veterinary science and education. His first professional role was in 2002 as an Animal Health Expert at Abichu and Gna District Office of Livestock and Fisheries, where he served for one year. Between 2003 and 2005, he worked as a private veterinary druggist, gaining practical experience in veterinary medicine. In 2011, he joined Gode ATVET College as an instructor, followed by a position of Assistant Researcher II at Ethiopian Agricultural Research Institute (EARI), Assosa Agricultural Research Center from 2012 to 2013. In 2014, he began lecturing at Addis Ababa University, Salale Campus. Since 2015, he has been serving as a Lecturer and later as Assistant Professor at Salale University, where he continues to contribute to both teaching and research in the field of veterinary science.

Throughout his career, the author has demonstrated a strong commitment for the advancement of veterinary education and research, with a particular focus on veterinary epidemiology, zoonoses and food safety. He has published 10 articles in reputable international journals, listed as follows;

1. **Tesfaye D**., Bekele M., Fufa A., Hika W.Fanos T., Muluken T., Ephrem S. and Fanta D. Occurrence, molecular characterization, and antimicrobial susceptibility of sorbitol non-fermenting *Escherichia coli* in lake water, fish and humans in central Oromia, Ethiopia. *Scientific Reports*. 2024, 14:12461. https://doi.org/10.1038/s41598-024-61810-z.

2. **Tesfaye D. B**., Getahun E.A. and Fanta D.G. Hygienic Assessment of Fish Handling Practices along Production and Supply Chain and its public health implications in Central Oromia, Ethiopia. *Sci Reports*. 2022, 12:13910. https://doi.org/10.1038/s41598-022-17671-5.
3. **Tesfaye D**., Fufa A. and Gezahegne M.K. A Preliminary Study on Public Health Implications of Avian Tuberculosis in Selected Districts of the Oromia Region, Ethiopia. Vet Med Int 2021, https://doi.org/10.1155/2021/6331599.
4. **Tesfaye D**., Fufa A., Gezahegne M.K. and Gobena A. Epidemiology of Avian Tuberculosis in Selected Districts of Oromia Region, Ethiopia. Vet Med Int 2022, https://doi.org/10.1155/2022/6933701.
5. **Tesfaye D.**, Berhanu D., Aman B. A Preliminary Study on Major Diseases of Cattle Circulating in North Shewa Zone, Oromia, Ethiopia. *Vet Med Animal Sci*. 2023, 6(1): 1124.
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9. Baradin A., **Tesfaye D**.., Alemnesh Y. Assessment on Challenges of Hide and Skin Production and Marketing in North Shewa Zone, Oromia, Ethiopia. *Journal of Biology, Agriculture and Healthcare 2022,, 12 (20):* www.iiste.org ISSN 2224-3208 (Paper) ISSN 2225-093X.
10. Yilma T., Alemayehu A., Shibeshi Z., **Tesfaye D**., Workneh T. Characterization of Goat Production System in Shifting and Permanent Farming Systems in Western Ethiopia. *International Journal of Livestock Research*, 2016 6(7): 24-37.doi:10.5455/ijlr.20160718105920.