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BAHIR DAR UNIVERSITY

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCES

SCHOOL OF ANIMAL SCIENCE AND VETERINARY MEDICINE

Department of Veterinary Science

Master of Science in Veterinary Public Health

Detection of *Salmonella* and *Escherichia coli* with its Associated Virulence Factors along the Fish Value Chain in Bahir Dar City, Ethiopia

MSc thesis

By:

Temesgen Sendekie

June, 2023

Bahir Dar, Ethiopia

BAHIR DAR UNIVERSITY

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MASTER OF SCIENCE IN VETERINARY PUBLIC HEALTH

Detection of *Salmonella* and *Escherichia coli* with its Associated Virulence Factors along the Fish Value Chain in Bahir Dar City, Ethiopia

A Thesis Submitted to Department of Veterinary Science, Bahir Dar University, in Partial Fulfillment of the Requirements for the Degree of Master of Science (MSc) in Veterinary Public Health

By:

Temesgen Sendekie

Advisor: Habtamu Tassew (DVM, MSc, Assoc. Prof)

Co-advisor: Belayneh Getachew (DVM, MSc, PHD, Assoc. Prof)

June, 2023

Bahir Dar, +Ethiopia

THESIS APPROVAL SHEET

We hereby certify that we have examined this thesis entitled "Detection of *Salmonella* and *Escherichia coli* with its Associated Virulence Factors along the Fish Value Chain in Bahir Dar City, Ethiopia" by Temesgen Sendekie Ayalew. We recommend that the thesis is approved for the degree of "Master of Science in Veterinary Public Health".

Board of Examiners

Tadesse Eguale (DVM, MSc, PHD, Assoc. Prof)	_____	_____
External examiner's name	Signature	Date
Tiliksew Bialfew (DVM, MSc, Assis. Prof)	_____	_____
Internal examiner's name	Signature	Date
Birhan Agmas (DVM, MSc, Assis. Prof)	_____	_____
Chair person's name	Signature	Date

DECLARATION

This is to certify that this thesis entitled "**Detection of *Salmonella* and *Escherichia coli* with its Associated Virulence Factors along the Fish Value Chain in Bahir Dar City, Ethiopia**" submitted in partial fulfillment of the requirements for the award of the degree of Master of Science in "**Veterinary Public Health**" to the Graduate Program of College of Agriculture and Environmental Sciences, Bahir Dar University by **Temesgen Sendekie** (ID. No. 1300404) is an authentic work carried out by him under our guidance. The matter embodied in this project work has not been submitted earlier for award of any degree or diploma to the best of our knowledge and belief.

Name of the Student Temesgen Sendekie Ayalew (DVM)

Signature & date _____ 19/06/2023

Name of the Supervisors

1) Habtamu Tassew (DVM, MSc, Assoc. Prof) (Major Supervisor)

Signature & date _____ 19/06/2023

2) Belayneh Getachew (DVM, MSc, PHD, Assoc. Prof) Co-Supervisor)

Signature & date _____

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LIST OF ABBREVIATIONS

A/E	Attaching and Effacing
AIEC	Adherent Invasive *E. coli*
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
BPW	Buffered Peptone Water
CD	Crohn's Disease
CFS	Center for Food Safety
CFs	Colonization Factors
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CNS	Central Nervous System
CSA	Central Statistics Agency
DAEC	Diffusely Adherent *E. coli*
DEC	Diarrheagenic *Escherichia coli*
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
ECDC	European Centre for Disease Prevention and Control
EHEC	Enterohamerrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELFA	Enzyme-Linked Fluorescent Assay
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Eosin Methylene Blue
EPEC	Enteropathogenic *E. coli*
EPECa	atypical Enteropathogenic *E. coli*
EPECt	typical Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
FBD	Foodborne Disease
FDA	Food and Drug Administration

HACCP	Hazard Analysis Critical Control Point
HUS	Hemolytic Uremic Syndrome
IECs	Intestinal Epithelial Cells
IMVIC	Indole, MR-VP and Citrate Utilization Test-
LEE	Locus of Enterocyte Effacement
LT	Labile Toxin
MDR	Multidrug Resistance
MH	Mueller-Hinton Agar
MR-VP	Methyl Red and Voges-Proskauer test
NTS	Non typhoidal *Salmonella*
NVI	National Veterinary Institute
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
RV	Rappaport-Vassiliadis
SSNCISM	*Salmonella* Subcommittee of the Nomenclature Committee of the InternationalSociety for Microbiology
ST	Stable Toxin
STEC	Shiga Toxigenic *E. coli*
Stx	Shiga Toxin
TCC	Total Coliforms Count
TVC	Total Viable Count
TTSS	Type Three Secretion System
VRB	Violet Red Bile Agar
VTEC	Verocytotoxigenic *E. coli*
VTs	Verotoxins
WASH	Water, Sanitation, and Hygiene
WHO	World Health Organization
WOAH	World Organisation for Animal Health
XLD	Xylose Lysine Deoxycholate

Detection of *Salmonella* and *Escherichia coli* with its Associated Virulence Factors along the Fish Value Chain in Bahir Dar City, Ethiopia

Temesgen Sendekie[1], Habtamu Tassew[1], Belayneh Getachew[2]

[1] Department of Veterinary Science, College of Agriculture and Environmental Science, Bahir Dar University, Ethiopia

[2] National Veterinary Institute, Ethiopia

ABSTRACT

Fish meal is clearly a possible source of foodborne diseases with *Salmonella* and *Escherichia coli* are among the primary pathogens. A cross-sectional study was undertaken to identify *Salmonella* and *Escherichia coli* and its virulence genes along the stages of the fish value chain in Bahir Dar City, Ethiopia. Purposive sampling was used to collect 121 samples comprising of fresh fish, retailing fish, filleted and cooked fish, swabs from processing equipments and the hand of food handlers, and water samples. Both culture based and molecular methods were used for detection. There was a significant difference between the four stages of fish value chains towards all types of counts. *Escherichia coli* and *Salmonella* were isolated from 41 (33.88%) and 6 (4.96%) of the samples, respectively. The highest *E. coli* isolation rate was from retailing fish 16 (80%) at retailers whereas the highest *Salmonella* isolation rate was found from filleted tissue 2 (20%). At restaurants, 12 (30%) and 3 (7.5%) of the samples were positive for *E. coli* and *Salmonella*, respectively. Among the fish, *Labeobarbus* spp was the one in which the leading *Escherichia coli* isolation rate 8 (50%) was observed. All 41 *E. coli* isolates were resistant to amoxicillin/clavulanate whereas no resistance was shown for Gentamicin and Amikacin at a given dose. Two thirds of *salmonella* isolate and 95.12% of *Escherichia coli* were detected as they develop multi-drug resistance. The highest rate of resistance was recorded for Ceftazidime against all (n=6) isolates of *Salmonella* species while Ciprofloxacin and TMP-Sulfa were identified as having no resistance. From a total of 10 *E. coli* isolates tested by PCR, four were positive for two virulence genes (hlyA1 and eae). The study provides the presence of potential biological hazards along the value chain. Therefore, the hygiene of fish handlers and their working environment and proper fish cooking are highly valuable. One health campaign should be carried out on drug resistance, contamination of the lake, and fish safety issues.

Key words*; Bahir Dar, Fish, Hand swab, Retailers, Virulence genes, Lake Water,*

Chapter 1. INTRODUCTION

1.1. Background and Justification

In underdeveloped nations, fishing is a major contributor to livelihood, food security, and income. Fish has the potential to lessen the triple burden of malnutrition, which is a persistent problem in low- and middle-income nations (Hasselberg *et al.*, 2020).Due to its nutritional content, which includes high-quality protein, omega-3 fatty acids, necessary vitamins, and minerals, fish is becoming more and more recognized by people all over the world as a healthy dietary component (Iwamoto *et al.*, 2010).

When a food supply is ample, secure, sustainable, resilient, and sound, aquatic food security has been attained. A safe food supply is one that presents customers with little risk to their health. Scientifically proven safe levels of contamination with human pathogens, chemicals, or radionuclides indicate that a food supply is safe. Additionally, aquatic products must be harvested, handled, treated, processed, stored, and prepared in a way that prevents contamination or the development of microorganisms. Pathogens and biotoxins are the primary biological dangers to consumers of aquatic foods, and when taken in excess of threshold levels, they can cause illness (Jennings *et al.*, 2016).

The risk of consuming contaminated food affects billions of people worldwide. Numerous thousands of people get sick or pass away every year as a result of eating high-risk, unclean foods. Because of this, food safety is lifesaving. Mild gastroenteritis to neurological, hepatic, or renal disorders might all be considered diseases. The majority of serious and fatal foodborne infections are caused by bacteria that are present in food. Having access to safe food not only makes people and the general population healthier, but it also helps the economies of the areas where it is available. *Salmonella* and *E. coli* species together with *Staphylococcus, Clostridium, Campylobacter, Listeria*, *Vibrio*, and *Bacillus* are causative agents for more than 90% of food poisoning diseases (Olaleye and Abegunde, 2015; Fung *et al.*, 2018).

According to the World Health Organization (WHO), foodborne illnesses are prevalent throughout much of the world, causing at least 1 in 10 people to become ill each year and 420, 000 fatalities (WHO, 2022) with the highest mortality rate in Africa (Havelaar *et al.*, 2015). Due to their widespread distribution throughout the world, foodborne pathogens are considered a significant risk factor for public health in both developed and developing

countries (Ekici and Dümen, 2019). The top five causes of illness and mortality in the majority of developing nations are still gastrointestinal diseases, and eating unwholesome food plays a significant role in this unnecessary burden (Roesel and Grace, 2014).

The surfaces of handling equipment, as well as storage and washing water inside the production environment come into contact with the fish throughout the value chain, from capture to landing and processing. Microorganism contamination during this interaction could happen due to the water, the staff, or insufficient cleaning practices (Svanevik and Lunestad, 2011).

Foodborne infections can be found throughout the supply chain, and risk-based approaches place a lot of emphasis on figuring out where these pathogens come from, how they behave during the manufacturing and processing of meat, and where they emerge from. Greater understanding of the causes and locations of foodborne outbreaks is now possible thanks to recent advancements in molecular and genetic based technology, data analysis, and source attribution research. Non typhoidal *Salmonella* and enteropathogenic *Escherichia coli* were determined to be the two most common foodborne illnesses, with portions of Africa and South East Asia having the largest disease burden per capital (Fegan and Jenson, 2018).

More than 15% of instances of food poisoning that are reported each year could be caused by pathogenic *E. coli* and *Salmonella* serovars. Customers of restaurants and street food are at danger of contracting enteropathogens through poor hand hygiene and food handling, which may result in fecal contamination of foods by hands during meal preparation, especially in low-income nations (Takalkar *et al.*, 2010; de Freitas *et al.*, 2019; Nizame *et al.*, 2019). A fish's hygienic status is determined by the presence of *Enterobacteriaceae* such as *E. coli*, which serve as markers of possible pathogen presence (Jay *et al.*, 2005). As a result, the likelihood of contamination is greatly influenced by the health of the people handling the food, their personal hygiene, and their understanding and observance of food hygiene regulations (Mama and Alemu, 2016).

In Ethiopia only few studies have been conducted to reveal the occurrence of *Escherichia coli* and *Salmonella spp* in fish. The bacteria *E. coli*, *E. coli* O157:H7 (predominant and most virulent serotype of *Escherichia coli*) and *Salmonella* spp from fish were reported

(Anwar, 2007; Assefa *et al.*, 2019; Tilahun and Engdawork, 2020; Birhan *et al.*, 2022b; Halo *et al.*, 2022).

For the purpose of developing and putting into practice efficient treatment, control, and prevention methods, evidence of molecular-based assessment of bacterial profile and their antibiotic susceptibility pattern is crucial. Molecular approaches have become the confirming method for identification in many diagnostic applications, even if culture-based methods are still regarded as the gold standard for identification and diagnosis. Polymerase chain reaction (PCR), a Deoxyribonucleic acid (DNA) based technique, is quicker and more informative than culture-based techniques (Mangal *et al.,* 2016). Over the years few studies have been conducted on the detection of the hygienic status of fish value chain with a special emphasis to results of biochemical assessment.

1.2. Statement of the Problem

Fish processing takes place at Lake Tana mostly in a traditional system, on bare ground in unsanitary conditions for gutting and filleting with little to no consideration for safety. The fish are gutted and cleaned before being carried to local markets, restaurants, and hotels in Bahir Dar City. Fish processing is not meeting the criteria required for hazard analysis critical control point (HACCP) system compliance (Mengistu *et al.*, 2017a).

Fish meal is a possible source of foodborne zoonoses especially developing countries like Ethiopia due to the frequent practice of eating raw or lightly cooked fish meal, as well as the unfavorable facilities for slaughter, filleting, transport, and storage of fish (Haile and Getahun, 2018). Fish-borne zoonoses are among the country's most neglected fish diseases (Sorsa *et al.,* 2019). Foodborne infections such as *E. coli* O157:H7 and *Salmonella* spp. are frequently connected with foods (Elizaquível *et al.,* 2012).

In the study conducted at the upper Blue Nile River watershed in Northwest Ethiopia, *E. coli and Salmonella* are among the contaminants of fish in the study area, and their occurrence in fish could represent a risk to consumers (Birhan *et al.*, 2022b). Foodborne pathogens were identified using routine culture and biochemical test-based diagnostic techniques which implies there is a high need for advanced laboratory protocols to enhance the diagnosis and detection of foodborne disease (FBD) in Ethiopia (Belina *et al.*, 2021). Further studies such as molecular works on *Escherichia coli* is recommended to

show the public health importance of the pathogen (Halo *et al.*, 2022). Although biochemical asessment methods are relatively inexpensive and sensitive however the main drawback of these methods is their long analysis time and their labor intensiveness (Biswas, 2005; Zhao *et al.*, 2014), and low selectivity (Kim and Kim, 2021).

The study of virulence factors and the genes that encode them can provide explicit knowledge about the interactions that occur at the molecular level between these factors in enteric *E. coli* pathotypes and host proteins, indicating how they cause diseases and allowing for the implementation of preventive measures (Kaper *et al.*, 2004).

Therefore, using molecular techniques in addition to the conventional type will help to better identification of the foodborne bacteria along the fish value chain. According to the information currently available, only a few studies have been undertaken on the investigation of contamination of potential public health major bacterial infections of the fish value chain in Bahir Dar. Furthermore, the antimicrobial resistance (AMR) pattern of those possible pathogens in the fish value chain, as well as the source and level of contamination, needs also be addressed. Molecular identification of the common enterobacteria from the fish value chain will have a paramount significance to show the pathogenicity of bacterial fish diseases and having a plan to control and prevention strategy.

1.3. Objectives

1.3.1. General Objective

- To identify *Salmonella* and *Escherichia coli* with its common virulence genes and their antimicrobial resistance pattern along the stages of the fish value chain in Bahir Dar city, Ethiopia.

1.3.2. Specific Objectives

- To determine the bacterial load along each stages of the fish value chains in Bahir Dar City.
- To assess the prevalence of *Escherichia coli* along the stages of the fish value chains.
- To estimate the pevalence of *Salmonella* species along the fish value chains.
- To determine the antimicrobial susceptibility profile of *E. coli* and *Salmonella* isolates.
- To detect the virulence genes of *Escherichia coli* along the fish value chain.

1.4. Research Questions

1. How much is the level of bacterial load along the fish value chain in Bahir Dar City?
2. What is the prevalence of *Escherichia coli* along each stages of the fish value chain?
3. What is the prevalence of *Salmonella spp*. along each stage of the fish value chain?
4. What is the antimicrobial susceptibility profile of *Salmonella spp.* and *Escherichia coli* t commonly used drugs?
5. Which virulence genes are present among *E. coli isolates* along the fish value chain?

Chapter 2. LITERATURE REVIEW

Salmonella and *Escherichia coli* are among the predominant pathogens responsible for most meat and meat product related outbreaks (Omer *et al.*, 2018).When a healthy fish is caught, its muscle tissue is presumed to be sterile, but in reality, germs are typically present on the fish's skin, gills, and other external surfaces, as well as in the digestive tract (Painter *et al.*, 2013). *Escherichia coli* and other members of the coliform group are indicators of fecal contamination, unsanitary settings, or the presence of enteric pathogens in food. It is a significant pathogen because it signals the presence of feces-related contamination in food. It is regarded as an indicator bacteria in food safety and hygiene because of this feature (Uçar *et al.*, 2015). *Salmonella* spp. and enteropathogenic *E. coli* contamination of food goods has detrimental effects on both health and the economy. Typhoid fever, paratyphoid fever, and food poisoning are all brought on by bacteria under the genus *Salmonella* (Butaye *et al.*, 2006).

2.1. *Escherichia coli*

2.1.1. General Characteristics

Escherichia coli is a gram-negative, facultatively anaerobic rod that bears the name of the German-Austrian pediatrician Theodor Escherich. Escherich used his own anaerobic culture techniques and Hans Christian Gram's novel staining approach to extract a wide range of bacteria from newborn fecal samples. The common colon bacillus was initially given the name *Bacterium coli* commune by Escherich. *Escherichia coli* was first given that name by Castellani and Chalmers in 1919, but it wasn't formally accepted until 1958 (Etymologia, 2015).

Escherichia coli is a common cause of gastrointestinal illness in both humans and animals. It can cause an illness ranging from mild diarrhea to situations that resemble dysentery in immunosuppressed patients or when the gastrointestinal walls are conquered (Ryan and Ray, 2004).

2.1.2. Taxonomical Classification

Escherichia coli, one of the 30 members of the bacterial family of *Enterobacteriaceae,* is a coliform bacterium and is one of the 6 types of *Escherichia* species (*E. adecaroxylate, E. blattae, E. fergusonii, E. hermannii* and *E. vulneris*). It is a gram-negative, non-spore-forming, facultative, anaerobic, rod shaped, mesophilic bacterium that grows in 7-45°C (Croxen *et al.,* 2013).

Escherichia coli can be divided into more than 190 serogroups (serotypes) based on its surface antigens (i.e., somatic O antigen and flagellar H antigen), with the serotype of an *E. coli* isolates being determined by a particular combination of O and H antigens (for example, O157:H7). In many instances, a heat-labile, acidic polysaccharide capsule (K-antigen) "masks" the O antigen, which is a component of the lipopolysaccharide layer. Although some serogroups may be involved in more than one category of *E. coli* pathotypes, certain clinical symptoms are frequently characteristically associated with specific serogroups (Schmidt, 2019).

According to their pathogenicity profiles (virulence factors, clinical symptoms, and phylogenetic profile), enteric *E. coli* infections are generally categorized into 6 pathotypes: Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohamerrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC, including Shigella sp), and Diffusely Adherent *E. coli* (DAEC) are some types of enteropathogenic *E. coli* (Clements *et al.*, 2012).

Later, 9 distinct enteric *E. coli* pathotypes have been identified; they are known to cause illnesses ranging from gastrointestinal problems to infections of the urinary tract. There are seven pathotypes in all, including the conventional six pathotypes and a novel pathotype called Adherent-Invasive *E. coli* (AIEC), which primarily causes diarrhea and intestinal problems. To mediate their virulence and pathogenesis, these pathotypes use a variety of virulence factors and effectors that interfere with the functions of host cells (Pakbin *et al.*, 2021). Shiga toxin 1 and 2 *(*stx1 and stx2) [*Shiga toxigenic E. coli* (STEC)], stx1, stx2, and eae (EHEC), eae [atypical Enteropathogenic *E. coli* (EPECa)], eae and bfp [typical Enteropathogenic *E. coli* (EPECt)], enterotoxin genes LT (labile toxin) and ST (stable toxin) and colonization factors (ETEC), ipaH and ial (EIEC), and aggR, aap, and AA probe (EAEC) are the genes used for genetic characterization of these groups (Croxen

et al., 2013). The pathotypes have been proposed based on their infective mechanisms, including toxin production, adherence effects, and tissue damage. It is also evidenced that environmental stresses, including bacterial exposition to antibiotics and disinfectants, contribute to this evolution (Enciso-Martínez *et al.*, 2022).

2.1.3. The pathotypes of *Escherichia coli*

Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *Escherichia coli* are defined as diarrheagenic strains of *E. coli* that can produce attaching and effacing lesions on the intestinal epithelium. All the EPEC strains are *eae* positive; typical EPEC strains are both *eae* and *bfp*A positive, while atypical strains are *bfp*A negative. There is no vaccine currently for prevention mechanism against EPEC infections (Mare *et al.*, 2021)

Enterohaemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) is a subset of the *E. coli* pathotype STEC (Shiga toxigenic *E. coli*). These bacteria produce Shiga-like toxins, which are also known as verotoxins due to their action against cultivated Vero cells (Alberto *et al.*, 2022). The presence of genes that produce the Shiga toxin (Stx), which is carried by a prophage, characterizes Shiga toxigenic *E. coli* (STEC). A pathogenicity island (PAI) shared with EPEC, the locus of enterocyte effacement (LEE) is present in the majority of EHEC strains. The enterohaemolysin gene is a plasmid-borne gene that is present in EHEC strains. Therefore, common EHEC strains use lysogenic phage, plasmid, and PAI acquisitions to show the main methods of virulence gene acquisition *(*Denamur *et al*., 2021).

A protein called intimin, which is responsible for the bacteria's close attachment to intestinal epithelial cells, causes attaching and effacing lesions in the intestinal mucosa, and facilitates the bacteria's attachment and colonization in the intestinal wall, is one of the many putative virulence factors that *E. coli O157* produces in addition to its toxin (Dhaka *et al*., 2016). The locus of enterocyte effacement (LEE) is a pathogenicity island that contains the chromosomal gene eaeA which codes for intimin. Haemolysin, which is produced by the hlyA gene and has the ability to lyse red blood cells and release iron to

assist *E. coli* metabolism, is another element that may also have an impact on the virulence of *E. coli O157* (Elvis *et al*., 2020).

Enterotoxigenic *Escherichia coli (ETEC)*

In underdeveloped nations, enterotoxigenic *Escherichia coli* (ETEC) is a major contributor to diarrhea in children and tourists. It's capacity to create important virulence factors, like enterotoxins and colonization factors (CFs), which bind to specific receptors on epithelial cells and cause diarrhea (Zhang *et al*., 2022). In areas with limited resources, enterotoxigenic *E. coli* (ETEC) frequently causes diarrhea by producing heat-labile (LT) and/or heat-stable (ST) enterotoxins (Feng *et al*., 2020).

Enteroinvasive *Escherichia coli* (EIEC)

Although this may be related to the methods employed to detect these organisms, EIEC appears to be a less frequent cause of diarrheal sickness than other *E. coli* infections. They cause a diarrheal illness that is identical to shigellosis. EIEC appears to be the main factor in occasional occurrences of diarrheal illness. *Shigella* spp. and the EIEC are closely related, and the EIEC uses genetic material identical to *Shigella* to encode virulence components such as the type three secretion systems (TTSS). The EIEC enters the intestinal cell, multiplies intracellularly, and extends into neighboring intestinal cells via virulence proteins known as "invasins." Cell death and occasionally bloody diarrhea result from this process. The majority of EIEC disease manifests clinically as watery diarrhea and is difficult to recognize at the bedside from the many other causes of diarrhea (Poolman, 2017).

Enteroaggregative *Escherichia coli* (EAEC)

Children in low-income nations are most commonly affected by enteroaggregative *Escherichia coli* (EAEC; formerly known as "EAggEC"), which is also linked to traveler's diarrhea in children and adults in middle- and high-income countries. Since EAEC is so diverse, not all strains are pathogenic. Although there is evidence of foodborne transmission of EAEC in the literature, there is currently no proof of a zoonotic reservoir (Jenkins, 2018).

Diffusely Adherent *Escherichia coli* (DAEC)

The term "diffusely adherent *Escherichia coli*" (DAEC) pathogroup refers to a diverse group of *E. coli* strains that are capable of infecting otherwise healthy people with diarrhea due to the presence of genes encoding for Afa/Dradhesins. It causes distinctive changes in epithelial cells that lead to diarrheal sickness and epithelial damage, which may also hasten the onset of other intestinal illnesses (Meza-Segura and Estrada-Garcia, 2016).

Adherent Invasive *Escherichia coli* (AIEC)

The adherent-invasive *Escherichia coli* (AIEC) pathobiont bacteria can adhere and invade intestinal epithelial cells (IECs), as well as to survive and replicate within macrophages. It is among types of pathotype of *Escherichia coli* involved in the pathogenesis of Crohn's disease (CD), abnormally colonizing the intestinal mucosa of patients. Numerous studies have been conducted on AIEC in recent years to identify some genes associated with AIEC pathogenicity, comprehend the effects of AIEC infection on the gut, and as a result, their role in CD (Chervy *et al.*, 2020).

2.1.4. Pathogenesis and Public Health Importance of *Escherichia coli*

More than 30% of diarrheal infections in children under the age of five are caused by diarrheagenic *Escherichia coli* (DEC), a significant bacterial agent for infants, children, and overseas visitors (Salleh *et al.*, 2022). The most studied and dangerous serotype is *E.coli* O157:H7, a pandemic clone capable of causing severe and highly significant public health concerns. After entering the human gut, EHEC causes attaching and effacing (A/E) lesions (pedestal-like structures) on the mucosal epithelium, allowing close contact between the bacteria and the cell and the subsequent production of Shiga toxins. A pathogenicity island known as the locus for enterocyte effacement (LEE) encodes a total of 41 genes that are implicated in the development of A/E lesions. Inhibiting protein synthesis, the Shiga-like toxins Stx1 and Stx2 can kill cells, slough off the mucosa, cause inflammation, lead to intestinal vasculitis, and finally result in bloody diarrhea. In people, EHEC can develop into a potentially fatal disorder known as HUS (Hemolytic uremic syndrome), which results in anemia and acute renal failure (Alberto *et al.*, 2022).

Along with renal disorders, Verotoxins (VTs) are also to blame for systemic difficulties affecting the central nervous system (CNS). Doctor Kiyoshi Shiga, who discovered

Shiga's bacillus as the source of a dysentery outbreak in Japan in 1897, is honored by the toxin's name (Terajima *et al*., 2017). The global significance of *E. coli* O157:H7 as a public health issue has increased. The primary natural host of VTEC is ruminants, which are typically healthy hosts for pathogens (WOAH, 2018).Only stx1 and stx2 appear to be significant in human infections out of the six verotoxins found in enterohemorrhagic *E. coli* (EHEC). The predominant serotype of this family is *E. coli* O157:H7. Shigellosis-like diarrheal disease is brought on by enteroinvasive *E. coli* (EIEC). Traveler's diarrhea and baby diarrhea in underdeveloped nations are frequently brought on by enterotoxigenic *E. coli* (ETEC). These strains release either a heat-stable toxin (ST) or a heat-labile toxin (LT). A significant contributing factor to baby diarrhea is enteropathogenic *E. coli* (EPEC) (MS *et al.*, 2018).

Shiga toxins are protein molecules that attach to eukaryotic surface cells and block protein synthesis, which results in the death of the host cells. Another virulence component called intimin is encoded by the attaching and effacing eae gene. According to reports, intimin helps bacteria connect to intestinal epithelia during colonization, which causes sores and diarrhea. Another virulence factor for *E.coli* O157:H7is enterohaemolysin. This protein toxin disrupts the erythrocytes' cell membranes and is used to identify *E. coli* strains that produce the shiga toxin (Lupindua, 2018) as shown in table 1.

Table 1. The features of the commonest virulence genes of *Escherichia coli* with respective pathotypes (FDA, 2020)

Properties/Symptoms	**EPEC**	**EHEC**
Toxin	*No*	Shiga or Vero toxin *(*Stx or VT*)*
Intimin	*Yes*	*Yes*
Enterohemolysin	*No*	*Yes*
Stool	Watery, Bloody	Watery, very bloody
Fever	*Yes*	*No*
Intestine involved	Small	Colon
Serology	O26, O111 & others	O157:H7, O26, O111 & others
Infective dose	High	Low

Constipation and stomach pains are signs of STEC-related illnesses, which can occasionally lead to hemorrhagic colitis (bloody diarrhea). The infection may cause a life-threatening condition like hemolytic uraemic syndrome (HUS) in a tiny percentage of individuals (especially young children and the elderly). Patients with STEC disease are not given antibiotics as part of their medical therapy, which could potentially raise their chance of developing HUS later. Additionally, STEC has been isolated from water sources and it has been discovered that it may persist for months in manure and water-trough sediments (WHO, 2018a).

2.1.5. The occurrence of *Escherichia coli* in Fish and Fish products

Due to poor living conditions (inadequate water supplies, poor environmental hygiene and sanitation, and inadequate education), low- and middle-income countries in Africa, Asia, and Latin America are the most affected regions. Diarrheal infections are more common and can be fatal in these countries (Croxen *et al.*, 2013) as shown in table 2.

Table 2. Studies done on *Escherichia coli* in Ethiopia and abroad

Countries	Sampling site	Samples size	Identification Techniques	Prevalence	References
Ethiopia	Fresh and filleted	510	Biochemical tests	2.4%	(Anwar, 2007)
Northwest Ethiopia	Food chain	180	Biochemical tests	20%	(Halo *et al.*, 2022)
			Molecular test for stx1 and 2	72.22% & 51.85%	
Mekelle, Ethiopia	Retailer Shops	96	Biochemical tests	9.4%	(Awot *et al.*, 2019)
Hawassa, Ethiopia	Fresh fish	19	Biotechnical tests	21%	(Eromo *et al.*, 2016)
		343	slide agglutination test	23.3%	(Tilahun and Engdawork, 2020)
Zeway Lake, Ethiopia	Fresh Fish	100	Biochemical Tests	12%	(Haile and Getahun, 2018)
Morocco	Ready to eat	60	Biochemical Tests	30%	(Badri *et al.*, 2009)
			Molecular test for Virulence genes	0%	
Ghana	Value Chain	65	Biochemical Tests	6.1 %	(Aboagye, 2016)
India	Value Chain	18	Biochemical Tests	55.56%	(Sahu *et al.*, 2016)
Tanazania	value chain	780	Matrix-assisted laser desorption/ionization time-of-flight (MALDITOF)	8.6%	(Rabia *et al.*, 2017a)
Northeast Algeria	Retailer	100	Molecular detection	32%	(Dib *et al.*, 2018)
Seoul, Korea	Retailer	971	Biochemical Tests	7.6%	(Ryu *et al.*, 2012)

2.2. *Salmonella* Species

2.2.1. General Characteristics

Salmonella was named after Daniel E. Salmon, a veterinarian who first identified *"Bacillus choleraesuis"* (as it was known at the time) (Salmon and Smith, 1885) before changing its name to *"Salmonella choleraesuis*" in 1900 (SSNCISM, 1934). *Salmonella* is a widespread bacterium that can persist in soil, water, and food for several weeks (WHO, 2018b). *Salmonella* species is a bacterium that causes serious foodborne infections. It is not biological contamination that was originally noticed in fish, but it can enter the fish production chain through improper handling, poor hygiene, or contact with contaminated

water. Since fish, an important food item in international commerce and frequently exported to numerous countries might operate as a vehicle for *Salmonella* transmission throughout the production chain, fish microbiological safety is a concern for consumers, industries, and regulatory authorities worldwide (Fernandes *et al.*, 2018).

2.2.2. Taxonomic Classification

Salmonella includes a sizable and closely related community of pathogens with clinical and veterinary importance. Global public health difficulties are brought on by a number of diseases caused by this genus, including typhoid fever and salmonellosis (a variety of disorders including gastroenteritis). *Salmonella*'s nomenclature system is extremely complicated and constantly changing, making it difficult for doctors, veterinarians, and microbiologists to understand (Ryan *et al.*, 2017). *Salmonellae* are widespread bacteria that affect both humans and animals, although their nomenclature is debatable. Despite the complexity of the *Salmonella* nomenclature, *Salmonella enterica* was officially recognized as the type species of the genus *Salmonella* in 2005. *Salmonella bongori* is the additional species in the genus *Salmonella* that has been accepted and recognized (Agbaje *et al.*, 2011).

Salmonella enterica is classified into six subspecies: *enteric* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). The vast majority of these infections in humans and domestic animals are caused by subspecies I (Popoff *et al.*, 2004; Lu *et al.*, 2022). Serovars *S. Typhimurium* and *S. Enteritidis* are responsible for the majority of human salmonellosis illnesses linked to fish eating (Fernandes *et al.*, 2018).

Salmonella enterica is divided into more than 2,500 serotypes based on the antigenic variants (O, H1, H2, and Vi), of which over 1531 serovars are associated with the host-specific, clinically significant *Salmonella enterica subsp. Enteric* (Porwollik *et al.*, 2004).

2.2.3. Pathogenicity of *Salmonella*

The serotype involved and the state of the human host both affect how severe a *Salmonella* infection is in people. Immunosuppressed patients, elderly persons, and young children are more vulnerable to *Salmonella* infection than healthy people. Almost all *Salmonella* strains are harmful because they can enter, multiply, and survive in human host cells, causing potentially lethal illnesses (Eng *et al.*, 2015).

According to their capacity to cause particular human diseases, *Salmonella* species can be divided into typhoid and non-typhoid (NTS) types (Hendriksen *et al.*, 2011; Gal-Mor *et al.*, 2014). These two groups cause quite diverse diseases and immunological reactions in people despite sharing many genetic traits. The foundation for the variety of disease symptoms is starting to be explained by comparative analysis of the genomes of various *Salmonella* serovars. The ability to replicate intestinal gastroenteritis and enteric fever in mice has advanced, making it easier to study the bacterial and host-mediated mechanisms underlying salmonelloses. The comprehension of *Salmonella* pathogenesis, host immunity, and the molecular basis of host specificity will be improved by knowing the genetic and molecular pathways responsible for variations in illness outcomes (Gal-Mor *et al.*, 2014).

When *Salmonella* species other than *Salmonella Typhi* and *Salmonella Paratyphi* are responsible for enteric illnesses, they are typically referred to as "salmonellosis" (ECDC, 2023). Non-typhoidal *Salmonella* (NTS) infections in people are steadily rising, which is a significant global health issue (Jacob *et al.*, 2020). *Salmonella* species are primarily spread through tainted food and water (Ryan and Ray, 2004; Butaye *et al.*, 2006). This disease primarily comes from animals, and eating animal products is the major way for people to contract it (Ferrari *et al.*, 2019). The number of bacteria in the meal and the immunological health of the infected person are the main determinants of the incubation period and the symptoms (ECDC, 2023).

2.2.4. *Salmonella* in Fish

Salmonella contamination in the natural aquatic environment, in aquaculture, or during processing may be the cause of the presence of *Salmonella* in seafood. Concerns are also being raised by the identification of *Salmonella* serovars that are multi-resistant to antibiotics (Amagliani *et al.*, 2012). *Salmonella* may face difficult conditions for long-term survivability due to the natural environment surrounding irrigation water sources like rivers and lakes. Its viability may be affected by changes in physicochemical parameters (temperature, salts, pH, oxygen), the availability of nutrients, interactions with other microbes, and exposure to ultraviolet light (Wanjugi and Harwood, 2013). *Salmonella* outbreaks in water bodies are frequently traced to sewage discharges, heavy rain, or related surface run-off occurrences. Therefore, it has been demonstrated that natural or

non-recycled water sources including rivers and irrigation canals serve as reservoirs of viable *Salmonella (*Martínez *et al., 2017).*

If not for contaminated water or inappropriate handling, *Salmonella* was not previously reported to be biological contamination in fish (de Souza Sant'Ana, 2012). *Salmonella* can momentarily persist in the digestive tract of fish. Additionally, the bacterium was discovered in the fish feces. As a result, this is crucial for bacterial spread and environmental pollution (Santos, 2015). Several freshwater fish species, including tilapia, can have *Salmonella* in their stomachs, which can then invade their internal organs and muscle tissue (Nesse *et al.*, 2005).

2.2.5. Epidemiology of *Salmonella*

Although incidence rates vary by country, it is the most prevalent foodborne illness in both developing and industrialized nations (Addis *et al.*, 2011) as shown in table 3 for some of the studies.

Table 3. Summary of studies on *Salmonella* detection in Ethiopia and worldwide

Study Area	Stage of Samples	Sample size	Identification Techniques	Prevalence	References
Northwest, Ethiopia	Value chain	140	Biochemical Tests and Serology	36.4%	(Birhan *et al.*, 2022a)
Upper Blue Nile, Ethiopia	Value chain	396	Biochemical Tests	6.82%	(Birhan *et al.*, 2022b)
Hawassa, Ethiopia	Raw fish	78	Biochemical Tests	58%	(Eromo *et al.*, 2016)
Haramaya, Ethiopia	Value Chain	310	Biochemical Tests	14.5%	(Sufiyan *et al.*, 2022)
Vietnam	Value Chain	101	Agglutination Test	36.6%	(Nguyen *et al.*, 2016)
Saudi Arabia	Retailers	223	Biochemical Tests	39.9%	(Elhadi, 2014)
Nigeria	Farm and Market	200	Biochemical Tests	11.5%	(Raufu *et al*., 2014)
China	Retailer	231	…	18.6%	(Yang *et al*., 2015)
Brazil	…	50	…	4.0%	(Duarte *et al.*, 2021)
		116	Biochemical Tests	3.4%	(Esposto *et al.*, 2007)
India	Value Chain	18	Biochemical Tests	49.99%	(Sahu *et al*., 2016)

2.3. Antimicrobial Resistance of *E. coli* and *Salmonella*

Antibiotics are substances that are frequently used to treat bacterial illnesses in humans as well as many agricultural sectors, such as raising cattle, growing plants and crops, and aquaculture (Thapa *et al.*, 2020). Because more resistant and multi-resistant *Salmonella* serovars are being isolated, there is a problem with antibiotic abuse (Fernandes *et al.*, 2018). Resistance to at least three separate classes of antimicrobials was used to define multidrug resistance (MDR) (Magiorakos *et al.*, 2012).

Escherichia coli can spread quickly through the food chain and water in several habitats. It has been demonstrated that *Escherichia coli* exchanges genetic material with other

bacterial species, and it is feasible that this organism could transfer antibiotic resistance genes to bacterial pathogens that cause disease in humans (Alexander *et al.*, 2010). Drug resistance genes found in *Escherichia coli* have also been demonstrated, which may complicate the treatment of diarrheal disorders (Nakhjavani *et al.*, 2013). The use of antibiotics in veterinary medicine to treat bacterial infections in those animals as well as in animal feed to stimulate the growth of food animals is the main factor contributing to the formation of *Salmonella* with antimicrobial resistance (Hyeon *et al.*, 2011).

2.4. Detection of Foodborne Pathogens

There are a variety of biochemical and biological detection methods that are employed for the identification of harmful bacteria in food. The enzyme-linked immunosorbent assay (ELISA), the enzyme-linked fluorescent assay (ELFA), the lateral flow assay, and magnetic separation techniques comprise the immunologically based biochemical detection method, whereas the biological method is subdivided into two categories: culture-dependent and culture-independent methods. Methods that depend on culture are more time-consuming and labor-intensive. It takes too long to collect samples from food items (with pathogens affected), serially dilute them, and then inoculate them on media that are most suited for bacterial growth (Saravanan *et al.*, 2021).

Many different biochemical assays can be used to identify the infections. Microbes have been identified using chemical traits such as the indole production test, methyl red, triple sugar iron agar, Voges-Proskauer, citrate utilization, catalase, oxidase, starch hydrolysis, carbohydrate fermentation, and nitrate reduction test. Nucleic acid-based approaches and immunological-based methods are introduced and are in use to deliver with better accuracy and specificity (Singh *et al.*, 2013).

The development of molecular-based techniques made it simpler for microbiologists to accomplish their objectives. Rapid and multiplex detection is attributed to molecular-oriented techniques like PCR (Hameed *et al.*, 2018). Molecular biologists use the sensitive Polymerase Chain Reaction (PCR) method to amplify DNA. The addition of nucleotides is assisted by the taq polymerase, which is connected with this method. The success of this technology contributed to the large-scale, known-size, and fragmented amplification of DNA. The issue with tissues' capacity limitations has been resolved. A cutting-edge isothermal technology for the identification of several infections is a real-time polymerase

chain reaction. For the quick detection of cider and other fermented foods, multiplex PCR has been applied (Cousin *et al.*, 2019).

2.5. Prevention and Control

The discovery of common fecal coliforms like *E coli* and *Salmonella* spp. in processed fish points to the possible risk of pathogenic bacteria and/or their toxins making their way into human food supplies through contaminated and/or diseased fish products. Because of improper handling, poor hygiene, and contact with contaminated water, fish products in the fish production chain can get contaminated and cause human infection by pathogenic fish bacteria and food poisoning. Fish harvested from the research lakes must therefore be handled, processed, and consumed with caution by producers, consumers, and all other stakeholders (Dissasa *et al.*, 2022).

To identify and control foodborne issues that endanger human health, all nations and relevant international organizations must work together. Even if their biology, epidemiology, and analysis are complex, the majority of foodborne illnesses may be avoided. The public's health depends critically on consumers' and food producers' adherence to the standards generally recognized as safe practices (Ekici and Dümen, 2019). There is a significant risk of contamination throughout the entire value chain for fisheries and aquaculture. Water, sanitation, and hygiene measures can assist fisheries and aquaculture employees and communities' health and nutritional status, as well as the safety of the food products produced (World-Bank, 2022).

Controlling bacterial contamination is crucial from fish capture and handling through processing, as germs that develop on fish early in the process may stick around and negatively impact the final product's quality and safety. Based on the evaluation of the aforementioned characteristics, regular surveillance of the aforementioned crucial points along the pelagic fish production chain is a valuable measure to maximize the quality and consumer safety of pelagic fishery products (Svanevik *et al.*, 2015).

Objectives for food safety are based on preventative measures such as using safe raw materials, following appropriate production methods and having processes with essential control points for hazard assessments. The World Health Organization (WHO) and other concerned organizations identified indicators for enhancing general hygiene, and the

majority of them were under the control of producers and consumers, including poor general hygiene, consuming raw products, utilizing contaminated materials, and contamination through diseased persons (Robinson, 2014). The five principles of safer food include cleanliness, separating raw from cooked food, thorough cooking, maintaining safe food temperatures, and using safe raw materials and water (WHO, 2018b).

2.6. Fish Value Chain in Lake Tana, Ethiopia

Fisheries are recognized as a key tactic in Ethiopia's effort to achieve food security and a means of subsistence. Over 7,400 km2 of lakes and 7,000 km of rivers give the nation a producing capacity of 51,500 tons per year. But only between 30% and 38% of this potential is now utilized, and it barely makes up 0.02% of its gross domestic product (Demilew and Abebe, 2021). Since Ethiopia lacks a maritime shoreline, all of its fisheries are in fresh water, in its numerous lakes, rivers, and reservoirs. Ethiopia has a fairly low per capita intake of fish, but demand is steadily increasing due to population growth, rising incomes, and a change in preferences (Janko, 2014).

A value chain is a series of connected businesses (operators) carrying out tasks (functions) to enhance the value of a good from its initial production through its processing and marketing to its final sale to customers (Cheffo *et al.*, 2021). According to a study conducted in Lake Tana (Ayenew, 2016), Producers, wholesalers, retailers, collectors, and consumers/processors of the product are some of the different actors in the area's fish value chain, which is made up of various fish market channels. According to the study, among the five marketing channels available in the area, the producer channel predominated, followed by urban collectors, wholesalers, processors, and consumers.

At Lake Tana, fish are typically processed using a traditional manner, typically on bare ground in unhygienic conditions for gutting, filleting, or drying with little to no consideration for safety. The fish are carried and taken to local markets, eateries, and hotels in the neighboring town after being gutted and cleaned. A number of ladies travel from house to home selling fish they have purchased straight from fishermen in the lake's southern region. These ladies are the most marginalized segment of the local fishery industry (Mengistu *et al.*, 2017a)

Chapter 3. MATERIAL AND METHODS

3.1. Description of Study Area

Bahir Dar has an altitude of 1,830 meters above sea level and is characterized by a tropical climate with an average temperature of 29°C. It is located approximately 560 km northwest part of Addis Ababa (the capital city of Ethiopia)at 37° 10′ E longitude and 11° 38′ N latitude (Feleke, 2022). The city is the capital of the Amhara Region in Ethiopia (Figure 1). Administratively, Bahir Dar is a Special Zone. Bahir Dar is one of the leading tourist destinations in Ethiopia, with a variety of attractions in the nearby Lake Tana and Blue Nile River. The city is divided into nine sub-cities and 32 rural kebeles, comprising an area of 213.55 km^2. As data taken from Ethiopian Central Statistics Agency (CSA) indicated, the study area had a total population of 348,429 in 2017 (Getu and Bhat, 2022).

Lake Tana is the largest lake in Ethiopia. It embraces 50% of the country's fresh water. The resources are exclusively significant for the surroundings, industry, domestic and agricultural purposes (Worku, 2017). At 1,830 m altitude, Lake Tana is situated on the basaltic Plateau of the north-western highlands of Ethiopia covering an area of 3,050 km^2. This lake and adjacent wetlands provide directly and indirectly a livelihood for more than 500,000 people. The main tributaries to the lake are Little Nile River, Megech River, Gumara River, and the Rib River. The Blue Nile is the only outflowing river (Vijverberg *et al.*, 2009).

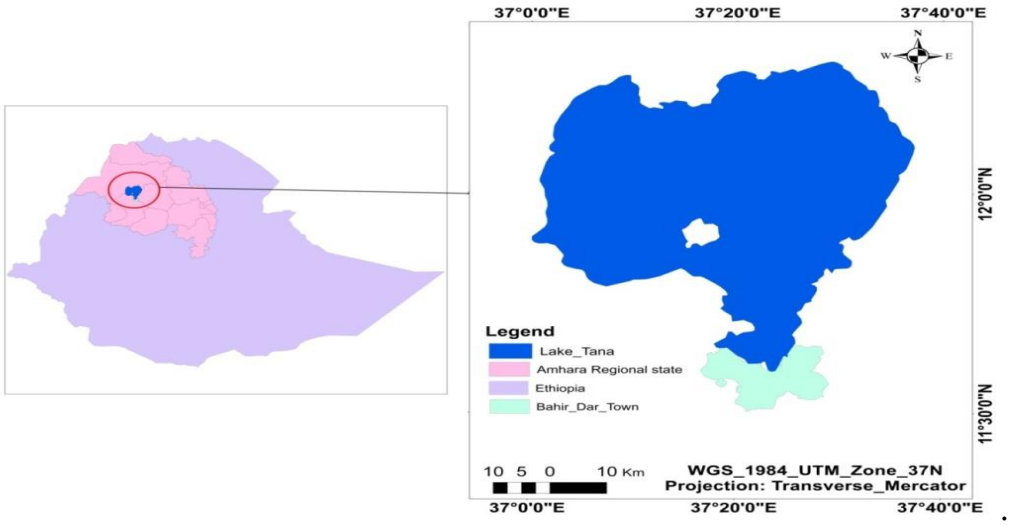

Figure 1. Arc GIS product for Map of the Study Area (Bahir Dar City and Lake Tana), (2023)

The fishes of Lake Tana show impressive diversity and unprecedented uniqueness. Fish production from capture fisheries including Nile Tilapia (*oreochromis niloticus*), Large African Barbs (*Labeobarbus* spp), and African Catfish (*clarias gariepinus*) from the lake and its influent and effluent rivers has not yet been able to satisfy the ever-growing local demand for fish. Most of the fisheries development activities are characterized by the traditional reed-raft gillnet fishery and some motorized boat gillnet fishery (Mengistu *et al.*, 2017b). The marketplace is very poor in terms of infrastructure which is liable for contamination and spoilage. The structure of the fish open market in Bahir Dar lacks facilities like unavailability of waste disposal which creates a big problem of bad smell; poor processing facilities with no or low water supply in the site (Cheffo *et al.*, 2021).

3.2. Study Design and Sampling Strategy

From February 2022 to November 2022, a cross-sectional study was undertaken at Bahir Dar landing sites of Lake Tana and the respective fish value chain in the city of Bahir Dar, Ethiopia. The two landing sites (Bata and Shimbit Mikael) and ten restaurants were selected with purposive sampling based on their better capacity to supply the harvested fish for the city and their higher number of consumers to serve, respectively. Each visit to the landing sites resulted in the sampling of 5-6 harvested fish as a pooled sample from randomly selected containers carried on the boat. At the restaurant, swabs of both personnel's hands and equipments including knives and cutting boards to be involved in fish preparation were sampled. The swab of knives and cutting boards were mixed into a single transport media as a single sample, representing one sample of the overall sample size. Furthermore, during the visit to the lake, a total of 10 water samples were collected from five fish harvesting sites of the lake at morning time.

3.3. Sample Collection and Preparation

Ten visits were carried out for the collection of proposed samples along the fish value chain (landing sites, retail markets, and restaurants in addition to water samples from the lake) randomly. The route of fish distribution was unable to be traced clearly due to a lack of consistent marketing channels. Therefore, following a connected marketing channel was difficult other than sampling the fish, from the landing site, retail market, and restaurant and water from the lake on the same day. To accommodate the existing fish value chain in Bahir Dar City, seven types of samples were included. These sample types

were fresh fish, retailing fish, filleted fish, cooked (ready-to-eat) fish, Swabs from the working environment including swabs from equipments (knives and cutting board in the kitchen), and food handlers and water samples from the lake were included.

A total of 121 samples comprising 51 fresh fish, 20 retailing fish, 20 filleted and cooked fish (each 10), 20 swabs from processing equipments and hand of food handlers (each 10) and 10 water samples were collected from two fish landing sites in the southern gulf of Lake Tana, retail fish markets, and 10 fish restaurants in the city as illustrated in Figure 2. During each visit, each stage (segment) of the value chain was represented by at least 10th of its total sample size.

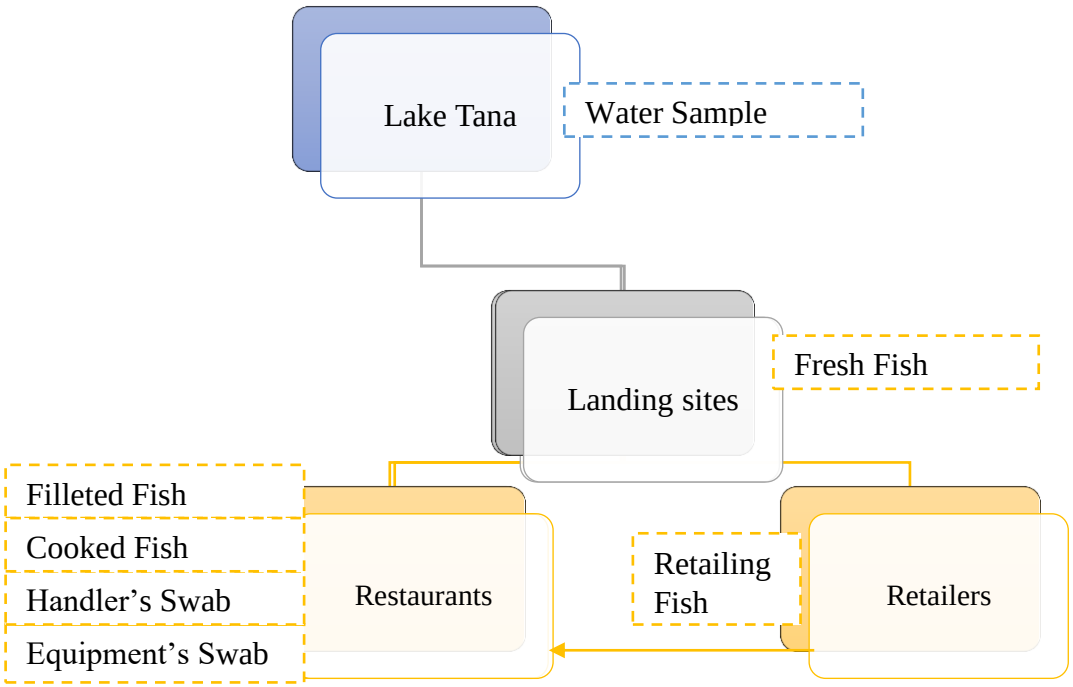

Figure 2. The schematic illustration of sampling channels with respective sample types

Fresh fish were purchased at the landing site during their immediate arrival from both motorized and reed boats either of early in the morning or afternoon and then put into separated and labeled plastic bag. Excision was made on the muscle along with the skin cover on both sides of the fish, above the lateral line, and between the dorsal fin and caudal fin by scalpel ensuring that a sufficient quantity of tissue. In the laboratory, 25g of fish sample was weighed and blended with a stomacher after mixing with 225 ml of sterile buffered peptone water (BPW) for 2 min to make a homogenate. Retailing fish were

collected from retailers who were selling fish handled by unhygienic apparatus at open market.

The swab samples were collected with a sterile cotton swab using an aseptic technique. For the processing of the swabs from hand of fish handlers and from processing equipments (knife and cutting board), each swab were then placed in a sterile tube containing 10 ml BPW, and the screw cap was closed tightly. To sample the water, the plastic bottles were submerged into the lake, filled and secured the lid. Water samples of 250 ml were collected from approximately 20 cm below the water surface of Lake Tana and transferred into sterile plastic bottles. Each sample was kept in the ice box with an ice pack and brought to the nearby biotechnology laboratory of Bahir Dar University for bacteriological analysis under aseptic conditions within three hours after collection.

3.4. Bacterial Enumeration

3.4.1. Total Viable Count (TVC)

Total viable count (TVC) was used to determine the total microbial load of fish. Using separate sterile pipettes, decimal dilutions of 10^{-1} to 10^{-8} of food homogenate were prepared by transferring 1 ml of the previous dilution to 9 ml of sterile peptone water. The dilutions were mixed by shaking with a vortex. Then 1 ml of appropriate dilution was transferred into separate, appropriately labeled, and sterilized Petri dishes. Then, plate count agar was cooled to 45°C, and around 20 ml of the medium was added to each plate within 15 min of the original dilution. After that, the sample dilutions and agar medium were mixed thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on a flat-level surface. Then, the agar was left to solidify for 15 minutes and the solidified Petri-dish was inverted and incubated promptly for 24 h at 37°C. Finally, the number of colonies was counted with a colony counter for each plate having colonies between 30 and 300 and multiplied by the dilution factor to calculate the total colony forming units per gram of (CFU/g) the sample (Whitman *et al.*, 2015).

3.4.2. Total Coliforms Count (TCC) and *Escherichia coli* Count

Violet red bile (VRB) agar was used to estimate TCC instead of total plate count agar unlike TVC to determine total coliform bacteria. Then after following the same procedure

with TVC, it was incubated promptly for 24h at 35 °C. Finally, the number of purple-red colored colonies were counted and multiplied by the dilution factor to calculate the total colonies forming units per gram of sample. For *E. coli* count, Eosin Methylene Blue (EMB) agar was used with the same aforementioned procedures for TVC and TCC, and green metallic sheen colonies were counted as *E. coli* against the standard (Table 4).

Table 4. Bacterial Load Standard

Food type		**Satisfactory**	**Boarder line**	**Unsatisfactory**	**Reference**
Cooked food	Total Viable count (TVC)	$<10^4$	10^4-10^7	$\geq 10^7$	(CFS, 2014)
Ready to eat fish	*E. coli*	<20	20 - 10^2	$> 10^2$	

3.5. Isolation of the Presumptive Bacteria

3.5.1. Isolation of *Salmonella* species

Aseptically 25g sample was mixed with 225 ml of sterile Buffered Peptone water in a sterile plastic bag and blended with a stomacher for 2 min. The BPW homogenate then was incubated for 24 hrs at 37 °C as a pre-enrichment step before inoculation of selective media. After the incubation of the pre-enrichment broths, 0.1 ml of each cultured medium was transferred to a Rappaport Vassiliadis (RV) broth. Rappaport-Vassiliadis (RV) was used for the first selection step and the inoculated media were incubated at 41.5°C for 24 h. Then a loopful of colonies from the edge of a migration zone was streaked onto Xylose Lysine Deoxycholate (XLD) agar plate. The suspected colonies having been pink with a black center and cultured on nutrient agar and biochemically confirmed by the Urease test, Triple Sugar Iron Agar slants, and IMViC tests (Indole, MR-VP, and Simmons Citrate agar tests). The result with Urease negative, Red slant/Yellow butt with gas production and H_2S on TSI test, Indole negative, MR positive, VP negative, and Citrate positive were considered as presumptive *Salmonella*.

3.5.2. Isolation of *Escherichia coli*

After homogenizing the sample in the stomacher, the sample in a buffered peptone water was incubated for 24 hours at 37^oC with the same procedure for the isolation of *Salmonella* species. A loopful of cultured suspension were then streaked on differential

media (Mac Conkey agar plates) for primary isolation and incubated aerobically at 37°C for 24 hours. The plates were observed for the growth of *E. coli* (pink colony surrounded by a dark pink area). A single isolated colony was picked and sub-cultured on Eosin Methylene Blue (EMB) agar and observed for the formation of a green metallic sheen. Morphologically similar and dominant bacterial colonies were selected and streaked onto nutrient agar plates for 24 h at 37 °C to obtain pure cultures for further processing. Pure cultured colonies were identified by biochemical tests IMViC. The result with Urease negative, Yellow slant/Yellow butt with gas production on TSI test, Indole positive, MR positive, VP negative, and Citrate negative were considered as presumptive *Escherichia coli*. The presumptive isolates were then stored at -20°C for further molecular detection (PCR).

3.6. Antimicrobial Sensitivity Test

The antibiotic sensitivity test was done by using the Kirby-Bauer disk diffusion method (Bauer, 1966). Eleven chemotherapeutic agents (antibiotic discs) for *Escherichia coli* isolates and six discs for *Salmonella* isolates were selected and used as described by the CLSI (Clinical and Laboratory Standards Institute) guideline with their availability (CLSI, 2020). The Commercial discs with Amoxicillin/Clavulanate (30µg), Amikacin (30µg), Azithromycin (15µg), Cefotaxime (30µg), Ceftazidime (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Nalidixic Acid (30µg), Trimethoprim-Sulfamethoxazole (25µg), Tetracycline (30µg) for *Escherichia coli* isolates and Ampicillin(10µg) Cefotaxime (30µg) Ceftazidime (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg) and Trimethoprim-Sulfamethoxazole (25µg) for *Salmonella* isolates were used for the susceptibility test.

The Mueller-Hinton (MH) agar medium was prepared according to the manufacturer's recommendations. The MH agar medium was tested for pH to be between 7.2 and 7.4 at room temperature. Around Five colonies of an isolate were picked using a sterile inoculating loop and suspended in 4-5 ml of sterile saline and vortex to create a smooth suspension matching 0.5 McFarland standard. The sterile swab was dipped into the inoculum tube and swabbed onto MH agar and allowed to dry for 3-5 minutes. The antimicrobial-impregnated discs were placed on the surface of the agar, using forceps, and were incubated at 37°C air incubator for 18 hours.

Following incubation, the zone sizes were measured to the nearest millimeter using a ruler. The susceptibility or resistance of the organism to each drug tested was determined using the published CLSI (Clinical and Laboratory Standards Institute) 2020 guidelines. The zone size of inhibition for each drug was indicated on the recording sheet as susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart (CLSI, 2020) (table 5).

Table 5. The interpretation standard of the zone size of inhibition

Antimicrobial agent	**Disc Content**	**Zone diameter for *E. coli/ Salmonella* (mm)**		
		Susceptible	Intermidiate	Resistant
Amoxicillin/Clavulanate	20/10µg	≥18	14-17	≤13
Amikacin	30µg	≥17	15-16	≤14
Azithromycin	15µg	≥13	-	≤12
Cefotaxime	30µg	≥26	23-25	≤22
Ceftazidime	30µg	≥21	18-20	≤17
Ceftriaxone	30µg	≥23	20-22	≤19
Ciprofloxacin	5 µg	≥26/≥31	22-25/21-30	≤21/≤20
Gentamicin	10µg	≥15	13-14	≤12
Nalidixic Acid	30µg	≥19	14-18	≤13
Trimethoprim-Sulfamethoxazole	25µg	≥16	11-15	≤10
Ampicillin	10µg	≥17	14-16	≤13

3.7. DNA Extraction

Genomic DNA extraction and purification were performed as per the protocol given by Genomic DNA Purification Kit (Qiagen, Germany) available at National Veterinary Institute (NVI) for *E. coli* isolates. A simple bacterial DNA extraction method was done involving centrifugation and boiling. The bacterial isolates grown in nutrient broth were

harvested and centrifuged at 15,000rev/min for 5 minutes. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and mixed by the vortex. The bacterial pellets were lysed by boiling in a water bath at 95°c for 10 minutes and then centrifuged to recover DNA template at the supernatant. The purity of extracted the DNA was examined with 2% agarose gel. The extracted DNA was stored at-20°C for the PCR procedure.

3.8. Polymerase Chain Reaction (PCR)

Genomic DNA samples were used to analyze the common virulence genes of the presumptive *E. coli* isolates by using specific primers. The DNA of selected presumptive isolates was then subjected to PCR. Polymerase Chain Reaction assay was carried out in 22µl reaction volume in a PCR master mix (thermo scientific, Denmark) containing 4µl of primers (2µlfor each forward and reverse), RNase free water 3µl, IQ supermix 10µl [which includes Taq DNA polymerase (0.05U/µL), reaction buffer, 4mM $MgCl_2$, and 0.4mM of each dNTP], Template (DN A) 5 µl.

The selected 10 *E. coli* isolates were subjected to PCR for the detection of 3 pathogenic genes (EVT1, eae, and hlyA) (table 5).

Table 6. Oligonucleotide sequence of the primers used in the detection of virulence genes of *E. coli*

Primers	**Sequence**	**Target**	**Product Size**	**Reference**
EVT_1 Forward	5'-CAACACTGGATGATCTCAG -3'	Vero Toxin 1 (stx_1 family)	349bp	(Hessain *et al.*, 2015)
EVT_2 Reverse	5'-CCCCCTCAACTGCTAATA-3'			
EAE_1 Forward	5'-AAACAGGTGAAACTGTTGCC -3'	eae	350bp	(Khan *et al., 2002)*
EAE_2 Reverse	5'-CTCTGCAGATTAACCTCTGC-3'			
HlyA1 Forward	5'-GGTGCAGCAGAAAAAGTTGTAG -3'	EHEC hlyA	1551bp	(Khan *et al.*, 2002)
hlyA4 Reverse	5'-TCTCGCCTGATAGTGTTTGGTA-3'			

3.9. Agarose Gel Electrophoresis

Amplified PCR products were analyzed by gel electrophoresis at 120 volts for 80 minutes in 2% agarose containing 4µl Gel red (Biotium, USA). It was run in TAE (Tris-acetate-EDTA) Buffer by loading 3 µl of the PCR product (mixed with loading dye) on gel wells stained with 2ul of Ethidium bromide stock solution (10mg/ml). A parallel lane was also loaded with a 10 µl marker DNA ladder of 100bp plus (fermantas, USA). The products were visualized with UV illumination and imaged with a gel documentation system (UVtec 08 100554, France). The expected size of the PCR products flanked by the primer pairs were around 350bp for eae, 1551bp for hlyA gene, 349 bp for VT_1.

3.10. Data Management and Analysis

The STATA/ SE (special edition) 17 and Microsoft Office Excel 2007 were used as statistical tool for data processing and analysis. Data collected were entered in an Excel spread sheet. Descriptive statistical analysis was used for variables under study, particularly with frequency distributions. Bacterial count data were transformed into logarithm values (log10 Cfu/ml) before statistical analysis. To establish statistically significant differences between means of microbial loads in stages of the value chain, the one-way analysis of variance (one-way ANOVA) was applied by using STATA/SE17.

3.11. Ethical Considerations

For sample collection, an ethical clearance certificate was received from the ethical board of the College of Agriculture and Environmental Sciences, Bahir Dar University. The entire sample collection was done after informed verbal consent by each participant about the objectives of the study, benefits, and their rights. Then their voluntarism was kept throughout the whole journey of the research.

Chapter 4. RESULTS

4.1. Bacterial Enumeration

The mean total viable count (TVC), total coliform count (TCC), and *E. coli* count of the samples were 8.44 log_{10} CFU/g, 5.99 log_{10} CFU/g, and 4.05 Log_{10} CFU/g, respectively. The cooked fish (ready to eat) in restaurants harbored the least mean TVC (6.21log_{10} CFU/g) and mean TCC (4.60log_{10} CFU/g) and the second least mean *E. coli* count (3.07log_{10} CFU/g) next to mean *E. coli* count of filleted fish 2.93log_{10} CFU/g (Table 7).

Table 7. Bacterial counts from different samples

Sample Type	**Mean TVC (Log_{10} CFU/g or ml)**	**Mean TCC (Log_{10} CFU/g or ml)**	**Mean *E. coli* count (Log_{10} CFU/g or ml)**
Fresh Fish	8.58	6.19	4.45
Retailing Fish	8.88	6.11	4.02
Filleted Fish	8.90	5.45	2.93
Cooked Fish	6.21	4.60	3.07
Lake Water	9.95	9.21	6. 65
Total	**8.44**	**5.99**	**4.05**

The mean count of the TVC, TCC, and *E. coli* count at restaurant (consumer) level revealed that 7.56log_{10} CFU/g, 5.03log_{10} CFU/g, and 3.01log_{10} CFU/g, respectively. The result of bacterial enumeration showed that an ascending order from TVC to TCC and *E. coli* count (Figure 3).

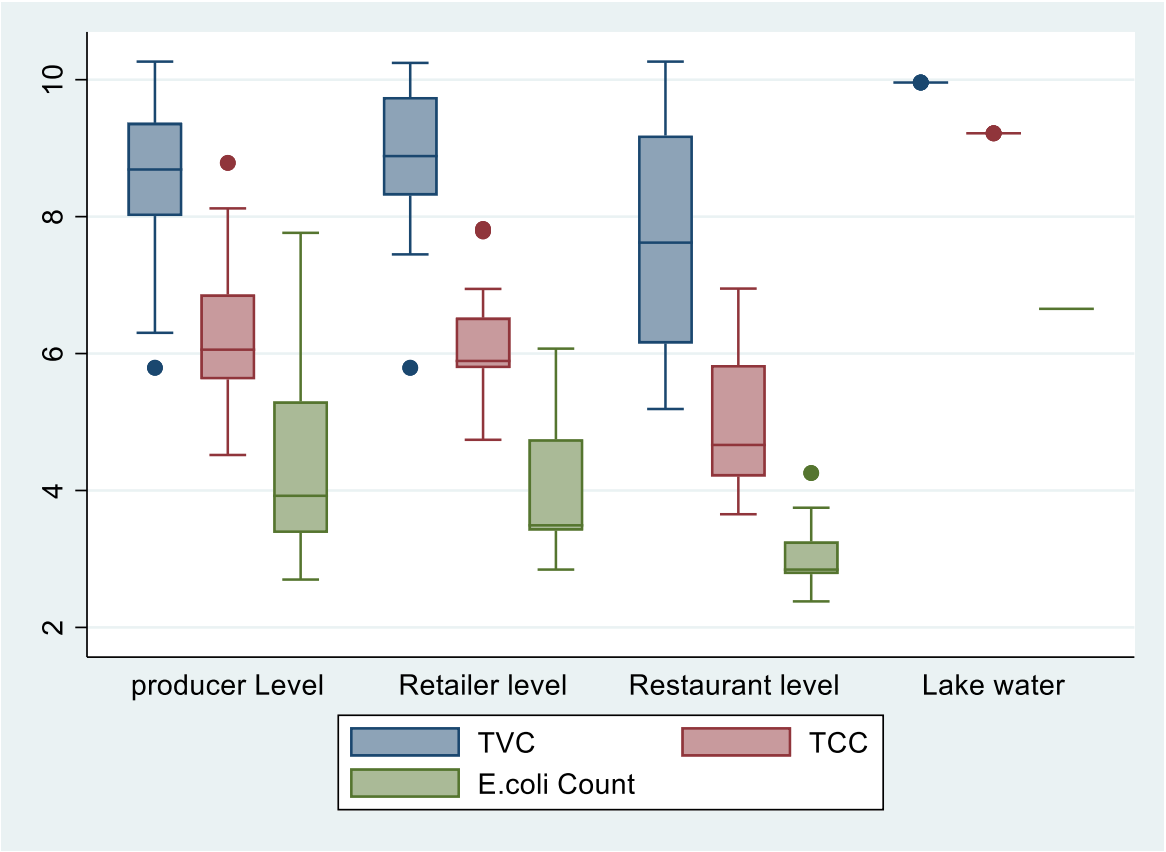

Figure 3. A box plot distribution of bacterial count data set across the channels of the fish value chain

The highest mean was recorded from the lake for all TVC, TCC, and *E. coli* count. The second highest mean count resulted at the retailer level for TVC and restaurant level for both TCC and *E. coli* count. There was a significant difference between the four stages of fish value chains(producers, retailers, restaurants, and the lake) towards all TVC, TCC, *and E. coli* count with P=0.000, P=0.000, and P=0.000, respectively (Table 8).

Table 8. One-way ANOVA output of stages of food chain on TVC, TCC and *E. coli* count

Types of Count	Source of variation	Sum of squares	Degree of freedom (df)	Mean square (S^2)	F-Ratio	Prob> F
TVC	Between	24.6419277	3	8.21397589	6.65	**0.0004**
	Within	106.278034	86	1.2357911		
	Total	130.919962	89	1.47101081		
TCC	Between	41.5537649	3	13.851255	16.18	**0.0000**
	Within	76.1991702	89	.856170451		
	Total	117.752935	92	1.27992321		
***E. coli* Count**	Between	31.1981652	3	10.3993884	7.36	**0.0004**
	Within	70.6732634	50	1.41346527		
	Total	101.871429	53	1.92210243		

4.2. Isolation Rate of *Escherichia coli* and *Salmonella*

Out of the total 121 samples examined by biochemical tests in this study, *E. coli* and *Salmonella* were isolated from 41 (33.88%) and 6 (4.96%) of the samples, respectively. The result in Table 9 has shown that the highest rate of *E. coli* isolate was recorded from retailing fish (80%, 16) at retailers and the lowest rate at the lake water (10%, 1) and for *Salmonella* isolate, the highest rate was from filleted tissue (20%, 2). At restaurants (Table 10), 30% (12) and 7.5% (3) of the samples were positive for *E. coli* and *Salmonella*, respectively. The same *Salmonella* spp isolation rate of 15% (3) was recorded at retailers and restaurants. Among fish species (Table 11), *Labeobarbus spp* was the one with the leading *Escherichia coli* isolation rate (50%, 8) while the unidentified mixed fish samples were examined for the highest *Salmonella* isolates (52%, 3), (Table 11). From the four categories of the origin of fish the unknown site (untraceable source) had the maximum *Escherichia coli* 41.85% (24) and *Salmonella* 10.71% (6) isolation rate. Around 35% of fish harvested at Bahir Dar Gulf of the Lake were detected as *E. coli* positive while no *Salmonella* isolate was detected.

Table 9. The frequency of *E. coli* and *Salmonella* isolates across sample type

Sample Type	**Total examined**	***E. coli***		***Salmonella***	
		Positive	**Percentage**	**Positive**	**Percentage**
Fresh Fish	51	12	23.53	0	0.00
Retailing Fish	20	16	80.00	3	15.00
Filleted Fish	10	6	60.00	2	20.00
Cooked Fish	10	3	30.00	0	0.00
Equipment Swab	10	2	20.00	0	0.00
Handler's Swab	10	1	10.00	1	10.00
Water	10	1	10.00	0	0.00
Total	**121**	**41**	**33.88**	**6**	**4.96**

Table 10. The frequency of *E. coli* and *Salmonella* isolates across the stages of the value chain

Channels of the fish value chain	**Total examined**	***E. coli***		***Salmonella***	
		positive	**Percentage**	**Positive**	**Percentage**
Producer	51	12	23.53	0	0.00
Retailer	20	16	80.00	3	15.00
Restaurant	40	12	30.00	3	7.5.00
Lake	10	1	10.00	0	0.00
Total	**121**	**41**	**33.88**	**6**	**4.96**

Table 11. The frequency of *E. coli* and *Salmonella* isolates across the fish species

Species	**Total examined**	***E. coli***		***Salmonella spp***	
		positive	**Percentage**	**Positive**	**Percentage**
Oreochromis niloticus	43	15	34.88	1	2.32
Labeobarbus spp	16	8	50.00	2	12.50
Clarias gariepinus	10	4	40.00	0	0.00
Unidentified	52	14	26.92	3	5.77
Total	**121**	**41**	**33.88**	**6**	**4.96**

4.3. Antimicrobial Sensitivity Test

4.3.1. *Escherichia coli* isolates

The overall antimicrobial sensitivity test findings for presumptive *E. coli* isolates were presented in Table 12. Taken together, all 41 *E. coli* isolates were resistant to amoxicillin/Clavulanate. Among the 11 antimicrobial discs tested, Ciprofloxacin (n=6), Cefotaxime (n=22), Ceftazidime (n = 2), Ceftriaxone (n=4), and Nalidixic Acid (n=28) were detected as intermediate level. All isolates were susceptible for Gentamicin and Amikacin at a given dose.

Table 12. Antimicrobial sensitivity pattern of *E. coli* Isolates

Antimicrobial disc	**Resistance Isolates**	**%**	**Intermediate Isolates**	**%**	**Sesceptible Isolates**	**%**
Ciprofloxacin	5	12.2	6	14.63	30	73.17
Azithromycin	13	31.71	0	0	28	68.29
Tmp-Sulfa	5	12.2	0	0	36	87.8
Tetracycline	36	87.8	0	0	5	12.2
Amox/Clav	41	100	0	0	0	0
Cefotaxime	4	9.76	22	53.66	15	36.59
Ceftazidime	39	95.12	2	4.88	0	0
Ceftriaxone	22	53.66	13	31.71	6	14.63
Gentamicin	0	0	0	0	41	100
Amikacin	0	0	0	0	41	100
Nalidixic Acid	1	2.44	28	68.29	12	29.27

The antimicrobial sensitivity test was analyzed for multidrug resistance rate (MDR). All 41 isolates showed a resistance against at least two discs and with a maximum MDR against six drugs by three of the isolates (Table 13). The MDR rate for the *Escherichia coli* isolates was 95.12%.

Table 13. MDR Result of *E. coli* Isolates

Level of MDR	**Number of Isolates**	**Percentage (%)**
No Resistance	0	0.00
Resistance to one drug	0	0.00
Resistance to two drugs	2	4.88
Resistance to three drugs	13	31.71
Resistance to four drugs	10	24.39
Resistance to five drugs	13	31.71
Resistance to six drugs	3	7.32
Total	**41**	**100**

4.3.2. *Salmonella* isolates

The highest rate of resistance was recorded for Ceftazidime against all (n=6) isolates of *Salmonella* species while Ciprofloxacin and TMP-Sulfa were identified as having no resistance (Figure 4).

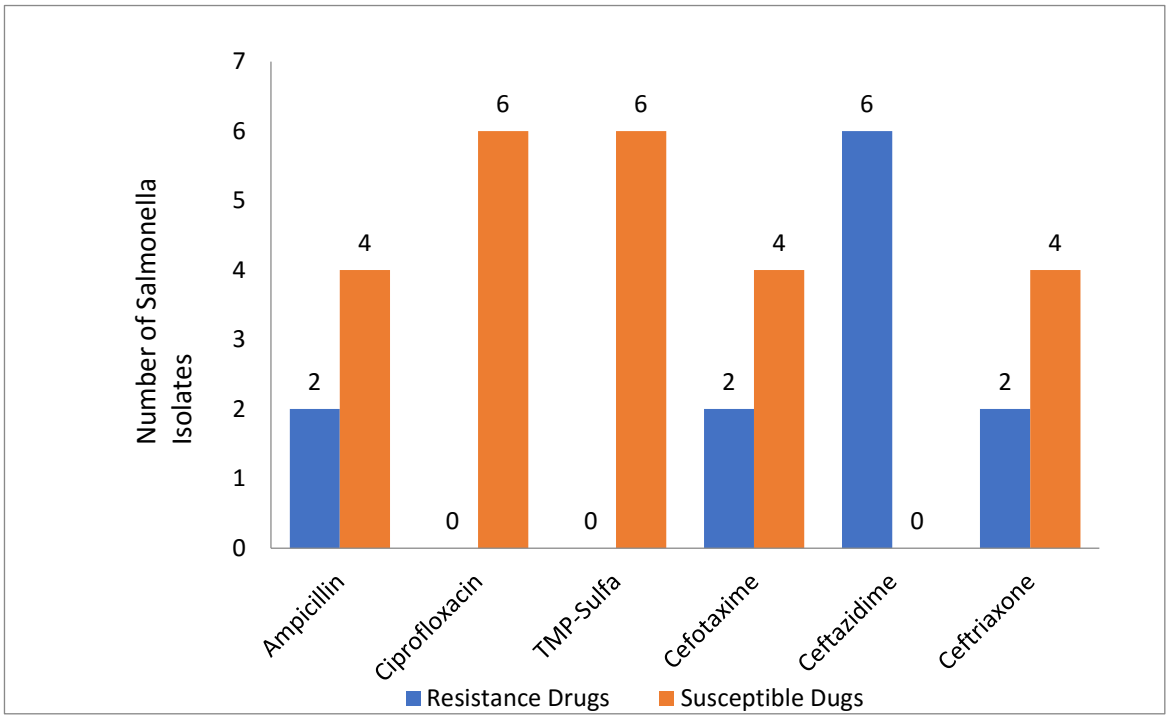

Figure 4. The Antimicrobial sensitivity test result of *Salmonella* Isolates

One-third of Salmonella isolates detected as they develop MDR (Table 14).

Table 14. The MDR Result of *Salmonella* Isolates

Level of MDR	**Number of Isolates**	**Percentage (%)**
No Resistance	0	0.00
Resistance to one drug	4	66.67
Resistance to four drugs	2	33.33
Total	**6**	**100**

4.4. Virulence Detection

4.4.1. *Escherichia coli* isolates

From the total 10 isolates tested by PCR, four were positive for two virulence genes (hlyA1 and eae) of *Escherichia coli*. Among the three virulence genes of interest of *Escherichia coli* for this study, only the two are detected (Table 15) whereas no VT1 gene were identified. A single *Escherichia coli* isolate was detected with the two virulence genes.

Table 15.The description of positive samples for detected virulence genes

Sample Id_	**Gene detected**	**Sample Type**	**Stages of Fish value chain**	**Species of Fish**
50	hly A1	Retailing Fish	Retailer	Nile Tilapia
55	hly A1	Cooked Fish	Restaurant	Unidentified
68	eae	Filleted Fish	Restaurant	Unidentified
72	hly A1and eae	Filleted Fish	Restaurant	Unidentified

The molecular work for 10 *Escherichia coli* isolates revealed the presence of intimin gene (eae) in the two isolates and hemolysin A (hlyA) in the three samples as shown in the Figure 5 below.

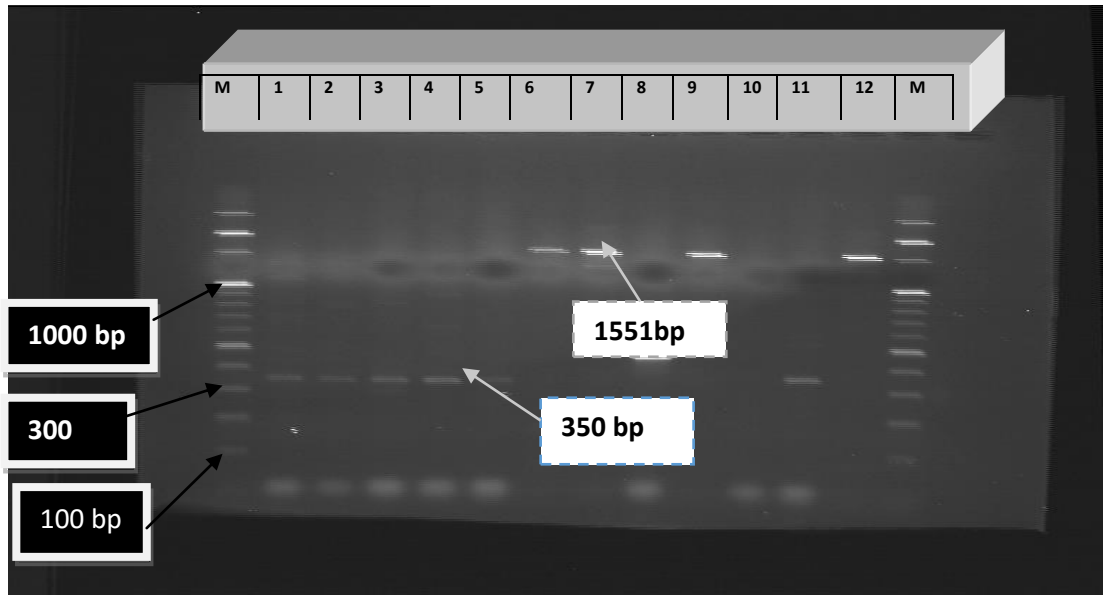

Figure 5. Agarose gel electrophoresis of virulence genes of the potential pathogenic *E. coli* isolates

The first and the last lane denoted by "M" (molecular ladder started at 100bp),
Lane 1 to 9: for tested samples,
Lane 10: is a negative control,
Lane 11: is a positive control of eae gene - 350 bp,
Lane 12: positive control of Hly A1 gene-1551 bp.

Chapter 5. DISCUSSION

The current study revealed that the mean total viable count (TVC) of the processed samples was 8.44 log_{10} CFU/g. This was much higher than the study undertaken in Turkey with a count range of 2.68-2.92 log_{10} CFU/g and 2.79-2.87 log_{10}cfu/g analyzed in the morning and evening, respectively (Kocatepe *et al.*, 2013). The study conducted in Sri Lanka (Ariyawansa *et al.*, 2016) with TVC of 6.30 log_{10} CFU/g, in Nigeria (Amuneke *et al.*, 2020) ranged from 6.30log_{10} CFU/g to 6.97 log_{10} CFU/g and in Norway (Svanevik *et al.*, 2015)5.7- 6.7 log_{10}cfu/g were lower than the present study.

The result of TCC was 5.99 log_{10} CFU/g is also higher than those of studies carried out in Turkey (Kocatepe *et al.*, 2013) and Norway (Svanevik *et al.*, 2015) with the count of an average coliform count was 2.25-2.34log_{10} CFU/g and 0.6-1.0 Log $_{10}$ CFU/g, respectively. *Escherichia coli* count of the current study resulted in average count of 4.05 Log_{10} CFU/g which is higher than the mean count obtained in Sri Lanka (Ariyawansa *et al.*, 2016) reported 3.74log_{10} CFU/g and below. The higher count might be a result of unhygienic handling and processing methods, such as utilizing filthy tools and containers with unwashed hands, undercooking, and/or post-cooking contamination at the level of consumers. In addition, the higher count in the study area raises questions about the safety of the water used for washing the fillet. It is well-recognized that the total coliform bacteria and *E. coli* count are indicators of poor hygiene and fecal contamination in food (Ekici and Dümen, 2019).

The mean count of the TVC across the four channels of the fish value chain in Bahir Dar city investigated that 8.59 log_{10} CFU/g, 8.88 log_{10} CFU/g, and 7.56 log_{10} CFU/g for samples at the level of producers, retailers, and restaurants, respectively. The highest count was at the level of retailers followed by at producer level and then at restaurants. The TVC mean count was higher but the load pattern along the sites of the value chain was in line with the result studied in Unguja Island at producer 5.85log_{10} CFU/g, retailer 5.96log_{10} CFU/g and consumer/restaurants 4.79log_{10} CFU/g and Pemba Island Producer level 5.74log_{10} CFU/g, retailer level 5.86log_{10} CFU/g and restaurant level l4.79log_{10} CFU/g in Tanzania (Rabia *et al.*, 2017b).

The water sample from the lake was analyzed and found mean TVC 9.95 log_{10} CFU/ml, TCC 9.95 log_{10} CFU/ml, and *E. coli* count 6.65 log_{10} CFU/ml which was quite higher than

the study in Norway with TVC (2 log CFU/ml), and TCC (0) (Svanevik *et al.*, 2015), in Sri Lanka with TVC (6.14$\log_{10}$ CFU/g), TCC (<4.20 $\log_{10}$ CFU/g and *E. coli* count (<3.74 $\log_{10}$ CFU/g) (Ariyawansa *et al.*, 2016) and in Nigeria TVC (5.15and 4.23log cfu/ml) (Amuneke *et al.*, 2020).

The bacterial count for cooked ready-to-eat fish in restaurants possessed the mean TVC (6.21 $\log_{10}$ CFU/g) which was higher than the study conducted in the upper Blue Nile watershed, Ethiopia reported as 4.90 $\log_{10}$ cfu/g (Birhan *et al.*, 2022b) and the *E. coli* count (3.073 $\log_{10}$ CFU/g) was lower than the findings of the study in Northwest, Ethiopia which is reported as 4.44 $\log_{10}$ cfu/g (Halo *et al.*, 2022). According to the standard set by Microbiological Guidelines for Food Safety, the mean TVC finding for cooked fish showed that it was at the borderline (between 4-7 $\log_{10}$ CFU/g) and the mean *E. coli* count was unsatisfactory (>2 $\log_{10}$ CFU/g) for consumers (CFS, 2014). The mean TCC of cooked fish (2.07 $\log_{10}$ cfu/g) was much higher than the mean TCC found by the study done in the upper Blue Nile watershed (2.07 $\log_{10}$ cfu/g) (Birhan *et al.*, 2022b). Persons who are doing as retailers and processors with less awareness about hygiene and sanitation are important for the contamination of the fish (Kumar *et al.*, 2005).

The retailing practice in the city is mostly a backyard marketing system done in the general market with other commodities and/or by carrying the fish and walking along the roads for searching the purchasers. The one-way ANOVA result of all counts (TVC, TCC, and *E. coli* count) produced a significant difference along the sites of the value chain. The result has shown that the highest rate of *E. coli* isolate was recorded from retailing fish 80% (16) at retailers. This might be due to most of the retailers (vendors) having no convenient vehicle to transport, no special equipment to keep uncontaminated such as refrigerators, traditional marketing system. These and other many unsafe handling of the fish by the marketer is conducive to microbial contamination and multiplication. There is a strong likelihood of getting higher coliform counts in the samples where there is an untreated sewerage system from the surrounding city, livestock rearing around the lake, or agricultural production being practiced around tributary rivers (Vaz-Pires *et al.*, 2008).

The presence of *Escherichia coli* is an indicator of fecal contamination along the stages of the fish value chain (Uçar *et al.*, 2015). Cattle, birds and, man around the fish production and processing can be used as a potential source of contamination. In an unhygienic

environment, the flies are also one among the vectors for the contamination (Cardozo *et al.*, 2018; Ekici and Dümen, 2019). The ability to trace the microbial source in the fish value chain is made possible by the fact that this bacterium can live in soil, water, and fish. This fact also sheds light on the nature of the contamination and the potential route of this bacterium's spread (Hoffmann *et al.*, 2016).

Out of the total of 121 samples examined by biochemical tests in this study, *E. coli* was isolated from 41 (33.88%). This finding was higher than the study reported in the southern gulf of lake Tana (2.4%) (Anwar, 2007), Northwest Ethiopia (20%) (Halo *et al.*, 2022), Hawassa (21%) (Eromo *et al.*, 2016) and (23.3%) (Tilahun and Engdawork, 2020), Mekelle (9.4%) *(Awot et al., 2019),* Lake Zeway (12%) (Haile and Getahun, 2018), Morocco (30%) (Badri *et al.*, 2009), India (29.34%) (Gupta *et al.*, 2013) and, Ghana (6.1 %) (Aboagye, 2016), Tanzania (8.6%) (Rabia *et al.*, 2017a), Seoul, Korea (7.6%) (Ryu *et al.*, 2012). The result was however almost in line with the study undertaken in Northeast Algeria (32%) (Dib *et al.*, 2018) but lower than the report in India (55.56%) (Sahu *et al.*, 2016), Egypt (42.2%) (Al Qabili *et al.*, 2022) and Sri Lanka (68%) (Ariyawansa *et al.*, 2016).

The isolation rate of *Salmonella* 6 (4.96%) in this study was lower than the findings in different parts of Ethiopia (36.4%) (Birhan *et al.*, 2022a), (58%) (Eromo *et al.*, 2016), (14.5%) (Sufiyan *et al.*, 2022), Nigeria (*11.5%*) *(Raufu et al., 2014),* Vietnam (36.6%) (Nguyen *et al.*, 2016), China (18.6%) *(Yang et al., 2015),* India (49.99%) (Sahu *et al.*, 2016), Saudi Arabia (39.9%) (Elhadi, 2014) but it was almost in agreement with the reports in Ethiopia (6.82%) (Birhan *et al.*, 2022b) and Brazil (4.0%) (Duarte *et al.*, 2021) and (3.4%) (Esposto *et al.*, 2007).The variation in the proportion of the *Escherichia coli* and *Salmonella* between the studies might be attributed to the difference in identification technique, sample type, site of sampling, fish species, and sample size, and most importantly the hygienic status of fishing, marketing, and processing environment (handler, equipments, and water).

Isolating pathogenic bacteria from fish indicate cross-contamination. This can happen from fish washing water, surfaces where fish are stored, or fish-transportation containers. Pathogens and coliform bacteria found in fish imply a contaminated environment (Uçar *et al.*, 2015), inadequate post-harvest management, and improper fishing techniques

(Ariyawansa *et al.*, 2016). The unhygienic handling practices may impose the potential risk of fishborne diseases (Bedane *et al.*, 2022). Information about the cleanliness of environments, such as lakes, rivers, ponds, and fish farms, is provided through the microbial assessment of fish (Novoslavskij *et al.*, 2016).

Cooked (ready-to-eat fish) was among the sample type that was analyzed for the presence of *Escherichia coli* and it resulted in 3 (30%) isolation. These had and implication of inadequate heating during cooking or post-cooking contamination. This indicates there is a provision of *Escherichia coli* contaminated food to consumers at the restaurants. Among the 10 analyzed handler's swabs at fish restaurants, one (10%) was positive for *Salmonella*. This might be because they don't wash their hands after having close contact with some animals. If they don't wash their hands well before handling food, they risk contaminating it (FDA, 2019). The result showed that the hygienic handling of fish could be taken as hazardous for consumers.

Out of the total 10 water samples taken from the lake the10% (n=1) *Escherichia coli* isolation rate has resulted. The report of the current study from the lake water was less than the study conducted in Cote d'Ivoire 51 (45%) (Kambire *et al.*, 2017). This might be because of the number of water samples, the hygiene of the water bodies, and the identification technique. Pathogenic bacteria or alterations in the environment's natural micro flora could be a key sign of potential contamination (Novoslavskij *et al.*, 2016). The presence of *E. coli* in water shows fecal contamination (Barbosa *et al.*, 2016).

One of the main causes of antimicrobial resistance is the incorrect use of antibiotics in the food chain, which is a global concern. The current finding of the antimicrobial sensitivity test resulted that all 41 *E. coli* isolates were resistant to amoxicillin/Clavulanate. Among the 11 antimicrobial discs tested, Ciprofloxacin (n=6), Cefotaxime (n=22), Ceftazidime (n = 2), Ceftriaxone (n=4), and Nalidixic Acid (n=28) were detected as intermediate level. This study found that all isolates were susceptible for Gentamicin and Amikacin at a given dose. The resistant rate against the *Escherichia coli* was in agreement with a study in Northwest Ethiopia for Gentamycin (100%) (Halo *et al.*, 2022) but different rate was recorded for sulfamethoxazole/trimethoprim and tetracyclines.

The resistant rate of amoxycillin/clavulanic and gentamycin were almost in line but not for ceftriaxone, ciprofloxacin, and tetracycline with the report in Egypt conducted sampled from edible shellfish (Al Qabili *et al.*, 2022). The report of the other study in Egypt was in agreement with the current study for the resistant rate of amoxicillin/clavulanic acid, and tetracyclines at a percentage of 100% followed by sulfamethoxazole/trimethoprim but it was higher for nalidixic acid and Ciprofloxacin (Bakry *et al.*, 2021). In this study, 95.12% of *Escherichia coli* isolates were found to be multidrug resistant. Globally, fish and shellfish have been identified as harboring *E. coli* that is resistant to several clinically significant families of antibiotics, including cephalosporins, aminoglycosides, ciprofloxacin, tetracycline, etc (Boss *et al.*, 2016). The highest rate of resistance and susceptibility was recorded for Ceftazidime against all (n=6) isolates of *Salmonella* species while Ciprofloxacin and TMP-Sulfa were identified as no resistance detected. The MDR investigation of the current study resulted of the study showed that 2 *Salmonella* isolates were detected with 3 and more antimicrobials. This was in line with the study conducted from Fish Value Chain, Northwest Ethiopia from Fish Value Chain, Northwest Ethiopia (Birhan *et al.*, 2022a) with a report of 2 isolates that were resistant for three antibiotics.

The emergence of multi-drug-resistant (MDR) *Salmonella* serotypes is having a great impact on the efficacy of antibiotic treatment, and an increasing prevalence of MDR strains may lead to an increase in mortality rates of *Salmonella* infections (Eng *et al.*, 2015). Rampant and uncontrolled use of antibiotics results in the emergence of resistant bacterial strains. Such resistant strains on the food chain possibly increase the risk of antibiotic resistant foodborne pathogens. Antibiotic resistant bacteria can reach humans either directly via contact with the infected animal or indirectly via the food chain through the consumption of contaminated food (Thapa *et al.*, 2020).

The virulence detection study found that 4 *E. coli* isolates harbored virulence gene eae (n=1) at the restaurant, hlyA at retailer (n=1) and restaurant (n=1) and both eae and hly A (n=1) at restaurant. The study conducted by Al Qabili and his colleague from edible fish in Egypt detected eaeA gene which was in agreement with the current study but the additional stx genes were also identified which is not detected in the present study (Al Qabili *et al.*, 2022) whereas no pathogenic virulence gene was detected at the study in fish

in Cote d'Ivoire (Kambire *et al.*, 2017) and Morocco (Badri *et al*., 2009).The eae gene's existence indicates a potential contributing factor to diseases that attach and expel the intestinal epithelial lining (Dhaka *et al*., 2016). After confirming the coexistence of the stx gene, which is only present in STEC, it is critical to identify the eae gene from an *E. coli* isolate to discriminate between EPEC and STEC pathotypes (Goffaux *et al.*, 1997; Naylor *et al.*, 2005; Parussolo *et al.*, 2019; Sirous *et al.*, 2020).

Enterohaemolysin (hlyA) was the other virulence gene discovered in the current investigation. The hlyA gene might be directly related to how pathogenic the STEC strain is and how likely it is to infect individuals with more severe diseases (Okechukwu *et al.*, 2020). Enterohemolysin causes epithelial and endothelial cell injury (Bielaszewska *et al.*, 2014) and it can lyse erythrocytes to release iron, which supports the metabolism of *E. coli (*Elvis *et al.*, 2020*).*

Chapter 6. CONCLUSION AND RECOMMENDATIONS

The study was concerned on providing evidence about the presence of the two enteric foodborne pathogens (*E. coli* and *Salmonella*), in the fish value chain. The bacterial load counts and identification of the pathogens along the fish value chain revealed that there is a serious hygienic issue from the producer to the consumer level, with a special concern at the site of retailer. The high bacterial load recorded from the lake water sample was an indicator of contamination of the lake from the surrounding area. Moreover, the study showed that the possible sources of contamination of fish meat in the city were the lake water itself, the equipment used for fish handling and processing, and/or the fish handlers themselves. The present study also showed multi-drug resistant *Salmonella* and *E. coli* including pathogenic *Escherichia coli* with intimin and enterohemolysin (eaeA and hlyA) virulence genes were detected. The findings of this study indicated that there is alarming biological hazards for fish handlers, consumers, researchers, and health organizations concerned with safeguarding public health.

Based on the above conclusion the following recommendations are made

- The fish handlers and consumers should always be alerted to maintain their personal hygiene and sanitation.
- The fish should be consumed after proper cooking.
- Additional molecular work should be conducted on the detection of drug-resistant genes, characterization of pathotypes of *E. coli,* and confirmation of *Salmonella* serotypes.
- The health impact of consuming unhygienically handled and improperly cooked fish in the city with regard to *Salmonella* and pathogenic *E. coli* should be addressed.
- A regular fish safety monitoring framework should be designed and implemented.
- Interdisciplinary (One Health) approach should be carried out against drug resistance, contamination of the lake, and fish safety issues.

Chapter 7. REFERENCES

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4. Selective and Differential Medium for Enterobacteriacea

4.1. Mac Conkey Agar

Ingredients	Gms / Litre
Peptone	20.000
Lactose	10.000
Bile salts	5.000
Sodium chloride	5.000
Neutral red	0.070
Agar	15.000
Final pH (at 25°C)	7.5±0.2

Procedures

Suspend 55.07 grams in 1000 ml distilled water. Heat to boiling with gentle swirling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Avoid overheating. Cool to 45-50°C and pour into sterile Petri plates. The surface of the medium should be dry when inoculated

5. Basal media

5.1. Nutrient Agar

Ingredients	Gms / Litre
Peptone	5.000
HM Peptone B #	3.000
Agar	15.000
Final pH (at 25°C)	6.8±0.2

Procedures

Suspend 23.00 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. If desired, the medium can be enriched with 5 - 10% v/v sterile defibrinated bloods. Mix well and pour into sterile Petri plates

5.2. Nutrient Broth

Ingredients	Gms / Litre
Meat extract	1.000
Peptone	5.000
Sodium chloride	5.000
Yeast extract	2.000
Final pH (at 25°C)	7.4±0.2

Procedures

Suspend 13 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

6. Selective Medium for Detecting and Counting *Escherichia coli*

6.1. EMB (Eosin Methylene Blue) Agar

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Dipotassium phosphate	2.000
Lactose	5.000
Sucrose	5.000
Eosin - Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH (at 25°C)	7.2±0.2

Procedures

Suspend 35.96 grams in 1000 ml distilled water. Mix until suspension is uniform. Heat for boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. AVOID OVERHEATING. Cool to 45-50°C and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue color) and to suspend the flocculent precipitate.

7. Selective medium for *Salmonella*

7.1. Xylose-Lysine Deoxycholate Agar (XLD Agar)

Ingredients	**Gms / Litre**
Yeast extract	3.000
L-Lysine	5.000
Lactose	7.500
Sucrose	7.500
Xylose	3.500
Sodium chloride	5.000
Sodium deoxycholate	2.500
Sodium thiosulphate	6.800
Ferric ammonium citrate	0.800
Phenol red	0.080
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Procedures

Suspend 56.68 grams in 1000 ml purified / distilled water. Heat with frequent agitation until the medium boils. DO NOT AUTOCLAVE OR OVERHEAT. Transfer immediately to a water bath at 50°C. After cooling, pour into sterile Petri plates. It is advisable not to prepare large volumes that will require prolonged heating, thereby producing precipitate. Note : Slight precipitation in the medium may occur, which is inheritant property of the medium, and does not affect the performance of the medium.

8. Media for Biochemical Tests

8.1. Triple Sugar Iron Agar

Ingredients	**Gms / Litre**
Peptone	10.000
Tryptone	10.000
Yeast extract	3.000
HM Peptone B#	3.000
Lactose	10.000

Sucrose	10.000
Dextrose (Glucose)	1.000
Sodium chloride	5.000
Ferrous sulphate	0.200
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000
Final pH (at 25°C)	7.4±0.2

Procedures

Suspend 64.52 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the medium to set in sloped form with a butt about 1 inch long.

8.2. Urea Agar Base

Ingredients	**Gms / Litre**
Dextrose	1.000
Peptic digest of animal tissue	1.500
Sodium chloride	5.000
Monopotassium phosphate	2.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.8±0.2

Procedures

Suspend 24.51 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50 ml of sterile 40% Urea Solution (FD048) and mix well. Dispense into sterile tubes and allow to set in the slanting position. Do not overheat or reheat the medium as urea decomposes very easily.

8.3. Tryptophan Medium

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Sodium chloride	5.000
DL-Tryptophan	1.000
Final pH (at 25°C)	7.5±0.2

Procedures

Suspend 16 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

8.4. MR-VP Medium

Ingredients	Gms / Litre
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2

Procedures

Suspend 17 grams in 1000 ml of distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

8.5. Simmons Citrate Agar

Ingredients	Gms / Litre
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25°C)	6.8±0.2

Procedures

Suspend 24.28 grams in 1000 ml distilled water. Heat, to boiling, to dissolve the medium completely. Mix well and distribute in tubes or flasks. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

9. Media for Antimicrobial Sensitivity Test

9.1. Mueller Hinton Agar

Ingredients	**Gms / Litre**
Acicase	20.000
Agar	17.000
Final pH (at 25°C)	7.3±0.1

Procedures

Suspend 38.50 grams in 1000 ml purified / distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

10. Polymerase Chain Reaction

Add A 22µl reaction volume in a PCR master mix with a negative control (20 µl of sterile deionized water), and a positive control (5 µl of known DNA sample). Perform the amplification of the reaction mixtures in Eppendorf thermal cycler with a temperature program consisting of the initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°c for 45 minutes and polymerization at 72°C for 1 minute. Conduct the final elongation step at 72°C for 7minutes and then keep under 4°C until the machine got off.

11. Agarose Gel Electrophoresis

11.1 Preparation of Agarose gel (2%)

1. Weigh out 2g of agarose into a 250 ml conical flask.
2. Add 100 ml of 1x TAE buffer.
3. Swirl to mix.
4. Dissolve the agarose using a microwave oven for about 1-2 minutes.
5. Leave it to cool for 5 minutes. (While the agarose is cooling, prepare the gel casting tray ready on a level surface).

6. Add 2µl of EtBr to the agarose solution and mix well. (when the solution stops fuming).
7. Pour the gel slowly into the gel casting tray.
8. Remove any air bubbles away to the side using the comb.
9. Insert the comb and check whether it is correctly positioned.
10. Leave to set for at least 30 minutes with its lid on.
11. After it becomes completely solidified, carefully remove the combs and place the gel in the proper position in the electrophoresis chamber.
12. Pour 1x TAE buffer into the gel tank to submerge the gel so that there is about 2-3 mm of buffer over the gel.
13. Carefully pipette out 3µl of DNA sample over a parafilm strip on which 3µl of loading dye has been placed. (For Ladder DNA: Pipette 10µl of Ladder DNA into the gel)
14. Mix well with a pipette.
15. Load the samples one by one into the wells.

11.2. Running the Gel

1. Place the lid of the apparatus.
2. Connect the electrodes to the power supply. Make sure the positive and negative electrodes are properly connected.
3. Turn voltage to 50 V; after the DNA has moved out of the well, set the voltage to 120V.
4. Make sure that the current is running through the buffer by looking for air bubbles forming from each electrode.
5. Let the power run until the blue dye approaches 3/4th of the gel.
6. Turn off the power and disconnect the wire from the power supply.
7. Remove the lid of the electrophoresis chamber.
8. Using gloves, carefully remove the tray with the gel.
9. Observe it with the help of a gel documentation system.

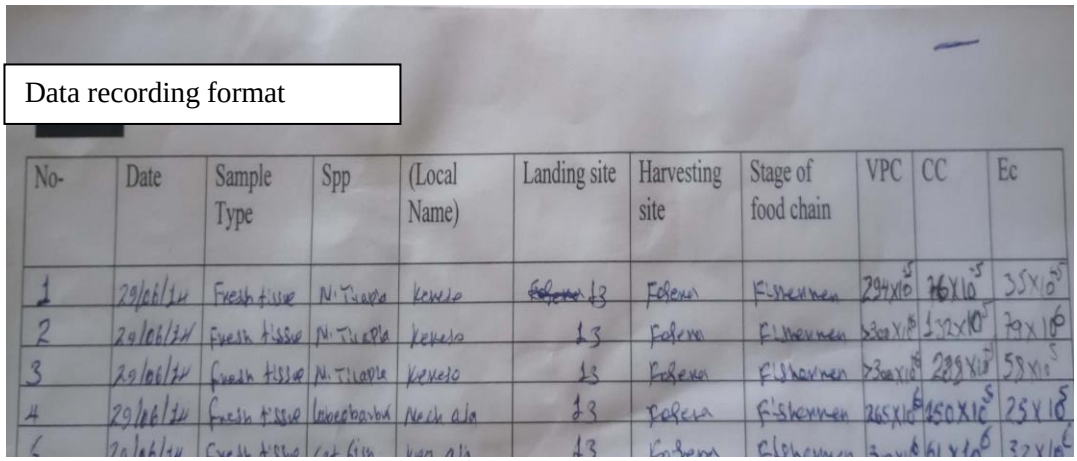
Appendix 2. Pictures of Laboratory equipment, sample collection, laboratory work and results



Hand Swab sampling



Retailing fish sample collection



Data recording format

No-	Date	Sample Type	Spp	(Local Name)	Landing site	Harvesting site	Stage of food chain	VPC	CC	Ec
1	29/06/14	Fresh tissue	N. Tilapia	Kereso	~~Fofera~~ 13	Fofera	Fishermen	294×10^{-5}	76×10^{-5}	35×10^{-5}
2	29/06/14	Fresh tissue	N. Tilapia	Kereso	13	Fofera	Fishermen	$>300 \times 10^{-5}$	132×10^{-5}	79×10^{-6}
3	29/06/14	Fresh tissue	N. Tilapia	Kereso	13	Fofera	Fishermen	$>300 \times 10^{-5}$	288×10^{-5}	58×10^{-5}
4	29/06/14	Fresh tissue	Labeobarbus	Nech aja	13	Fofera	Fishermen	265×10^{-6}	150×10^{-5}	25×10^{-5}
6	29/06/14	Fresh tissue	Cat fish	Kea aja	13	Fofera	Fishermen	[illegible]	61×10^{-6}	32×10^{-6}



Homogenizing the sample with Stomacher



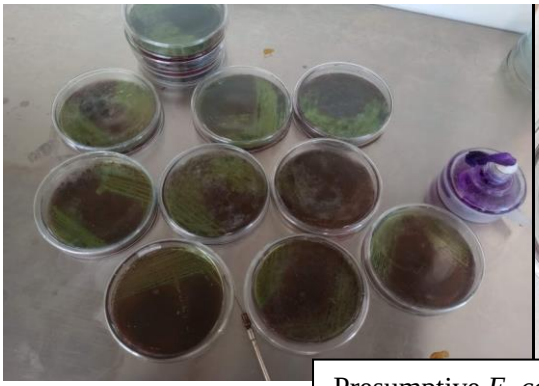
Media preparation for biochemical tests

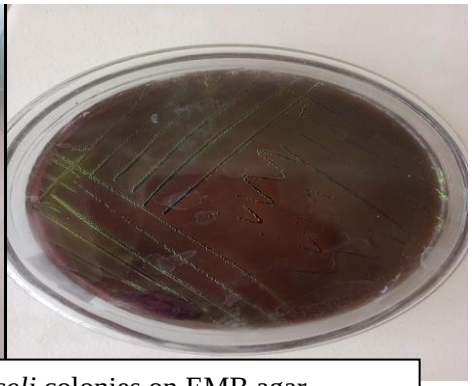


Total Coliform Counting with colony counter

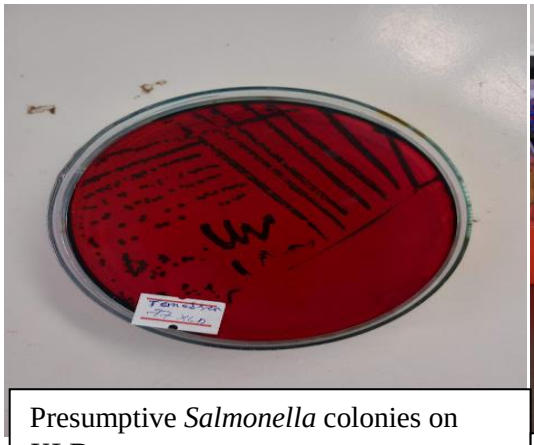


culturing in the Biosafety Level 2

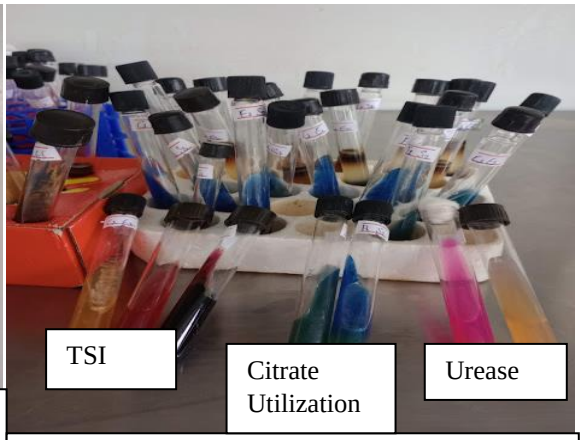




Presumptive *E. coli* colonies on EMB agar



Presumptive *Salmonella* colonies on XLD agar



Some of biochemical test results





Antimicrobial sensitivity test result measurement