



**PHENOTYPIC, MOLECULAR DETECTION AND ANTIBIOGRAM ANALYSIS
OF LISTERIA MONOCYTOGENES AND STAPHYLOCOCCUS AUREUS IN
CAPTURE FISHERIES IN SOUTH CENTRAL RIFT VALLEY LAKES OF
ETHIOPIA**

**WALLAGA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
SCHOOL OF VETERINARY MEDICINE**

MSC THESIS

BY

ADDISU DABE

**JUNE, 2026
NEKEMTE, ETHIOPIA**



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**JUNE, 2026
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APPROVAL SHEET FOR SUBMITTING FINAL THESIS

As members of the examining board of the final MSc thesis open defence, we certify that we have read and evaluated the thesis prepared by **Addisu Dabe**, entitled: " **Phenotypic, Molecular Detection and Antibigram Analysis of *Listeria Monocytogenes* and *Staphylococcus Aureus* in Capture Fisheries in South Central Rift Valley Lakes of Ethiopia**" and recommend it to be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

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Certification of the final thesis

I hereby certify that all the corrections and recommendations suggested by the board of examiners are incorporated into the final thesis entitled: " **Phenotypic, Molecular Detection and Antibigram Analysis of *Listeria Monocytogenes* and *Staphylococcus Aureus* in Capture Fisheries in South Central Rift Valley Lakes of Ethiopia**"." by Addisu Dabe.

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DECLARATION

This is to certify that this thesis, entitled: “**Phenotypic, Molecular Detection and Antibioqram Analysis of *Listeria Monocytogenes* and *Staphylococcus Aureus* in Capture Fisheries in South Central Rift Valley Lakes of Ethiopia**” was accepted in partial fulfilment of the requirements for the award of the degree of Master of Science by the school of graduate studies, Wallaga University, through the school of Veterinary Medicine; done by **Addisu Dabe**, was a genuine work carried out by him under my guidance. The assistance and help received during the course of this investigation have been duly acknowledged. Therefore, I recommend that it be accepted as fulfilling the research thesis requirements.

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STATEMENT OF THE AUTHOR

I declare that this thesis is my authentic and real work, that all sources and materials used for this thesis has been properly acknowledged. This thesis has been duly submitted in partial fulfilment of the requirements for MSc degree at Wallaga University, school of veterinary medicine and is deposited at the university/college/school library to be made available to borrowers under rules of the library. I sincerely declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate and allow access to users. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in a whole or in part may be granted the head of major department or the dean of the school when in his or her judgment the proposed use of material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

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LIST OF ABBREVIATIONS

AFNOR	Association Française De Normalisation
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
LLS	Listeriolysin
MLST	Multilocus Sequence Typing
PARC	Pan African Rinderpest Campaign
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RTE	Ready To Eat
SNNPR	Southern Nations, Nationalities, And Peoples' Region
SNPs	Single-Nucleotide Polymorphisms
SPSS	Statistical Packages For Social Science
USDA	United States Department Of Agriculture
WGS	Whole Genome Sequencing
WHO	World Health Organization

ABSTRACT

Listeria monocytogenes and *Staphylococcus aureus* contamination may result from inadequate quality control procedures during food processing, handling, and packaging. The objective of this study was to isolate and characterize *Staphylococcus aureus* and *Listeria monocytogenes* in fish samples taken from major fisheries in South Central Ethiopia. Using sterile methods, 300 fish tissue samples were methodically taken from Lake Abijata, Lake Ziway and Lake Hawassa. Bacteriological identification focused on *Listeria monocytogenes* and *Staphylococcus aureus*. Presumptive identification of *L. monocytogenes* was performed using colony morphology on Listeria Selective Agar following enrichment in Listeria Enrichment Broth (Half-Fraser). However, no *L. monocytogenes* isolates were recovered from 300 samples examined, suggesting possible low prevalence, seasonal effects. Sixteen isolates (5.33%) were identified as *S. aureus* based on characteristic colony morphology (yellow/golden-yellow colonies and yellow zone formation), beta-hemolysis on blood agar, Gram-positive cocci in clusters, and biochemical confirmation including catalase, coagulase, DNase, mannitol fermentation, and beta-hemolysis consistent with standard descriptions. All 16 *S. aureus* isolates were confirmed by MALDI-TOF MS, achieving scores between 2.15 and 2.48 (mean 2.31 ± 0.09), exceeding the ≥ 2.00 threshold. Real-time PCR (qPCR) targeting the *sec* gene (enterotoxin C) further validated species identity, with all isolates testing positive. Regarding sources, *s. aureus* detection was highest in live fish (7/150, 4.67%), moderate in market and ready-to-eat (RTE) fish (6/100, 6.0% and 3/50, 6.0%, respectively). Organ distribution in live fish showed greatest recovery from gills (42.86%), followed by muscle (28.57%), while intestine and kidney showed lower detection (14.29% each); spleen was negative. Antibioqram testing of selected isolates revealed resistance to penicillin and ampicillin (100%), with multi-drug resistance detected in 40% of tested isolates, highlighting antimicrobial resistance risks associated with *S. aureus* in fish. Based on these findings, the study recommends strengthening hygiene and handling practices through targeted training of fish handlers and processors; maintaining continuous microbiological surveillance of fish products and processing environments

Keywords: Fish, *Listeria monocytogenes*, *Staphylococcus aureus*, South Central Ethiopia.

1. INTRODUCTION

Fishery and aquaculture are rapidly growing industries in Ethiopia, with annual fish catches increasing by fivefold in the past twenty years (Anteneh *et al.*, 2023). The Ethiopian government is taking further action to boost aquaculture production through its "bounty of basket" project, which sees the establishment of large-scale intensive and small-scale semi-intensive commercial fish farms between 2024 and 2034. The recently launched National Blue Economy Strategy (2023-2027) also focuses on fisheries and aquaculture, with a major goal of promoting sustainable practices and conserving aquatic resources (MoTL, 2023). Aquatic initiatives are now seen as essential elements of rural and agricultural development plans (Ayana, 2022). With these initiatives in place, Ethiopia's fish catches are expected to exceed the estimated 100,000 tons per year in the coming years (Tesfaye and Wolff, 2014). The rapid expansion of aquaculture plays a substantial contribution to human nutrition, alleviating poverty, and promoting sustainable development (Alayu *et al.*, 2015). However, such expansion initiatives should be coupled with strong aquatic biosecurity and robust early warning system to sustain the striving aquatic sectors (Aich *et al.*, 2022; Ernst and Peeler, 2020; Natea, 2019; Rothuis *et al.*, 2012; Wang *et al.*, 2023). Emerging diseases are the major bottleneck to the development of the aquaculture industry (Stentiford *et al.*, 2017).

Globally, the risk of disease outbreaks, as well as the emergence and reemergence of aquatic diseases, is exacerbated by production growth, the domestication of new aquatic animal species, and increased trade (Surachetpong *et al.*, 2020; OIE, 2021). Emerging and re-emerging infectious diseases pose a major threat to fish farms and wild fisheries, costing \$6 billion per year globally (Wrobel *et al.*, 2019). As demand for fish rises and aquaculture trade intensifies, so too does the risk of environmental contamination, disease transmission, and new fish-borne zoonosis (Hutson *et al.*, 2023; Ziarati *et al.*, 2022). Emerging zoonotic pathogens of fish are a major food safety concern with serious public health and economic consequences (FAO, 2021). Climate change and the growing demand for raw, undercooked, and unusual foods have been identified as the primary causes of the rising incidence, geographic range, and frequency of zoonotic fish-related health issues (FAO, 2021; Hutson *et al.*, 2023).

Listeria monocytogenes is what causes listeriosis, a disease that affects both humans and animals and is one of the most significant new bacterial zoonotic illnesses in the world. One

of the most important pollutants in raw agricultural inputs and food processing is *Listeria monocytogenes*, an environmental foodborne pathogen (Tiensuu *et al.*, 2019). The mortality rate for these individuals can be as high as 20–30% (Choi *et al.*, 2018). In recent years, it has caused significant outbreaks associated with fresh produce, deli meats, and various RTE items globally (Bland *et al.*, 2022). The most common source of *L. monocytogenes* contamination worldwide is gravled and smoked fish, although listeriosis is frequently linked to the consumption of tainted RTE food products, including cheese, meat, and fish products (Lachmann *et al.*, 2022; Lopez-Valladares *et al.*, 2018; Lambertz *et al.*, 2012; Ricci *et al.*, 2018). In other nations, people are thought to be exposed to *L. monocytogenes* mostly through salmon and salmon products (Gillesberg Lassen *et al.*, 2016; Lunestad *et al.*, 2013).

Most patients with *Listeria monocytogenes* experience flu-like symptoms. However, the pathogen causes fetal-placental infection and central nervous system infection in the vulnerable population (elderly, immunocompromised, and pregnant patients (Lecuit *et al.*, 2020). *L. monocytogenes* can pass important barriers in a host, namely the intestinal epithelium, the blood–brain barrier, and the placenta, and subsequently spread to other organs (Chen *et al.*, 2009; Ireton *et al.*, 2021). Internalin, which is encoded by the *inlAB* operon, and pathogenic islands (LIPI-1, LIPI-3, and LIPI-4) are the primary genetic variables that determine the capacity to produce listeriosis infection. Important genes that encode adhesion, internalization, intracellular survival, and dispersion are concentrated in the *inlAB* operon and LIPI-1 (Quereda *et al.*, 2021; Vilchis-Rangel *et al.*, 2019; Quereda *et al.*, 2016). LIPI-4 is a cluster containing six genes encoding a sugar transport system involved in neural and placental infection (Maury *et al.*, 2016; Schiavano *et al.*, 2022).

Staphylococcus aureus is another critical foodborne disease that is extremely dangerous to the general public's health, especially in fish and seafood products. It is a Gram-positive bacterium known for producing heat-stable enterotoxins, which can lead to staphylococcal food poisoning (Basti *et al.*, 2003; Hayes, 1995). This bacterium can enter food through contact with contaminated surfaces or food handlers and thrives across a wide range of temperatures (7°C to 48.5°C) and pH levels (4.2 to 9.3) (Cleveland *et al.*, 2001). Staphylococcal food poisoning typically manifests rapidly, with symptoms such as nausea, vomiting, diarrhea, and abdominal cramps appearing within hours of consumption (Wen *et al.*, 2018). Though generally not life-threatening, the illness can cause severe discomfort, especially in vulnerable populations (Basti *et al.*, 2003).

The pathogenicity of *S. aureus* is attributed to various virulence factors, including adhesins, toxins, immunomodulators, and enzymes. Key toxins, such as hemolysins and Pantone-Valentine leukocidin (PVL), contribute to tissue damage and immune evasion (Lindsay, 2010). Genetic characterization through techniques like pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing (WGS) reveals diverse sequence types and clonal complexes among *S. aureus* isolates, highlighting their epidemiological significance (Baker *et al.*, 2018).

The germs that make up *Listeria monocytogenes* are diverse. Using PCR or MLST (multilocus sequence type), this disease can be classified into 14 distinct sero-groups that are common. However, those techniques have poor discriminatory power because they rely on only a few genes. The gold standard PFGE technique can distinguish between several strains, but it is time-consuming and necessitates great attention to detail to prevent mistakes. The characterization of core genome MLST (cgMLST) is presently the most suitable method because of whole genome sequencing (WGS) (Schiavano *et al.*, 2022). Because WGS offers the greatest discriminatory capability, it allows for the detection of virulence genes, their characterization, and their association with documented disease severity. Both *Listeria monocytogenes* and *Staphylococcus aureus* present significant challenges in ensuring the safety of fish and seafood products. Given their prevalence in aquatic environments and the potential for contamination during processing, effective control measures are vital. Preventative strategies should include strict adherence to good hygiene practices throughout the supply chain, proper food handling, and regular monitoring for these pathogens to mitigate public health risks.

1.1. Statement of the Problem

The increasing incidence of foodborne illnesses linked to contaminated fish products poses a significant public health risk, particularly in regions where fish consumption is common. In South Central Ethiopia, the consumption of catfish, a popular aquatic species, has been associated with various health concerns due to potential contamination with pathogenic microorganisms. Among these, *Listeria monocytogenes* and *Staphylococcus aureus* are notable for their virulence and ability to cause severe infections in humans (Feng *et al.*, 2020). Zoonotic *Listeria monocytogenes* poses a significant public health risk, particularly in regions where fish products are a staple in the diet (Dhama *et al.*, 2015).

Despite the importance of catfish as a dietary protein source, there is a paucity of research focusing on the phenotypic and genotypic characterization of these pathogens in catfish products within major fisheries in the region. This lack of data hinders the implementation of effective food safety measures and exposes consumers to health risks. Therefore, it is crucial to investigate the prevalence and characteristics of *listeria monocytogenes* and *staphylococcus aureus* in catfish captured from these fisheries, as this could inform public health policies and improve food safety standards.

Public health issues are raised by the possibility of *L. monocytogenes* and *Staphylococcus aureus* contamination due to inadequate quality control methods during food processing, handling, and packaging (Carpentier & Cerf, 2011; Oliver *et al.*, 2005). Numerous researchers from throughout the world have reported cases of human listeriosis illness caused by ready-to-eat food over time (Lianou & Sofos, 2007). Furthermore, environmental factors in South Central Ethiopia, such as water quality and fishing practices, may influence the presence and characteristics of *L. monocytogenes* and *Staphylococcus aureus*. Understanding the phenotypic and genotypic diversity of this pathogen in both fish products and linked water samples will provide insights into its transmission dynamics and potential reservoirs. Previous studies in other regions have reported contamination in different food categories or different fish sources, but the specific microbiological burden in the study area—together with rapid and accurate confirmation of isolates using MALDI-TOF—has not been adequately documented. Therefore, this study was conducted to generate baseline evidence to support targeted food safety monitoring and improved handling practices in the local fish value chain.

1.2. Objectives

1.2.1. General Objective

To isolate, phenotypically and molecularly identify, and determine the antibiogram profiles of *Listeria monocytogenes* and *Staphylococcus aureus* from capture fisheries in the South Central Rift Valley Lakes of Ethiopia.

1.2.2. Specific objectives

- ✓ To isolate and phenotypically identify *listeria monocytogenes* and *staphylococcus aureus* from cat fish in the south central rift valley lakes of Ethiopia.
- ✓ To determine the antimicrobial susceptibility profiles of *L. monocytogenes* and *S. aureus* isolates to selected antibiotics of veterinary and human importance using the disc diffusion method.
- ✓ To characterize the phenotypic and genotypic traits of *L. monocytogenes* and *S. aureus* isolates using standard microbiological and molecular techniques.

2. LITERATURE REVIEW

2.1. *Listeria* Species and *Staphylococcus aureus*

According to Jadhav (2015), *Listeria* species (spp.) are small, Gram-positive rods that belong to the class Bacilli, order Bacillales, and phylum Firmicutes. These bacteria are facultatively anaerobic, non-spore producing, and measure approximately 0.5 μm in width and between 1 μm and 1.5 μm in length (Wieczorek *et al.*, 2012). They are classified within the Clostridium-Bacillus-Lactobacillus sub-branch, which also includes *Brochothrix thermosphacta* (Schmid *et al.*, 2014). Due to their peritrichous flagella, *Listeria* species exhibit motility at temperatures between 24 and 28 degrees Celsius, but they become non-motile beyond 30 degrees (Indrawattana *et al.*, 2011). These species exhibit positive catalase activity, although some exceptions have been observed (Cepeda *et al.*, 2006). *Listeria* spp. are negative for oxidase, urea, and indole, and they can hydrolyze aesculin (De Vasconcelos Byrne *et al.*, 2016). They can withstand salt concentrations (NaCl) as high as 20% (weight/volume) and flourish in a pH range of 4.4–9.6 (Holch *et al.*, 2013). Their preferred growth temperature spans from $-0.4\text{ }^{\circ}\text{C}$ to $45\text{ }^{\circ}\text{C}$, with an ideal of $37\text{ }^{\circ}\text{C}$, and they are capable of enduring low water activities ($a_w < 0.90$) (Liu, 2006). These growth characteristics contribute to their versatility, allowing them to thrive under extreme conditions in food-processing facilities, posing a significant challenge for the food industry (Ducey *et al.*, 2006).

The genus *Listeria* consists of 20 species, including *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and others (Den Bakker *et al.*, 2010). This classification is based on various analyses, including 16S ribosomal RNA, DNA sequencing, and multilocus enzyme analysis, which are useful for surveillance and epidemiological studies of foodborne listeriosis outbreaks (Chasseignaux *et al.*, 2001). Among these species, only *L. monocytogenes* is pathogenic to humans and animals, while *L. ivanovii* primarily affects ruminants and has rarely been associated with human infections (Orsi *et al.*, 2011; Zhang *et al.*, 2004). *L. seeligeri* and *L. welshimeri* have been implicated in sporadic cases of human listeriosis (Orsi *et al.*, 2011).

Although the phenotypes of *Listeria* species are similar, biochemical tests such as hemolysis, acid synthesis from D-xylose, L-rhamnose, mannitol, motility, and alpha-methyl-D-mannoside can differentiate them (Orsi *et al.*, 2011). The Christie–Atkins–Munch–Peterson test can also be used to distinguish *Listeria* species based on increased hemolysis in the presence of *Staphylococcus aureus* (FDA *et al.*, 2008).

Most *Listeria* species are environmental contaminants, primarily found in soil. However, they have also been discovered in sewage, water, and decomposing vegetation (Willaarts *et al.*, 2013). Various animals, including ruminants, birds, marine life, insects, and crustaceans, have been shown to harbor *Listeria* spp. (Van Vuuren, 2001). These species can be isolated from 1% to 7% of the intestinal material of healthy animals (Borucki and Call, 2003). Humans can also serve as carriers, with *Listeria* spp. isolated from 5% to 10% of healthy adults and 1.3% of younger individuals (Churchill *et al.*, 2006). Due to their ubiquitous and resilient nature, *Listeria* spp. can enter the food supply chain and contaminate a wide range of food products (Leong *et al.*, 2014).

In contrast, *Staphylococcus aureus* is a significant pathogen known for its ability to cause a variety of infections, including skin infections, pneumonia, and food poisoning. Its pathogenicity is largely due to various virulence factors, including enterotoxins responsible for foodborne illness (Kühn *et al.*, 2016). *S. aureus* can evade host immune responses through mechanisms such as producing protein A, which binds to the Fc region of antibodies and inhibits opsonization and phagocytosis (Foster, 2005).

Once inside the host, *S. aureus* adheres to tissues via surface proteins that facilitate adhesion to extracellular matrix components (Wardeck *et al.*, 2018). The bacterium can produce a range of toxins, such as hemolysins and leukocidins, leading to cell damage and contributing to tissue destruction. Furthermore, the ability of *S. aureus* to form biofilms on medical devices and tissues complicates treatment, as biofilms provide a protective environment that enhances bacterial persistence and resistance to antibiotics (Hall & Mah, 2017).

2.2. Pathogenicity of *Listeria monocytogenes*

Listeria monocytogenes has a unique capacity for internalization into host cells, which is critical for its infectious ability (Carvalho *et al.*, 2014). This pathogen can spread to various organs by overcoming significant barriers in the human host, including the placenta, blood-brain barrier, and intestinal epithelium (Chen *et al.*, 2009). The infection process involves several stages: adhesion and invasion of host cells, internalization, lysis of vacuoles, intracellular multiplication, and intercellular spread to adjacent cells (Vazquez-Boland *et al.*, 2001). Upon ingestion through contaminated food, *L. monocytogenes* demonstrates resilience against high acidity, bile salts, and host-derived inflammatory attacks and proteolytic enzymes (Jeyaletchumi *et al.*, 2012). After surviving this harsh environment, the pathogen

adheres to and enters phagocytic and non-phagocytic cells facilitated by surface proteins known as internalins (Carvalho *et al.*, 2014).

The pathogenicity of *Listeria monocytogenes* is further characterized by its ability to persist within phagocyte cells, employing mechanisms to evade immune responses (Wilson *et al.*, 2018). After internalization by macrophages, the bacterium escapes from the phagosomal vacuole with the aid of phosphatidylinositol-specific phospholipase (plcA) and a cytolysin called listeriolysin O (LLO). This allows *L. monocytogenes* to replicate in the cytoplasm, utilizing nutrients from the host. An actin-based motility machinery facilitates the organism's movement across the cytoplasm to neighboring cells, allowing for spread without exposure to extracellular immune surveillance (Neves *et al.*, 2013). The bacterial surface protein actin polymerization protein (ActA) is critical for this intracellular movement (Bonazzi, Lecuit, and Cossart, 2009).

In contrast, *Staphylococcus aureus* is another significant pathogen known for its ability to cause a wide range of infections, including skin infections, pneumonia, and food poisoning. Its pathogenicity is largely attributed to the production of various virulence factors, including enterotoxins, which are responsible for foodborne illnesses (Kühn *et al.*, 2016). *S. aureus* can evade the host immune response through several mechanisms, including the production of protein A, which binds to the Fc region of antibodies, inhibiting opsonization and phagocytosis (Foster, 2005).

Upon entering the host, *S. aureus* can adhere to host tissues via surface proteins that promote adhesion to extracellular matrix components (Wardeck *et al.*, 2018). Once established, the bacterium can produce a range of toxins, such as hemolysins and leukocidins, which damage host cells and contribute to tissue destruction. The ability of *S. aureus* to form biofilms on medical devices and tissues further complicates treatment, as biofilms provide a protective environment that enhances bacterial persistence and resistance to antibiotics (Hall and Mah, 2017).

2.2.1. Virulence Factors of *Listeria monocytogenes* and *Staphylococcus aureus*

The pathogenicity of *Listeria monocytogenes* is attributed to a wide range of virulence factors that operate at various stages of the human infection cycle (Jeyaletchumi *et al.*, 2012). Most of *L. monocytogenes*' virulence factors are grouped into genomic islands known as Listeria

pathogenicity island-1 (LIPI-1) along the chromosome (Denes *et al.*, 2014). Whole genome sequencing (WGS) has identified additional pathogenicity islands, LIPI-3 and LIPI-4, which also carry significant virulence factors. LIPI-1 contains six key virulence factors, including internalin (Hain *et al.*, 2012), phosphatidylinositol-specific phospholipase (Gilmour *et al.*, 2010), actin polymerization protein, and metalloprotease (encoded by *mpl*) (Jeyaletchumi *et al.*, 2012). The expression of these genes is regulated by the positive regulatory factor A (PrfA) (Rabinovich *et al.*, 2012).

The incidence of human listeriosis and the number of high-profile outbreaks resulting in fatalities have significantly increased in many countries (De Castro *et al.*, 2012). This rise is largely due to changing consumption behaviors, with many individuals consuming ready-to-eat (RTE) foods (Mateus *et al.*, 2013). Additionally, the globalization of food trade and demographic changes, such as an aging population and the prevalence of immune-compromising conditions, have heightened the risk of listeriosis (Wang *et al.*, 2012). The advent of sequencing methodologies for detecting and typing listeriosis outbreaks has also led to an increase in reported cases (Zuber *et al.*, 2019). Epidemiological studies indicate that human listeriosis is predominantly reported in high-income and industrialized countries, attributed to effective surveillance systems for foodborne diseases (Grace, 2015).

Incidences of listeriosis were particularly high in Greece (0.3), Sweden (0.6), Norway (1.0), France (0.6), and Scandinavia (0.2), with the higher intake of smoked fish in these nations and an increase in cases among individuals over 60 years of age explaining this trend (Food Authority, 2013). Surveillance reported 198 listeriosis-related deaths across 18 Member States. Furthermore, between 2010 and 2011, the EU conducted a comprehensive baseline study in 26 Member States to ascertain the prevalence of *L. monocytogenes* in food products sold in retail establishments (Food Authority, 2013). A total of 13,088 food samples, including smoked or gravad fish (3,053), meat products (3,530), and various cheese types (3,452), were tested. The prevalence varied among food products, with the highest recorded in fish (10.4%), followed by meat products (2.07%) and cheese (0.47%) (Food Authority, 2013).

On the other hand, *Staphylococcus aureus* is another significant pathogen known for its ability to cause a variety of infections, including skin infections, pneumonia, and food poisoning. Its pathogenicity is primarily attributed to the production of various virulence factors, including enterotoxins responsible for foodborne illness (Kühn *et al.*, 2016). S.

aureus can evade the immune response through several mechanisms, such as the production of protein A, which binds to the Fc region of antibodies, inhibiting opsonization and phagocytosis (Foster, 2005).

Upon entering the host, *S. aureus* adheres to host tissues via surface proteins that promote adhesion to extracellular matrix components (Wardeck *et al.*, 2018). Once established, the bacterium can produce a range of toxins, including hemolysins and leukocidins, which damage host cells and contribute to tissue destruction. The ability of *S. aureus* to form biofilms on medical devices and tissues complicates treatment, as biofilms provide a protective environment that enhances bacterial persistence and resistance to antibiotics (Hall and Mah, 2017).

2.3. *Listeria monocytogenes* and *S. aureus* in Animal Origin and Their Products

2.3.1. Prevalence of pathogens in fish products

Global Prevalence: Numerous studies have reported varying prevalence rates of *Listeria monocytogenes* and *Staphylococcus aureus* in fish products worldwide. These variations can be attributed to differences in geographical location, fish species, handling practices, and testing methodologies. Understanding the prevalence of these pathogens is crucial for assessing food safety and public health risks associated with fish consumption. *Listeria monocytogenes*, a bacterium associated with serious foodborne illness has been the subject of extensive research in fish products. A systematic review and meta-analysis revealed a pooled prevalence of 5.8% in raw fish and a significantly higher rate of 14.5% in ready-to-eat products. This indicates that while raw fish can harbor this pathogen, ready-to-eat fish products pose a greater risk due to potential contamination during processing and handling. In Northern Greece, a study reported an 11.5% prevalence of *Listeria monocytogenes* in fish samples, aligning with the aforementioned global findings (Zakrzewski *et al.*, 2023).

Further studies in Europe have documented the presence of *Listeria monocytogenes* in 1.6% of various fishery products, with particularly elevated rates in ready-to-eat fish, especially smoked varieties, which showed prevalence rates ranging from 34% to 60%. In Iran, the prevalence of *Listeria monocytogenes* in freshwater raw fish was reported at 7.6%, highlighting regional variations in contamination levels. Additionally, a study examining fish products in Giza markets found *Listeria monocytogenes* in 6.66% of frozen fish, 3.33% of

smoked fish, and 13.33% of ready-to-eat fish products, further emphasizing the need for vigilant monitoring of fish safety (Peratikos *et al.*, 2024).

In contrast, *Staphylococcus aureus*, another significant foodborne pathogen, has been detected at alarming rates in fish products. In a fish processing factory, *Staphylococcus aureus* was found in 74% of analyzed fish samples, indicating a critical concern for food safety in processing environments. Another research in Iran found that *S. aureus* was common in various seafood, showing an overall positivity rate of 27.83%. The highest levels of contamination were found in smoked fish (64.29%) and salted fish (55.71%), highlighting the dangers linked to certain processing techniques. In Poland, the occurrence rate of *S. aureus* in seafood samples was reported at 68.5%, further illustrating the widespread nature of this pathogen in fish products. Historical data from India also indicate that the prevalence of *S. aureus* in seafood has ranged from 9% to 23% over several decades, highlighting the persistent challenge of foodborne pathogens in this sector (Wu *et al.*, 2018).

Regional Prevalence: In Nigeria, recent studies have shown that *S. aureus* was present in 10.5% of fish samples, while *Listeria monocytogenes* was detected in a smaller percentage (Okoh *et al.*, 2020). In Ethiopia, research has underscored the necessity for more extensive surveys to assess the prevalence of these pathogens in locally consumed fish species (Abebe *et al.*, 2022). An assessment of fish retail areas in Addis Ababa revealed the presence of *Staphylococcus* spp. among other bacteria, indicating inadequate hygienic practices during fish handling and processing (Fatunla *et al.*, 2025). Furthermore, a review of food safety policies in Ethiopia emphasizes that unsafe food handling practices in traditional markets significantly increase the risk of contamination. Studies conducted in central Oromia also revealed unhygienic fish handling practices, such as washing filleted fish with lake water and the lack of cold chain facilities, exacerbating the risk of pathogen proliferation (Abebe *et al.*, 2022).

Several factors contribute to the prevalence of *Listeria monocytogenes* and *Staphylococcus aureus* in fish products. Poor hygiene and sanitation practices during processing and transportation are significant contributors to the introduction of *S. aureus* into seafood. Inadequate storage and refrigeration of perishable goods further exacerbate the risk of bacterial growth and food spoilage. Cross-contamination during storage and contact with contaminated surfaces can also lead to higher prevalence rates of these pathogens.

Additionally, environmental factors, such as the presence of animals and exposure to chemical and microbial contaminants in water sources, can significantly influence fish contamination levels (Kester *et al.*, 2024).

2.3.2. *Listeria monocytogenes* and *Staphylococcus aureus* in food-processing facilities

Listeria monocytogenes has been reported in various meat-processing facilities, including abattoirs, meat-processing plants, and butcheries (Carpentier & Cerf, 2011). Studies indicate that animals presented for slaughter are a significant source of initial contamination, which can lead to the introduction of *L. monocytogenes* into these facilities (Autio *et al.*, 2000; Churchill *et al.*, 2006; Lekkas, 2016; Nightingale *et al.*, 2004). For instance, Autio *et al.* (2000) characterized strains of *L. monocytogenes* from environmental samples in pig farms and found the same pulsotypes in carcasses, suggesting that contamination may occur during the production and processing cycles. Additionally, Nel, Van Vuuren, and Swan (2004) observed that animals frequently carry significant amounts of microorganisms, such as *L. monocytogenes*, on their surfaces and hooves, which may contaminate processing facilities without adequate hygiene practices.

In addition to animal sources, *L. monocytogenes* can enter meat-processing facilities through raw materials and personnel (El-Shenawy *et al.*, 2011; Pava-Ripoll *et al.*, 2012; Thévenot, Dernburg, & Vernozy-Rozand, 2006). Contamination can also arise from floors, floor drains, racks, and rollers within the facilities (Thévenot *et al.*, 2006). Once *L. monocytogenes* enters processing facilities, it is challenging to eradicate due to its ability to survive under various stress conditions such as low temperatures, pH fluctuations, and osmotic stress (Takahashi *et al.*, 2014). The bacterium's resistance to sanitation agents and its capacity to form biofilms further complicate control efforts (Carpentier and Cerf, 2011). Although cold storage (4 °C) is commonly used to inhibit bacterial proliferation, it can inadvertently support *L. monocytogenes* growth due to the bacterium's ability to thrive at low temperatures.

Sanitation practices significantly impact the frequency of *L. monocytogenes* in environments related to meat processing are not adhered to cleaning procedures, followed by disinfection, are crucial for reducing pathogen levels. However, even after cleaning, *L. monocytogenes* is frequently detected, indicating the persistence of certain strains and often inadequate cleaning (Cruz & Fletcher, 2011). Harborage sites—areas where sanitation agents are improperly applied—facilitate the survival of *L. monocytogenes* (Lourenço *et al.*, 2009). Studies have

shown that *L. monocytogenes* can withstand conventional sanitation agents, primarily due to biofilm formation, which allows it to persist in facilities for extended periods (Bremer, Monk, & Butler, 2002; Fouladynezhad *et al.*, 2013; Pan *et al.*, 2006).

Similarly, *Staphylococcus aureus* represents a significant risk in food-processing facilities. Known for its ability to produce enterotoxins, *S. aureus* can lead to food poisoning, particularly in meat products. This pathogen can be prevalent in various meat types due to poor hygiene practices during processing and handling. For instance, studies have reported that *S. aureus* was detected in up to 74% of meat samples analyzed; indicating serious food safety concerns (Wu *et al.*, 2018).

Staphylococcus aureus can enter processing environments through cross-contamination from equipment, surfaces, and personnel. Its ability to survive and thrive in such environments often stems from inadequate sanitation practices. The bacterium can also form biofilms on surfaces, including stainless steel and plastic, complicating eradication efforts. The persistence of *S. aureus* in food-processing facilities highlights the need for rigorous sanitation protocols and monitoring to mitigate the risks associated with this pathogen (Okoh *et al.*, 2020).

2.3.3. *Listeria monocytogenes* and *Staphylococcus aureus* in Retail Level

According to Sauders *et al.* (2016), the presence of *Listeria monocytogenes* in meat and meat products at the retail level does not always mean that contamination took place there. However, the persistence of strains and cross-contamination from personnel, surfaces, and equipment have been found to be important sources of *L. monocytogenes* in retail products. According to Gombas *et al.* (2003), compared to items that are pre-packaged at the abattoir, meat products that are handled, sliced, and packaged at retail establishments frequently have greater levels of *L. monocytogenes*. Food handlers' sanitary procedures have a major role in this predominance. To avoid cross-contamination or recontamination in retail establishments, meat handlers must practice good personal hygiene and make sure that contact surfaces and equipment are properly sanitized (Bogere and Baluka, 2014).

Similarly, *Staphylococcus aureus* poses a significant risk at the retail level. This pathogen is known for its capacity to produce enterotoxins that can lead to food poisoning, especially in meat products. Retail environments often present opportunities for *S. aureus* contamination due to inadequate hygiene practices. In a study examining various meat products, *S. aureus*

was detected in 74% of analyzed samples, highlighting serious food safety concerns (Jackson *et al.*, 2013). In Iran, a study showed that *S. aureus* was present in all seafood types, with an overall positivity rate of 27.83%. Notably, contamination rates were significantly higher in smoked fish (64.29%) and salted fish (55.71%) (Wu *et al.*, 2018). In Poland, the occurrence rate of *S. aureus* in seafood samples reached 68.5%, underscoring the widespread nature of this pathogen in meat products (Wu *et al.*, 2018).

2.3.4. *Listeria monocytogenes* and *Staphylococcus aureus* in Meat and Meat Products

Listeria monocytogenes: *Listeria monocytogenes* has been detected in a variety of meat products, highlighting the potential risks associated with the consumption of contaminated meats. For instance, a study by Vitas and Garcia-Jalon (2004) in Spain found *L. monocytogenes* in 34.9% of minced pork and beef products and 36.1% of poultry meat from 396 samples collected across 55 small meat-processing units. In Sweden, a survey analyzing 507 heat-treated ready-to-eat (RTE) meat products revealed that *L. monocytogenes* was present in 61% of heat-treated ham products, with lower rates found in turkey (12%), roast beef (9%), and sausage (7%) (Lambertz *et al.*, 2012).

In Estonia, a decade-long baseline survey conducted from 2008 to 2010 indicated that *L. monocytogenes* was prevalent in 18.7% of raw meat and meat products, and 2% in RTE meat products collected from various food-processing facilities (Kramarenko *et al.*, 2013). In Ireland, analysis of RTE meat samples between 2013 and 2014 found *L. monocytogenes* in 4.2% of meat products from poultry (Leong *et al.*, 2014). Additionally, Ismaiel, Ali, and Enan (2014) reported the presence of *L. monocytogenes* in beef carcasses, raw lean beef, frozen chicken meat, and camel meat. The pathogen has also been isolated in various meat products in studies conducted in India and Japan (Dhanashree *et al.*, 2003; Okutani *et al.*, 2004). In South Africa, Matle *et al.* (2019) reported *L. monocytogenes* in various meat and meat products, including 10.1% in raw intact meat, 19.5% in raw processed meat, and 13.5% in RTE meat products from cattle, pork, sheep, game, and poultry.

Staphylococcus aureus: Similarly, *Staphylococcus aureus* is another significant pathogen found in meat products. This bacterium is known for its ability to produce enterotoxins, which can lead to food poisoning. Studies have shown that *S. aureus* is prevalent in a range of meat types, with contamination often linked to poor hygiene practices during processing and handling. In a review of meat samples, *S. aureus* was detected in 74% of analyzed fish products, indicating a critical concern for food safety. A separate study in Iran reported *S.*

aureus in all seafood types, with an overall positivity rate of 27.83%, particularly high in smoked fish (64.29%) and salted fish (55.71%). In Poland, the occurrence rate of *S. aureus* in seafood samples was reported at 68.5%, illustrating the widespread nature of this pathogen in meat products. The presence of *S. aureus* in meat products can result from multiple factors, including cross-contamination during processing, inadequate cooking, and improper storage conditions. Additionally, the bacterium's ability to survive in various environments increases the risk of contamination throughout the meat supply chain (Thwala *et al.*, 2021).

2.4. Diagnosis

2.4.1. Diagnosis of *Listeria monocytogenes*

For the purpose of disease control and prevention, *L. monocytogenes* identification is crucial. Over time, the cold enrichment technique for detecting and isolating *L. monocytogenes* has given way to conventional and molecular technologies (World Organization for Animal Health 2014).

Conventional methods: To isolate and identify *L. monocytogenes* in food samples, a number of traditional techniques have been established. Antibody-based tests, enzyme-linked immunosorbent assays, culture-based procedures, and immune-capture techniques are all part of the traditional approach of isolating *L. monocytogenes* (Välimaa, Tilsala-Timisjärvi and Virtanen 2015). Among these techniques, culture-based tests are typically chosen for a variety of reasons, including their sensitivity, affordability, and continued status as the "gold standards" in comparison to other validated techniques (Barajas *et al.*, 2019). Furthermore, culture-based assays that yield pure colonies of the targeted organisms are helpful for managing outbreaks and conducting epidemiological surveillance (WHO & World Organisation for Animal Health 2014). Low resolution when it comes to differentiating bacterial strains is one of the disadvantages of culture-based techniques. These techniques are also time-consuming (Leong *et al.*, 2014), and phenotypic alterations due to environmental selection, contaminating bacteria, and atypical reactions by atypical strains might produce false-negative results (Välimaa *et al.*, 2015).

Selective agents and an enrichment process are used in culture-based approaches for the isolation and identification of *L. monocytogenes*. While the enrichment process enables the growth of *L. monocytogenes* to detectable levels and the repair of damaged or stressed cells, the selective agents serve to prevent other competing microflora (Chen *et al.*, 2017). There are three commonly used culture-based techniques for separating *L. monocytogenes* from

food due to international regulations and restrictions. These techniques include the One-Broth *Listeria* approach, the United States Department of Agriculture (USDA), and the International Standard (ISO) (Gasnov, Hughes & Hansbro 2005; Gómez *et al.*, 2013; Zhang *et al.*, 2004).

For the isolation of *L. monocytogenes* in a wide range of food and feed products as well as environmental samples, the ISO 11290 standard is advised (Liu *et al.* 2007). For the detection of *Listeria* spp. in ambient swabs and meat and poultry food products, the USDA approach is advised (Jeyaletchumi *et al.*, 2012). *Listeria* spp. are isolated using one Broth *Listeria* method that has been authorized by the Association Française de Normalization (AFNOR) from meat, seafood, veggies, and dairy products (Gasnov *et al.* 2005).

Conventional polymerase chain reaction: *L. monocytogenes* has been widely detected using the polymerase chain reaction (PCR) technique. The most prevalent and distinct genes of *L. monocytogenes*, including *hly*, *inlA*, *inlB*, *iap*, *plcA*, *plcB*, 16S and 23S rRNA genes, and *dth-18* delayed type hypersensitivity protein, are the targets of conventional PCR (Jadhav 2015). Because they are easy to use and can yield results quickly, conventional PCR techniques are more commonly employed than culture processes (Jeyaletchumi *et al.* 2012). Nevertheless, this PCR is unable to accurately identify low levels of *L. monocytogenes* (Jadhav 2015) or differentiate between living and dead cells, viable but not culturable cells, metabolically damaged, or stressed cells (Truter 2015). According to Quendera *et al.* (2016), an organism poses a risk to public health because a positive sample obtained using traditional PCR does not always indicate that the organism is alive and at the necessary concentrations.

Multiplex polymerase chain reaction: Another PCR-based technique that enables the simultaneous detection of numerous diseases or strains of the same species in a sample is multiplex PCR (Chen *et al.* 2017). The particular binding of the primer pair to the microorganism's target sequence determines the method's detection specificity. By focusing on distinct genes for each species, Ryu *et al.* (2013) created a multiplex PCR technique that can differentiate between five distinct *Listeria* species, including *L. monocytogenes*. According to Jadhav (2015), multiplex PCR can identify between 1 and 100 cfu/mL of *Listeria*; however, because it is unable to discriminate between living and dead cells, it may overstate the pathogen's abundance.

Real-time polymerase chain reaction: Unlike other PCR methods, real-time PCR allows for the observation of the amplicon as it builds up. The process tracks the fluorescence levels that

accumulate, which are based on the quantity of accumulated PCR product. The fluorescent molecule may be a non-specific DNA-binding dye or a target-specific probe labeled with a fluorescent dye and a quencher molecule. The technique is extremely sensitive, capable of detecting and tracking minimal quantities of target DNA, can be automated, and allows for quantification of bacterial load without any post-PCR processing. Nonetheless, its drawbacks include the potential for primer dimers to emit fluorescence, a strong reliance on primer concentration and design, and the necessity for strict quality controls.

Subtyping of *Listeria monocytogenes*: Subtyping is a method employed to differentiate various bacterial strains within the same species (Jeyaletchumi *et al.*, 2012). Subtyping methods are extremely helpful for identifying sources and tracking specific strains of *L. monocytogenes* linked to listeriosis outbreaks, as well as for assessing the population genetics, taxonomy, and epidemiology of this pathogen (Doumith *et al.*, 2004).

Serotyping: Based on the antigen-antibody interaction, serotyping is the initial technique used to distinguish strains of *L. monocytogenes* from one another (Ntivuguruzwa 2016). Using distinct combinations from somatic (O) and flagellar (H) surface antigens (antisera) reaction, *Listeria monocytogenes* is serotyped into 13 serotypes ($\frac{1}{2}$ a, $\frac{1}{2}$ b, $\frac{1}{2}$ c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) using the slide agglutination method (Doumith *et al.*, 2004). The value of this method in epidemiological studies is very limited because of poor discriminative powers (Gasnov *et al.*, 2005). Serotyping measures the phenotypic traits of *L. monocytogenes*, which are frequently subject to change and do not always accurately reflect the genotype of a microorganism. It is also linked to numerous disadvantages, such as the inability to produce consistent, dependable, and repeatable results (Liu 2006). Furthermore, serotyping is challenging, time-consuming, and requires high-quality antisera (Shaker & Hassanien 2015). *L. monocytogenes* and *L. Seeligeri* share antigens, which could result in the wrong serotype being identified (Liu 2006).

Genetic sub-typing methods

Polymerase chain reaction serogroup multiplex polymerase chain reaction assays: Borucki and Call (2003) then developed a multiplex PCR-based method to serotype *L. monocytogenes* into five PCR serogroups in order to overcome the drawbacks of slide agglutination and enzyme-linked immunosorbent assay (ELISA) serotyping. Serotypes $\frac{1}{2}$ a and 3a were represented by IIa, $\frac{1}{2}$ c by IIc, $\frac{1}{2}$ b by IIb, 3b, and 7, and IVb by IVb to 4b, 4d, and 4e (Doumith *et al.* 2004). This approach employs primer pairs that focus on five genes

(lmo0737, lmo1118, ORF2110, ORF2819, and prs) to characterize *L. monocytogenes* strains and categorize those PCR serogroups (Doumith *et al.* 2004; Nho *et al.* 2015). Nonetheless, this assay failed to differentiate between ½a and 3a, ½c and 3c, ½b, 3b and 7, 4a and 4c, as well as between 4b, 4d and 4e; however, since 3a, 3c, 3b, 7, 4a, 4c, 4d, and 4e are infrequently associated with human listeriosis, this method was deemed appropriate for quick identification of serotypes. While this approach is fast, reliable, and simple to apply, its discriminatory power is limited, resulting in low resolution for epidemiological typing (Nho *et al.* 2015).

Pulsed-field gel electrophoresis: PFGE is regarded as the 'gold standard' method for subtyping in source tracking and epidemiological studies of infections caused by *L. monocytogenes*. This is because of its strength, repeatability, and excellent discrimination power (Martín *et al.* 2014). However, it is labor-intensive and time-consuming; it often takes two to three days to finish and necessitates rather expensive equipment (Li *et al.* 2017). Few high molecular mass fragments are produced in PFGE since the genomic DNA is broken down (usually employing AscI and ApaI enzymes) with occasional cutting endonucleases (Kalpana & Muriana 2002). PFGE can be used to segregate these pieces according to size (Ruppitsch *et al.* 2015). DNA fragments smaller than 50 kb are typically separated by the PFGE because larger and more dispersed bands are produced by DNA fragments larger than 50 kb (Jersek *et al.*, 1999). Furthermore, it employs alternating electric fields that cause the DNA fragments to constantly shift orientation. High molecular weight DNA is resolved into distinct bands as a result (Hopkins, Arnold & Threlfall 2007).

Multiple-locus variable number of tandem repeat analysis: The concept of the multiple-locus variable number of tandem repeats analysis (MLVA) is to identify differences in the count of tandem repeats (VNTRs) at a specific locus in the genome's DNA. PCR amplification can be used to identify the VNTRs, which are brief DNA sequences with varying copy numbers. Next, agarose gels and/or DNA sequencing devices are used to measure the size of the PCR result. The number of repeats in each location is then ascertained using the PCR product sizes (Volpe Sperry *et al.* 2008). Thus, a multidigit, strain-specific code (profile) can be obtained by integrating the size differences from many repeat locus regions; these profiles can then be utilized for cluster analysis (Møretrø *et al.*, 2013; Murphy *et al.* 2007).

The MLVA became a preferred subtyping technique for bacterial isolates due to its reliability, speed, and significant discriminatory ability (Camargo *et al.* 2016). As stated by various authors (Chenal-Francisque *et al.*, 2015; Lindstedt *et al.*, 2008; Murphy *et al.*, 2007), MLVA may serve as an effective means for identifying *L. monocytogenes* strains and is an attractive preliminary screening technique for epidemiological studies and listeriosis monitoring. The primary disadvantage of MLVA, however, is that its results cannot be compared between laboratories due to its lack of reproducibility. Furthermore, VNTRs are unstable and may even change during standard laboratory sub-culturing, which has an impact on the MLVA method's reproducibility.

Multi-locus sequence typing: For *L. monocytogenes* typing, several multilocus sequence typing (MLST) techniques have been reported (Den Bakker *et al.* 2013; Roberts *et al.* 2018). To distinguish between subtypes, the MLST relies on several gene segments or genes (Jeyaletchumi *et al.*, 2012). Seven loci from housekeeping genes are amplified and then examined for nucleotide variations. When compared to the gold standard for *L. monocytogenes* typing, the PFGE approach has been demonstrated. Moreover, MLST is viewed as a costly and lengthy approach since it demands multiple sequencing reactions for each isolate, lacks multiplexing capability, and does not provide sufficient discriminatory power for the 4b *L. monocytogenes* serotypes (Jadhav 2015), which are frequently associated with outbreaks. Direct analysis of single-nucleotide polymorphisms (SNPs) may provide a more effective option for DNA sequence-based subtyping, considering that most sites sequenced for MLST are stable (Ducey *et al.* 2006).

Whole-genome sequencing: Although the aforementioned phenotypic and molecular approaches have many benefits, their selective power limits the amount of information they can provide about the pathogenic organism (Lekkas 2016). This obstacle can be overcome by the WGS, which can classify isolates into groups that are relevant to epidemiology. According to Fox *et al.* (2016), the WGS has been suggested as the new gold standard for subtyping *L. monocytogenes* strains linked to outbreaks. With the advent of high-throughput next-generation sequencing technology and a decrease in processing time, this method has significantly improved since its beginnings. Additionally, the technology is becoming easier to use and less expensive. WGS may quickly generate whole-genome sequence data in addition to epidemiological data, which can aid in identifying targets for potential assay development (Lekkas, 2016).

Before these methods can be used in food-borne pathogen detection, there are still significant obstacles that must be overcome, even if the use of WGS offers useful information that was not previously available in such a short amount of time (Lekkas, 2016). There is still a need for computer platforms that are user-friendly, strong enough to manage the enormous databases that are produced, and simple to understand. Furthermore, there is disagreement over how regulatory bodies like the FDA and CDC will use or preserve these data during inspections or outbreak. There will very certainly be some sequence data in the amount of data gathered that could be mistakenly interpreted as suggesting a health risk (Eruteya & Odunfa, 2014). The WGS approach's other drawbacks include the requirement for highly qualified bioinformatics specialists, which smaller businesses cannot afford, the absence of standardized and validated protocols similar to those already in place for PFGE, the absence of reference databases, and, finally, a significant resource investment, which has its own drawbacks (Orsi *et al.*, 2011). Furthermore, food-borne infections are typically discovered in extremely small and quantity, which presents further challenges for epidemiological research (Lekkas, 2016).

2.4.2. *Diagnosis of Staphylococcus aureus*

Staphylococcus aureus is diagnosed using a combination of culture-based and molecular methods. Culture-based methods typically involve inoculating samples onto selective media, such as mannitol salt agar, which inhibits growth of competing organisms while allowing *S. aureus* to form distinctive colonies (Foster, 2005). The identification of *S. aureus* can also be confirmed through biochemical tests, such as coagulase production, which distinguishes it from other coagulase-negative staphylococci (Kühn *et al.*, 2016).

Molecular methods, including PCR, have gained popularity for their rapidity and specificity. Conventional PCR can target specific genes such as *nuc* (nuclease) and *mecA* (methicillin resistance), providing quick identification of *S. aureus* and its resistance profile (Truter, 2015). Real-time PCR further enhances detection sensitivity and allows for quantification of bacterial load, which is crucial in outbreak scenarios (Hall & Mah, 2017).

Multiplex PCR methods can simultaneously detect multiple *S. aureus* strains or associated pathogens, improving diagnostic efficiency. The advent of WGS has revolutionized *S. aureus* subtyping, enabling comprehensive epidemiological tracking and characterization of strains linked to infections (Leong *et al.*, 2017).

2.5. Treatment of *Listeria monocytogenes* and *Staphylococcus aureus* infection

Since *L. monocytogenes* may infiltrate nearly every cell type, treating human listeriosis can be difficult (Dhama et al. 2015). Furthermore, the lengthy incubation time of *L. monocytogenes*, which causes the treatment period to fluctuate depending on the severity of the infection, renders human listeriosis treatment frequently ineffective. Nonetheless, human listeriosis has long been well treated with antibiotics (Al-Nabulsi et al. 2015).

Most antibiotics are generally effective against *Listeria monocytogenes*, although cephalosporin, fosfomicin, and fluoroquinolones do not work against this bacterium (Noll, Kleta & Al 2018). The absence or poor affinity of the enzyme catalyzing the last stage of cell wall formation is the origin of *L. monocytogenes*' inherent resistance to these antibiotics (Al-Nabulsi et al. 2015). The preferred antibiotic for treating human listeriosis is ampicillin or penicillin G in combination with an aminoglycoside such as gentamicin. Trimethoprim in combination with a sulfonamide, such as sulfamethoxazole-co-trimoxazole, is considered second choice of therapy (Kovacevic et al. 2013). Additionally, human listeriosis has been treated using tetracycline, erythromycin, and vancomycin (Rip 2011). However, *L. monocytogenes* has significantly expedited the development of bacteria toward resistance (Moreno et al. 2014).

2.5.1. Antimicrobial resistance in *Listeria monocytogenes*

The overprescription of medications in clinical settings, their extensive use as growth promoters for farm animals, and increased international trade and travel are all factors contributing to the acceleration of antimicrobial resistance in *L. monocytogenes*. These factors also encourage the spread of antimicrobial resistance across nations and continents (Moreno et al. 2014; Zhang et al. 2004). Strains of *L. monocytogenes* resistant to first-line antibiotics have been documented. Clinical strains of *L. monocytogenes* resistant to gentamicin were documented (Walsh et al. 2001). A strain of *Listeria monocytogenes* resistant to ampicillin was discovered in the US (Verraes et al. 2013).

Clinical isolates from various countries have also reported *Listeria monocytogenes* resistant to rifampin, sulfonamide, kanamycin, erythromycin, and streptomycin (Moreno et al. 2014; Zhang et al. 2004). Drug resistance has been noted in strains obtained from food and

environmental sources globally (Morobe *et al.*, 2009). Research done in Northern Ireland (Walsh *et al.*, 2001) revealed 0.6% of *L. monocytogenes* in retail food products. Additionally, the antimicrobial resistance of *L. monocytogenes* obtained from food and animal sources (n = 167) in the US was analyzed. The resistance to ciprofloxacin, tetracycline, sulfonamide and nalidixic acid were 1.8%, 9%, 73% and 100%, respectively (Zhang *et al.* 2004).

2.5.2. Treatment of *Staphylococcus aureus*

Staphylococcus aureus presents its own challenges in treatment, largely due to its ability to develop resistance to multiple antibiotics. The primary treatment for infections caused by *S. aureus* typically involves beta-lactam antibiotics, such as methicillin, but the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has complicated treatment (Kühn *et al.*, 2016). Resistance to methicillin is primarily conferred by the *mecA* gene, which encodes a penicillin-binding protein with low affinity for beta-lactam antibiotics (Fowler *et al.*, 2015).

In cases of serious infections, vancomycin is often used as a first-line treatment, although resistance to vancomycin has also been reported (Liu *et al.*, 2011). Other treatment options may include linezolid, daptomycin, and ceftaroline, which are effective against MRSA (Kühn *et al.*, 2016). However, treatment regimens may vary based on the site of infection, severity, and patient factors. The rise of antibiotic resistance in *S. aureus* is fueled by overuse and misuse of antibiotics in clinical settings, as well as in agriculture (Fowler *et al.*, 2015). Surveillance of *S. aureus* resistance patterns is crucial to inform treatment decisions and manage outbreaks effectively (Kühn *et al.*, 2016).

2.6. Control of *Listeria monocytogenes* and *Staphylococcus aureus*

Since there aren't many treatment options due to the pathogen's rapid development of antibiotic resistance, various alternative techniques and therapies have been investigated to lessen the presence of *L. monocytogenes* in foods. In food, food-processing facilities, and humans, bacteriophages have been used as biocontrol agents for *L. monocytogenes*, bacteriocins, and essential oils (Klumpp & Loessner 2013; Soni *et al.* 2014). Since probiotics improved host immunity, it has also been demonstrated that they suppress the growth of *L. monocytogenes* (Dhama *et al.* 2015).

During processing and packing, bacteriophages—viruses that can kill bacteria—were identified as potential biocontrol agents for *L. monocytogenes* in beef and meat products

(Strydom 2015). Without negatively affecting the final consumer's natural microbiota or other desirable bacteria in the meal, bacteriophages demonstrated a high degree of specificity in lysing strains of *L. monocytogenes* (Dhama *et al.* 2015). Additionally, bacteriophages are stable in long-term cold storage and have the ability to self-replicate (Strydom 2015). This implies that after processing, bacteriophages will still be effective against *L. monocytogenes*. Several commercial bacteriophage-based products, such as Phage LM-103a, phage LMP-102a, Strydom (2015), Ply511 phage, ListShield, ListexP-100, and ListexTM (Dhama *et al.* 2015), have been developed for the biocontrol of *L. monocytogenes* in food based on these desirable attributes (Leong *et al.* 2017). In various nations, these products have been shown to effectively combat *L. monocytogenes* in food and food facilities. For instance, ListexP-100 and ListShield are employed to manage *L. monocytogenes* in the Netherlands and the United States, respectively (Dhama *et al.* 2015). Nonetheless, other nations like SA continue to prohibit the use of bacteriophage-based products in food items and food-processing facilities (Strydom 2015).

Bacteriocins are antimicrobial peptides synthesized by ribosomes that can impair the integrity of the target cell membrane by creating pores in it. Bacteriocins like nisin are utilized in meat items to prevent the proliferation of *L. monocytogenes* (Rahimi *et al.*, 2012). Bacteriocins may inhibit either closely related species or a wide array of unrelated species. Nisin has recently been found to combat *L. monocytogenes* effectively, and there is a lack of data to thoroughly understand the possible use of other bacteriocins in controlling food-borne pathogens (Leong *et al.*, 2017). Additionally, essential oils derived from plant extracts may have antibacterial qualities that could lower *L. monocytogenes* survival in a variety of goods. These essential oils come from *Cinnamomum cuspidatum* and *Cinnamomum crassinervium* (Dhama *et al.*, 2015), as well as thyme, rosemary, and oregano (Hilliard *et al.*, 2018).

Controlling *Staphylococcus aureus* in food and clinical settings is critical due to its propensity to cause foodborne illnesses and infections. Prevention strategies primarily focus on hygiene practices, such as proper handwashing, sanitation of food preparation surfaces, and safe food handling techniques to minimize contamination (Kühn *et al.*, 2016).

In food processing, the use of bacteriocins, such as nisin and pediocin, has also been investigated for their effectiveness against *S. aureus*. These bacteriocins can inhibit the growth of *S. aureus* in various food products, providing an additional layer of protection against contamination (Fowler *et al.*, 2015). Probiotics may also play a role in controlling *S.*

aureus by enhancing gut microbiota and immune responses. Some strains of probiotics have been shown to inhibit the growth of *S. aureus* in vitro, suggesting potential for use in food products (Kühn *et al.*, 2016). Essential oils, similar to their use against *L. monocytogenes*, have demonstrated antibacterial activity against *S. aureus*. Oils derived from oregano, thyme, and tea tree have been reported to inhibit the growth of this pathogen in food contexts (Burt, 2004).

2.7. Resistance of *Listeria Monocytogenes* and *Staphylococcus Aureus* in Fish Samples

A key feature contributing to risk is *L. monocytogenes*' ability to grow at refrigeration temperatures and tolerate low pH and high salt environments typical of some food-processing settings. This tolerance means contamination in fish products can persist even during cold storage, which is commonly used to slow microbial growth. *L. monocytogenes* can persist in food-processing environments by forming biofilms and attaching to surfaces. In seafood processing facilities, biofilms can allow survival of the organism through cleaning cycles, increasing the chance of recurring contamination of fish batches (Tompkin, 2002). While resistance patterns vary by region and strain, *L. monocytogenes* infections can be difficult to treat in high-risk patient groups. Clinical management often relies on antibiotics that are effective against invasive listeriosis; however, resistance surveillance is important because resistance can emerge under selective pressures (Chakraborty *et al.*, 2009).

Staphylococcus aureus is commonly associated with skin and nasal carriage in humans and can be introduced into fish handling areas through contact with workers, contaminated equipment, or poor hygiene practices. The organism can survive certain processing conditions, and its stress tolerance can support persistence on fish surfaces and in contaminated environments. Like *L. monocytogenes*, *S. aureus* can form biofilms. Biofilms protect bacterial cells from environmental stresses and can reduce the effectiveness of sanitizers used during processing. This increases the risk of contamination continuing across production cycles. Some *S. aureus* strains exhibit resistance to beta-lactam antibiotics (e.g., methicillin-resistant *S. aureus*, MRSA). Although the relationship between MRSA in food and clinical cases depends on context, the possibility of resistant strains in food chains makes monitoring essential. In addition, *S. aureus* can produce heat-stable enterotoxins, which may remain after bacterial inactivation, complicating prevention strategies (Harris *et al.*, 2010).

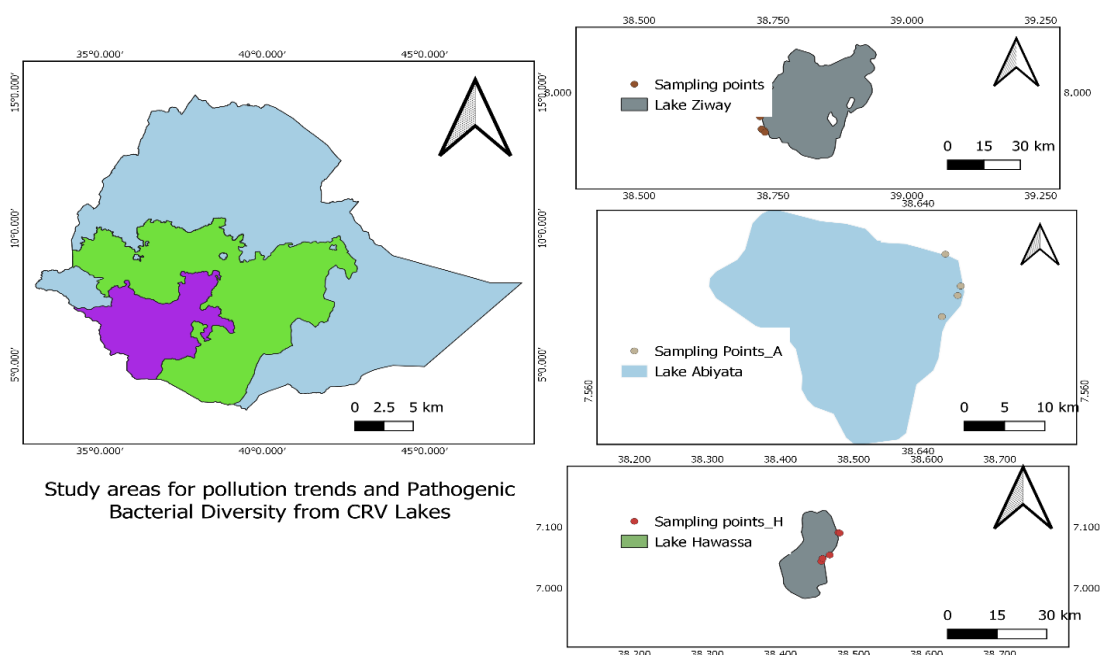
3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in purposively selected lakes of the Rift Valley, focusing on sites known for significant fish harvesting. The study was conducted in the selected major fisheries lakes and rivers in South Central Ethiopia. South Central Ethiopia is a diverse and vibrant region characterized by its rich cultural heritage, varied landscapes, and significant agricultural activities. The following lakes were sampled: At an elevation of 1636 meters above sea level, **Lake Batu** is located in the Adami Tulu Jido Kombolcha district of Ethiopia's Great East African Rift Valley Lakes, 165 kilometers south of Addis Ababa. Its open water area is 434 square kilometers, its shoreline is 137 kilometers long, and its minimum and maximum depths are 2.5 and 9 meters, respectively (Bayissa *et al.*, 2021). The Ziway watershed spans over 7300 km² and is located between latitudes 7°15'N and 8°30'N and longitudes 38°E and 39°30'E. The Meki River from the northwest and the Katar River from the east supply it with water, while the Bulbula River empties it into Lake Abijata (Desta and Lemma, 2017). With a brief wet season from March to May and a long rainy season from June to September, followed by a dry season from October to February, the region experiences an average annual rainfall of 700 mm. The region has a relative humidity of 60% with maximum and minimum temperatures of 27.2°C and 12.7°C, respectively (Desta, 2021). Tulu Gudo (4.8 km²), Tsedecha (2.1 km²), Funduro (0.4 km²), Debre Sina (0.3 km²), and Galila (0.2 km²) are the five larger islands that make up Lake Ziway (Dadebo *et al.*, 2013). The species found in Ziway Lake include Nile tilapia (*Oreochromis niloticus*), Barbus species, Catfish (*Clarias gariepinus*), Carp species, Redbelly tilapia (*Tilapia zillii*), African big barb (*Barbus intermedius*), African sharptooth catfish (*Clarias gariepinus*), and Carassius spp. (*Carassius carassius* and *Carassius auratus*) (Nakayama and Ishizuka, 2014; Esta *et al.*, 2017). **Lake Abijatta** is a basic lake located in Ethiopia. It is situated in the Main Ethiopian Rift valley to the south of Addis Ababa, within the Abijatta-Shalla National Park

Lake Hawassa is situated 1,680 meters above sea level, with a surface area of 90 km², a mean depth of 11 meters, and a maximum depth of 22 meters, in the southeast of Ethiopia at 6°33'–7°33' N, 30°22'–38°29' E (Worako, 2015). It is located amidst a number of lakes in the Rift Valley and is one of Ethiopia's major wet land resources. The lake is located west of Hawassa town, and both the city and the people who live in the whole watershed depend heavily on its survival and preservation. It encourages commercial fishing, and *Oreochromis*

niloticus (*Tilapia nilotica*), the lake's predominant fish, is the subject of a sizable commercial fishery (Sorsa *et al.*, 2016).



3.2. Study Design

From November 2025 to May 2026, a cross-sectional study was carried out at Ziway, Abijata, and Hawassa Lakes. The lakes were chosen due to their large fish harvest, which accounts for 84% of the nation's overall fish harvest (FAO, 2018). The number of leisure activities around the lake and the ease of access to public transportation transit areas determine the restaurants in each area.

3.3. Products Tested

Three hundred fish samples collected randomly from daily collected fish in different locations (Lake Hawasa, Lake Ziway, and the Lake Abijata) in South Central Ethiopia. The samples were collected from different sites: Captured catfish products from local fisheries, Ready-to-eat catfish dishes sold at markets or restaurants. The samples were transported into sterile plastic bags and transported in an ice box to the laboratory.

3.4. Sample Size and Sampling Procedure

Sampling was performed during a defined study period using a structured collection plan, and isolates were identified and confirmed using conventional microbiological methods and MALDI-TOF mass spectrometry. Data were recorded using standardized sample labeling

forms and transported under a maintained cold chain until laboratory analysis. A total of 300 samples were examined for the detection of both *Staphylococcus aureus* and *L. monocytogenes*. The sample distribution was as follows: 150 live fish, 100 market fish, and 50 RTE samples, collected from markets and retail outlets located within the study area. About 300 fish exhibiting indicative lesions (hemorrhages on the outer surface, at the base of the pectoral and tail fins, skin ulcers, abdominal swelling, unilateral or bilateral exophthalmia, protruding anus, and fin decay) were chosen for sampling using a purposeful sampling technique. Fish with suspicious lesions had their muscles, gills, intestines, spleens, and kidneys sampled.

3.5 Study Techniques

3.5.1. Sample collection and preparation

Samples Collection: All the fish were captured with gillnets featuring mesh sizes varying from (10 cm to 14 cm) that were employed for the exploratory fishing efforts at the lakes. For the Ziway and Abijata lakes, samples were transported alive in auto-clavable sterile plastic bags filled with water from the lake where they were caught. Following collection, the samples were sent to the The Batu Fishery and Other Aquatic Life Research Center conducted post mortem examinations; for Hawassa Lake, specimens were sent to the Hawassa University Biology Department laboratory for immediate post mortem analysis. To effectively isolate these pathogens in this study, fish tissue samples were collected with precision. Samples were obtained from various parts of the fish, including the skin, gills, and muscle tissue. This comprehensive approach allowed for a thorough assessment of potential contamination. For processed fish, both filleted and unfileted samples were included, providing a broader scope for analysis. The collection process adhered strictly to sterility protocols. Sterile equipment, such as swabs, dissection tools, and gloves, was utilized to prevent cross-contamination. It was crucial to ensure that the working environment was clean and that all tools were properly sterilized prior to use. During the collection, care was taken to avoid excessive damage to the fish. For instance, when sampling the gills and skin, a gentle scraping motion was employed to gather adequate tissue without compromising the overall integrity of the fish.

Once the desired samples were collected, they were placed in sterile bags immediately. Minimizing exposure to air and environmental contaminants during this process was vital to safeguard the quality of the samples. Transporting the samples required additional care; they

were stored on ice to maintain their integrity and prevent the growth of unwanted microorganisms. It was essential to ensure that the samples remained chilled until they were processed in the laboratory.

Homogenization: In this study, after the samples arrived at the laboratory, the next critical step involved homogenizing the collected fish tissue. This process facilitated the even distribution of any potential pathogens throughout the sample, enabling accurate detection during subsequent analyses. Preparation for homogenization took place in a laminar flow cabinet, providing a sterile working environment. All surfaces and equipment in the cabinet were disinfected to maintain sterility. The first step in the homogenization process involved accurately weighing 25 grams of the collected fish tissue. This specific weight was crucial for maintaining consistent dilution ratios, which are necessary for reliable results.

Following the weighing, 225 mL of a suitable diluent, such as sterile peptone water, was added to the fish tissue. The mixture was then thoroughly homogenized using a stomacher or a high-speed blender. The stomacher was particularly effective for achieving a uniform suspension of the fish tissue and diluent, ensuring that any bacteria present were evenly distributed throughout the sample. Homogenization was performed for a sufficient duration, typically 1-2 minutes, until a smooth and consistent mixture was obtained. After homogenization, the resulting mixture was ideally processed immediately. If immediate processing was not feasible, it was stored under appropriate conditions, such as refrigeration, to preserve its integrity. Any remaining homogenate was kept in sterile conditions to prevent contamination, ensuring the reliability of the subsequent analyses.

3.5.2. Field Examination (Clinical and P.M examination)

According to Austin and Austin (2016), sampled fish were clinically examined for gross external symptoms. Transecting the spinal cord beneath the skull killed the fish. The internal organ examination and autopsy were performed using the procedure outlined by Wu et al. (2019). Muscle, gills, kidney, and spleen were taken for molecular analysis and bacterial 25 culture. Initially, samples from the fish's muscles and gills were obtained aseptically, the fish's exterior body surface was checked for lesions, and the gills, tail, and fins were checked for obvious symptoms of infection. After the corpse was opened, the internal organs were carefully exposed using a ventral approach so as not to puncture any intestinal system. Samples of the kidney, spleen, and intestine were taken after the organs' surfaces were seared

with a hot scalpel blade if there were no obvious injuries. Two grams of each specimen were aseptically transferred into a 50 milliliter falcon tube that held 20 milliliters of PH 8.5 alkaline peptone water (Oxoid, England) that was kept cold at 4 degrees Celsius. Based on the quantity of fish stock retained by a particular seller, market availability, and the customer's preferred store based on unofficial data collecting in the area, all raw fish was bought and collected from traditional markets and supermarkets in the corresponding towns. RTE fish samples were gathered as takeout orders and stored in sterile plastic bags, whereas each market fish sample was promptly put in a clear, sterile polyethylene bag. A falcon tube with 20 milliliters of peptone water was filled with 2 grams of each specimen from market fish and RTE, which was then kept in an icebox. All of the fish, market, and RTE specimens were labeled, moved as quickly as possible to the aseptic laboratories at Batu and Hawassa University, and then transported to the Animal Health Institute (AHI) using electrical cooler jugs (iceboxes) for additional analysis.

3.5.3. Bacteriological examination (*Phenotypic Identification*)

The isolation and identification procedures followed a regular operating procedure of *Listeria monocytogenes* and *Staphylococcus aureus* from captured fish products and ready-to-eat fish samples. Aseptically, 2 g of each sample (muscle, gill, intestine, kidney, spleen) was homogenized and transferred into respective enrichment broths: *Listeria* Enrichment Broth (LEB) for *L. monocytogenes* and Tryptic Soy Broth (TSB) with 10% NaCl for *S. aureus*. Samples were incubated at 35°C for 24 hours. A loopful from each enriched homogenate was streaked onto selective media: PALCAM Agar for *L. monocytogenes* and Mannitol Salt Agar (MSA) for *S. aureus*, followed by incubation at 35°C for 24–48 hours. Single presumptive colonies were re-streaked onto fresh selective media to obtain pure cultures.

Pure colonies were incubated at 35°C after being injected into Brain Heart Infusion (BHI) broth for 18–24 hours. A loop-ful was then sub-cultured onto Nutrient Agar and incubated for a full day at 35°C to prepare stock cultures for further analysis. Biochemical identification was performed based on colonial morphology, Gram staining, and species-specific tests. Characterization followed guidelines from Bergey's Manual of Systematic Bacteriology and Buller (2004).

3.5.4. MALDI-TOF identification

Confirmed bacterial for quick and precise species-level identification, isolates were immediately submitted to Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF). Each identified isolate was stored at -20°C with 25% glycerol in triplicates for further molecular analysis.

3.5.5. DNA Extraction and molecular detection

Fish samples were homogenized using sterile equipment to ensure uniform distribution of microorganisms. Total genomic DNA was then extracted using a commercial DNA extraction kit or an alternative validated extraction method such as phenol–chloroform, following the manufacturer’s instructions step-by-step. After extraction, DNA was eluted in an appropriate volume (typically 50–100 µL) and stored under recommended conditions (e.g., 4°C for short-term use and –20°C for long-term storage). The extracted DNA was subsequently assessed for quality and concentration using spectrophotometric (e.g., A260/A280) or fluorometric measurements. If inhibition of downstream amplification was suspected (e.g., due to co-extracted inhibitors from food matrices), the DNA template was diluted appropriately and used for the molecular assay. Specific primers and a hydrolysis probe were selected based on previously published assays. For *Listeria monocytogenes*, the assay targeted the *iap* gene (also described as the invasion-associated protein / p60 gene). For *Staphylococcus aureus*, the assay targeted the *sec* gene (enterotoxin C gene). Primers and probes were prepared to the concentrations recommended in the original assay reports.

qPCR reactions were prepared by first making a master mix using a probe-based qPCR chemistry (e.g., TaqMan-style) at the recommended 2× or 4× concentration. Primers and the specific hydrolysis probe for each target were then added to achieve the final concentrations specified by the selected assay. Extracted DNA template was added to each reaction well, and reactions were set up in accordance with the recommended reaction volumes. To ensure assay reliability, appropriate controls were included in every run: a positive control containing DNA from a confirmed *L. monocytogenes* (*iap* target) strain and a confirmed *S. aureus* (*sec* target) strain (run separately or in the appropriate assay format), a no-template control (NTC) containing nuclease-free water instead of DNA to monitor for contamination, and an extraction blank processed alongside the samples to detect any cross-contamination introduced during DNA extraction. After setup, the qPCR instrument was programmed with

the thermocycling conditions specified for the primer/probe sets, including the initial activation/denaturation step followed by repeated cycles of denaturation and annealing/extension, with fluorescence data collected at the end of the extension step. The primer and probe sequences, amplicon sizes, and references are summarized in Table 1.

Table 1: DNA sequences used for qPCR primers and probes

Primers and probes	Sequences (5'→3')	Genes	Functions	Reference
Lmon-F	AGTGCTATTATTGCTGAAGC TCAAAA	<i>iap</i> (invasion associated protein)	p60 gene (invasion associated protein)	Gertrudes <i>et al.</i> , 2018
Lmon-R	TCCGTTACCACCCCATGAAT			
Lmon-PR	CACCTTGGAAAAGC			
Saur-F	CGTATTAGCAGAGAGCCAA CCA	<i>Sec</i>	Enterotoxin C gene	Gertrudes <i>et al.</i> , 2018
Saur-R	GTGAATTTACTCGCTTTGTG CAA			
Saur-PR	ACCCTACGCCAGATGA			

Antibiogram analysis: A total of 16 bacterial isolates were subjected to antibiotic sensitivity testing using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2020). The procedure involved fresh culture in Muller Hinton broth with turbidity adjusted to McFarland standard No. 0.5. Isolates were streaked onto Muller Hinton agar, and antibiotic disks were placed using an applicator, followed by incubation at 37°C overnight. The zones of inhibition were measured and interpreted as sensitive, intermediate, or resistant according to CLSI reference values (CLSI, 2020).

Fresh culture in Mueller Hinton broth with turbidity adjusted to McFarland standard No. 0.5 was used in the process. After streaking isolates onto Mueller Hinton agar and applying antibiotic disks using an applicator, the mixture was incubated at 37°C for the entire night. Using CLSI reference values, the zones of inhibition were assessed and classified as Sensitive (S), Intermediate (I), or Resistant (R). The following antibiotics relevant to aquaculture,

veterinary, and human treatment were tested: Amoxicillin-clavulanate (AMC, 30 µg), Penicillin (P, 10 µg), Ampicillin (AMP, 10 µg), Ceftriaxone (CRO, 30 µg), Gentamicin (CN, 10 µg), Streptomycin (S, 10 µg), Tetracycline (TE, 30 µg), Ciprofloxacin (CIP, 5 µg), Trimethoprim-Sulfamethoxazole (SXT, 25 µg), and Chloramphenicol (C, 30 µg). Finally, an antibiogram by measuring the diameter of each inhibition zone (in mm) produced by each antibiotic disc against a bacterial isolate, and then comparing that zone diameter to the CLSI breakpoint ranges.

3.6. Data Management and Analysis

For statistical analysis, field-collected data was checked for mistakes and correct coding. Using descriptive statistics using the statistical software SPSS version 22 such as frequencies and cross-tabulations (Chi-square test) were used to find missing data and plausible connections. A percentage of the total number of samples examined was used to represent the number of positive samples. Following the enrichment and isolation phases, data regarding colony counts and characteristics were recorded. For each selective agar plate, the number of colonies exhibiting yellow coloration on Mannitol Salt Agar (MSA) was documented, as this indicated the presence of *Staphylococcus aureus*. Additionally, any atypical colonies were noted for further investigation. Once the samples were identified using MALDI-TOF mass spectrometry, the resulting mass spectra were analyzed using specialized software. The software compared the spectra obtained from the isolated colonies against a reference database containing known bacterial profiles. The quick identification of *Staphylococcus aureus* and other staphylococcal species found in the samples was made possible by this comparison. To ascertain the frequency of *Listeria monocytogenes* and *Staphylococcus aureus* in the fish samples, quantitative analysis was carried out.

4. RESULTS

4.1. Clinical and Post-Mortem Findings

Fish suspected of having a bacterial infection displayed bleeding throughout their bodies, particularly at the base of their fins and tails. Fins rot, cloudiness in eyes, scale detachment, skin ulcers, and abdominal distention are among the clinical manifestations that have been noted. As seen in (Figure 2), these fishes had internal symptoms such as abdominal dropsy with reddish ascetic exudates, liver paleness and enlargement in some fish and congestion with necrotic patches in others, and spleen congestion, enlargement, and hemorrhagic enteritis in other fish.



A

B

A) Eroded scales, fin rot and redness in the skin. B) Shows abdominal dropsy with reddish ascetic exudates

4.2. Bacteriological Identification and Biochemical Characterization of *Listeria monocytogenes* and *Staphylococcus aureus*

For *Listeria monocytogenes*: Presumptive identification was performed using colony morphology on Listeria Selective Agar (Oxford) following enrichment in Listeria Enrichment Broth (Half-Fraser). Although appropriate culture media were used, no *Listeria monocytogenes* isolates were recovered from the 300 samples tested. This lack of detection may be explained by a low underlying prevalence, seasonal fluctuations, the inhibitory effects of competing microorganisms, or limitations in the sampling approach.

For *Staphylococcus aureus*: Presumptive identification was carried out on Mannitol Salt Agar (MSA). A total of 16 isolates (5.33%) were identified as *S. aureus*. On MSA, colonies appeared yellow to golden-yellow (2–3 mm) with a yellow zone. On blood agar, beta-hemolysis was observed. Microscopically, isolates were Gram-positive cocci in clusters. All 16 isolates were catalase-positive, coagulase-positive, DNase-positive, mannitol-positive, and beta-hemolytic, consistent with standard descriptions of *S. aureus* in Bergey's Manual of Systematic Bacteriology.

4.2.1. Hemolysis assay for *staphylococcus aureus*

The isolates' hemolytic activity was assessed for its significance as a virulence factor. On a blood agar base containing 5% sheep blood, *S. aureus* caused hemolysis. Accordingly, from the current study it was found that 7 out of 7 (100%) isolates from live fish, 6 out of 6 (100%) isolates from market fish, and 3 out of 3 (100%) isolates from RTE fish showed beta (β) hemolysis, respectively. No alpha (α) hemolysis was observed. The media shows distinct halos surrounding bacterial colonies as a result of the hemolysis pattern. Overall, 16 out of 16 (100%) isolates showed beta (β) hemolysis (Table X).

Table 2: Hemolytic characteristics of *Staphylococcus aureus* isolates from different sources

Hemolytic activity	Live fish (n=7)	Market fish (n=6) (n=16)	RTE (n=3)	Total
β (Beta)	7 (100%)	6 (100%) (100%)	3 (100%)	16
α (Alpha)	0 (0%)	0 (0%) (0%)	0 (0%)	0

4.2.2. MALDI-TOF MS confirmation

All 16 presumptive *Staphylococcus aureus* isolates identified by phenotypic and biochemical methods were subjected to MALDI-TOF MS analysis for definitive species confirmation. The MALDI-TOF scores ranged from 2.15 to 2.48 (mean: 2.31 ± 0.09), all significantly above the ≥ 2.00 threshold for confident species-level identification. Thus, all 16 isolates (100%) were confirmed as *Staphylococcus aureus* by MALDI-TOF MS. No *Listeria monocytogenes* isolates were available for MALDI-TOF analysis as none were recovered from phenotypic isolation. Molecular Detection.

4.2.3. Real-Time PCR Detection of *Staphylococcus aureus*

Real-Time PCR (qPCR) was used for molecular detection using specific primers and probes targeting the *sec* gene (enterotoxin C gene) for *Staphylococcus aureus*. Only the 16 culture-positive isolates were subjected to molecular confirmation.

4.2.4. Real-time PCR amplification

Real-time PCR was conducted utilizing a thermocycler (Applied Biosystems Model Real-Time 7500). Probe-based detection utilized TaqMan Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher) in a 20 μ l reaction comprising 10 μ l of 2 $\times$ TaqMan Fast Advanced Master Mix, 1 μ l each of forward and reverse primer (10 μ M), 0.5 μ l of probe (10 μ M), 2 μ l of DNA template, and 5.5 μ l of RNase-free water. Fluorescence data was acquired during the 40 cycles of denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds that followed the initial enzyme activation step at 95°C for 2 minutes in the PCR procedure. Each reaction had both a positive control and a no-template control.

4.2.5. Threshold and interpretation

A cycle threshold (Ct) value of 34 was chosen as the threshold cut-off value for classifying samples as positive or negative. Positive samples were those having a sigmoid-shaped amplification curve and a Ct value of less than 34. Samples were categorized as negative if their Ct value was more than 34.

4.2.6. Results of real-time PCR detection

A total of 16 culture-positive *Staphylococcus aureus* isolates were examined using real-time PCR targeting the *sec* gene. All 16 isolates (100%) were confirmed positive. The Ct values ranged from 19.2 to 33.8, with a mean Ct value of 26.4 ± 4.1 . Melting curve analysis showed typical melting profiles at 84.5–85.2°C, confirming specific amplification of the *sec* gene. No amplification was observed in the negative controls. Molecular detection was not done because no *Listeria monocytogenes* was found by culture.

Table 3: Real-time PCR detection results of *Staphylococcus aureus* isolates

Bacterial Species	Target Gene	Number Tested (n)	Number Positive (%)	Ct Value Range	Mean Ct ($\pm$ SD)	Melting Temperature (Tm) (°C)
<i>Staphylococcus aureus</i>	<i>Sec</i>	16	16 (100%)	19.2 – 33.8	26.4 ± 4.1	84.5 – 85.2

4.3. Detection of *Staphylococcus aureus* from Different Sources

4.3.1. Detection of *staphylococcus aureus* isolated from examined fishes based on the sources

Three hundred samples were gathered from various sources and cultured on selective media for *S. aureus* (Mannitol Salt Agar). Out of these, 16 (5.33%) isolates were tentatively recognized as *Staphylococcus aureus* through morphological and biochemical analysis. These isolates were additionally verified as *S. aureus* by qPCR (100% confirmation) based on particular primers and probe aimed at the *sec* gene (enterotoxin C gene). Additionally, all 16 isolates were confirmed by MALDI-TOF MS with scores ranging from 2.15 to 2.48.

Table 4: Detection of *Staphylococcus aureus* based on source

Source	Number Examined	Culture Positive (%)	qPCR Positive (%)	MALDI-TOF Confirmed (%)
Live fish	150	7 (4.67%)	7 (100%)	7 (100%)
Market fish	100	6 (6.00%)	6 (100%)	6 (100%)
RTE	50	3 (6.00%)	3 (100%)	3 (100%)
Total	300	16 (5.33%)	16 (100%)	16 (100%)

CP: Culture positive, qPCR: quantitative polymerase chain reaction, RTE: Ready-to-eat

4.3.2. Detection of *Staphylococcus aureus* isolated from examined fishes based on the organs

In the present investigation, organ location from live fish samples was used to identify *S. aureus*. As a result, the gill had the highest detection (42.86%), followed by the muscle (28.57%), and the Intestine and Kidney had the lowest detection (14.29%) (Table 5). The spleen did not contain any isolated *S. aureus*.

Table 5: Detection of *Staphylococcus aureus* in respect to the organs

Organ	Number of Culture Positive	Total qPCR Positive (%)
Muscle	2	2/7 (28.57%)
Gill	3	3/7 (42.86%)
Intestine	1	1/7 (14.29%)
Spleen	0	0/7 (0%)
Kidney	1	1/7 (14.29%)

4.4. Antibigram Analysis

Staphylococcus aureus isolates were subjected to antibiotic sensitivity testing using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2020). Five of these isolates were chosen at random for in-depth antibiogram profiling. Out of 16 *S. aureus* isolates, 5 were randomly selected for antibiogram testing against 10 antibiotics. All 5 isolates (100%) were resistant to Penicillin and Ampicillin. Resistance to Tetracycline was observed in 3 isolates (60%), and resistance to Streptomycin was observed in 2 isolates (40%). Multi-drug resistance (resistance to ≥ 3 antibiotic classes) was observed in 2 out of 5 isolates (40%).

Table 6: **Antibiotic Sensitivity Testing**

Antibiotic	Sensitive (n=5)	Intermediate (n=5)	Resistant (n=5)	% Resistant
Amoxicillin-clavulanate (AMC)	5	0	0	0%
Penicillin (P)	0	0	5	100%
Ampicillin (AMP)	0	0	5	100%
Ceftriaxone (CRO)	5	0	0	0%
Gentamicin (CN)	5	0	0	0%
Streptomycin (S)	3	0	2	40%
Tetracycline (TE)	2	0	3	60%
Ciprofloxacin (CIP)	5	0	0	0%
Trimethoprim-Sulfamethoxazole (SXT)	5	0	0	0%
Chloramphenicol (C)	5	0	0	0%

Summary of Antibigram Results

Bacterial Isolate (n=5)	Most Effective Antibiotic(s)	Least Effective Antibiotic(s)	Multi-drug Resistance Observed
<i>S. aureus</i>	Amoxicillin-clavulanate, Ceftriaxone, Gentamicin, Ciprofloxacin, SXT, Chloramphenicol (100% sensitive)	Penicillin (100% resistant), Ampicillin (100% resistant)	Yes (resistance to Penicillin + Ampicillin + Tetracycline + Streptomycin in some isolates)

5. DISCUSSIONS

Out of the 300 fish tissue samples used in this investigation, 16 samples (5.3%) had *Staphylococcus aureus*, but none had *Listeria monocytogenes*. In view of food safety, the consequences for public health, and parallels with recent literature, these findings are worth discussing. From the standpoint of food safety, the absence of *L. monocytogenes* in the fish samples is encouraging. However, it should be interpreted with caution. Meta-analytic data indicate that *L. monocytogenes* is not uncommon in fish and fish-processing environments: a systematic review found a pooled prevalence of about 5.8% in raw fish samples and substantially higher rates (14.5%) in ready-to-eat fish products (Zakrzewski *et al.*, 2024).

Moreover, previous studies in the seafood industry have shown that *Listeria monocytogenes* may persist in processing environments, including equipment surfaces and drains, which can lead to recurrent contamination. Such persistence has been linked to strains associated with human listeriosis (Hoffman *et al.*, 2003; Skowron *et al.*, 2019). Regional studies have also reported contamination in fresh seafood, with Momtaz and Yadollahi (2013) reporting about 7.72% prevalence in Iran and evidence of virulence-associated genes. These reports indicate that occurrence is influenced by geography, fish type, processing practices, and sampling context. In contrast, *L. monocytogenes* was not detected in the present study. This absence may be explained by factors such as effective hygiene and sanitation practices in sampled units, shorter time between processing and sampling (reducing opportunities for establishment), possible low initial contamination levels at the point of capture/handling, and/or limitations related to sample size and detection sensitivity. Therefore, while *L. monocytogenes* persistence is documented elsewhere, its absence in the current dataset suggests that the local processing conditions and handling practices may have minimized its introduction and survival.

Effective sanitation and handling procedures in the fish processing chain, low levels of environmental contamination, or possibly sampling bias (e.g., fish kinds, geographic source, or seasonal influences) could all have contributed to the unfavorable outcome. Improvements in control methods may also be responsible for the declining trend in *Listeria* prevalence in some settings, as shown by meta-regression from the same review. Moreover, a review of the seafood industry highlighted how rigorous sanitation, genetic surveillance, and control of processing environments can limit *Listeria* contamination (Rip *et al.*, 2024). Given that *L. monocytogenes* can be particularly dangerous (especially in vulnerable populations),

continued surveillance is important even when immediate prevalence is low. Regular environmental monitoring, particularly in ready-to-eat fish products, may be warranted.

In the present study, the observed prevalence of 5.3% for *Staphylococcus aureus* is relatively low compared to many regional and international reports, yet it remains consistent with the understanding that *S. aureus* contamination in fish and seafood products is a continuing food safety concern. For instance, a comprehensive study from Iran reported a 27.8% prevalence of *S. aureus* across a variety of seafood types—including fresh, smoked, salted, dried fish, and shellfish—and further identified multiple virulence genes such as *sea*, *seb*, *sec*, *sed*, *see*, and the methicillin-resistance gene *mecA* (Derke *et al.*, 2025). These findings demonstrate that in some production systems, *S. aureus* contamination is not only widespread but also potentially more hazardous due to the presence of toxin-producing and antibiotic-resistant strains.

Higher prevalence figures have also been reported in other geographical locations. For example, a study conducted in India documented *S. aureus* in 13.7% of seafood samples, including several methicillin-resistant *S. aureus* (MRSA) isolates, highlighting concerns about antimicrobial resistance within the seafood supply chain (Suresh *et al.*, 2022). Similarly, research carried out in Ethiopia found a 26% prevalence of *S. aureus* in fish and in hand-swab samples from fish handlers, underscoring the role of human handling as a major contamination source (Melese *et al.*, 2024).

Further evidence from a One Health investigation in Malaysia also reported notable levels of *S. aureus* contamination in fish markets, with isolates exhibiting resistance to commonly used antibiotics, suggesting that environmental conditions, hygiene practices, and cross-contamination contribute significantly to pathogen spread (Rahman *et al.*, 2023). The presence of MRSA in seafood, though variable across studies, has been increasingly documented, which raises substantial public health concerns due to the difficulty in treating infections caused by resistant strains (Elbashir *et al.*, 2023).

Geographically, prevalence varies. In India, researchers found *S. aureus* in about 13.7% of seafood samples, with 3% being methicillin-resistant *S. aureus* (MRSA) (Sivaraman *et al.*, 2023). In contrast, a more recent study in retail fish markets (e.g., *Mugil cephalus*) reported *S. aureus* in ~26.8% of samples, with risk factors linked to handling (e.g., contamination from knives and fish handlers) rather than the fish itself (Attia *et al.*, 2024).

Compared to these, your prevalence of 5.3% is modest, which may reflect better hygienic practices, lower cross-contamination, or differences in the sampling frame (e.g., species of fish, points of The detection of *Staphylococcus aureus* in fish samples, even at a relatively low prevalence, carries important public health implications. One of the major concerns associated with *S. aureus* contamination is its ability to produce a variety of heat-stable enterotoxins. These enterotoxins, when present in sufficient quantities, can lead to staphylococcal food poisoning, a common form of foodborne illness characterized by symptoms such as vomiting, abdominal cramps, and diarrhea. Because the toxins can remain active even after cooking, the mere presence of *S. aureus* in raw fish products is considered a potential food safety hazard, emphasizing the need for effective control measures throughout the handling and distribution chain (Mengesha *et al.*, 2025).

Equally concerning is the growing global issue of antimicrobial resistance. Numerous recent studies have reported the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in various seafood products. Although the present study did not assess methicillin resistance specifically, the increasing prevalence of MRSA in aquatic environments and food chains suggests that resistant strains could also emerge within local fish-handling systems. This possibility raises additional public health concerns, as MRSA infections can be more difficult to treat and may contribute to the wider dissemination of resistant bacteria through food consumption or cross-contamination (Derke, *et al.*, 2025).

6. CONCLUSION AND RECOMMENDATIONS

The current analysis showed that there was no prevalence of *Listeria monocytogenes* fish in any of the 300 samples. This implies that there is little chance of *L. monocytogenes* infection in the fish items under investigation. The lack of this virus could be the result of both favorable environmental conditions that restrict its occurrence and good hygiene standards during fish handling, processing, and storage. The effectiveness of contemporary quick diagnostic methods in enhancing precision and efficiency in food safety surveillance is demonstrated by the successful detection of isolates using MALDI-TOF. Overall, the results emphasize the significance of ongoing observation and enhanced hygiene interventions to protect public well-being and preserve the microbiological quality of fish products.

- Strengthen hygiene and handling practices by training fish handlers, processors, and vendors on proper personal hygiene, safe handling, and sanitation procedures to minimize contamination, especially from human carriers of *Staphylococcus aureus*.
- Regular microbiological testing of fish products should be implemented to ensure early detection of pathogens, including *Listeria monocytogenes*, even when current results indicate absence.
- Assess antimicrobial resistance profiles of *S. aureus* isolates by conducting studies that screen for methicillin-resistant *S. aureus* (MRSA) and toxin-producing genes.
- Support further research by expanding sampling to include a wider range of fish species, geographic locations, and stages of processing.

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8. APPENDICES

8.1 Pictures taken during study period

