IDENTIFICATION AND CHARACTERIZATION OF ENTEROBACTER SPECIES CAUSING DECAY OF ONIONS AND THEIR ANTIMICROBIAL SUSCEPTIBILITY

BY

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To the

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DECLARATION

I, Lukheli Elelwani, student number 17008303, hereby attest that I have, to the best of my knowledge and belief, complied with the University's policies and rules applicable to postgraduate research. I admit having read and comprehended said policies and rules. I certify that the research project I am submitting for the Master of Science in Microbiology degree at the University of Venda is entirely original to me and has never been submitted elsewhere for a different degree. All sources have been properly cited and acknowledged.

Lukheli.e	23 February 2025
Signature	Date

DEDICATION

This dissertation is dedicated to the following:

- The all-mighty God for giving me strength to keep on going
- To my Daughter (Maluta Rilinde) who always guided me not to give up on school and supported me in every possible way
- To my Mother (Lukheli gladys) who ensured that I go to school, who supported me financial, always have my back and gave me words of courage whenever I feel lost along the way
- To my Partner who never gave up on me even when I had little time for the relationship but supported me and walked this journey with me and accompanied me for late night Lab work with no complains.

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ABSTRACT

Background: Plants are susceptible to a range of health problems, and onions are no exception. While onions are relatively resilient, they are not immune to diseases and pathogens. Onions that are packed before being fully dried are prone to rapid decay. Research has shown that water can facilitate the transmission of pathogens and contribute to microbial contamination of fresh produce, including Salmonella and E. coli. Salmonella can enter the soil through agricultural practices such as pesticide application, the use of fertilizers derived from animal manure, and irrigation with contaminated water. Since irrigation water can come from various sources—such as municipal supplies, treated wastewater, rivers, or groundwater—farmers are advised to safeguard their water sources to reduce the risk of contamination.

Objective: To identify and characterize Enterobacter species causing decay of bulb onions and their antimicrobial susceptibility.

Methods: This study was carried out in the Vhembe district in the Limpopo province. The focus was on farms in various municipalities, including both commercial and subsistence farms, as well as the rivers surrounding these farms. Water, soil, and onion samples were collected. The presence of total coliform and E. coli was determined using the Colilert Quanti tray method. Collected samples were further analyzed for Enterobacteriaceae using membrane filtration and culture-based methods. Biochemical tests (Vitek-2-system) were used to identify and confirm the isolates. *E. coli* pathotypes were characterized using multiplex PCR. The characterization of strains from Enterobacter species was done using Sanger Sequencing.

Results: Measured temperatures ranged from 19.8°C to 25°C, with Farm 2 showing slightly elevated values. Electrical conductivity (EC) values were within acceptable limits (<540 μS/cm) across all farms, though Farm 3 exhibited higher total dissolved solids (TDS) levels, reaching up to 450 mg/L. The pH values across farms ranged from 5.0 to 6.4, lower than the recommended 6.5–8.5 for agricultural water. Elevated TDS and acidic pH in Farm 3 was observed. High levels of total coliforms (up to 2419.6 MPN/100 mL) and the presence of *E. coli* were detected in water and onion samples, particularly from Farms 2 and 3. PCR analysis identified multiple *E. coli* pathotypes, including EPEC, ETEC, EAEC, and EIEC, with greater diversity in Farms 2 and 3. Membrane filtration and culture methods confirmed the presence of gram-negative bacteria (GNB) in most samples. Pathogens such as Enterobacter cloacae, Klebsiella oxytoca, and Pseudomonas aeruginosa were isolated and confirmed with the Vitek 2 system. Enterobacter cloacae was consistently detected in onion samples, Antimicrobial Resistance: Antibiotic susceptibility testing revealed multidrug resistance among Enterobacter cloacae, Klebsiella oxytoca, and Pseudomonas aeruginosa. Resistance to ampicillin, colistin was noted. Most of the strains identified as Enterobacter cloacae by phenotypic methods were identified as *Enterobacter Ludwigii*.

These findings highlight the potential risks of waterborne pathogen transmission and the need for stringent water treatment and management practices to safeguard agricultural and public health.

***Keywords:** Enterobacteriaceae, Irrigation water, Onion decay, Soil, Vitek-2.*

LIST OF ABREVIATIONS

16S	16S rRNA (a target gene used in bacterial identification through sequencing)
AMR	Antimicrobial Resistance
API	Analytical Profiling Index
AST	Antibiotic Susceptibility Testing
BGLU	Beta-glucosidase (a biochemical test result)
BOD	Biological Oxygen Demand
CDC	Centers for Disease Control and Prevention
CSO	Combined Sewage Overflow
DNA	Deoxyribonucleic Acid
DWAF	Department of Water Affairs and Forestry
EC	Electrical Conductivity
E. Cloacae	Enterobacter cloacae (a species complex in which E. ludwigii is categorized)
E. Coli	*Escherichia coli*
EPEC	Enteropathogenic Escherichia coli
ETEC	Enterotoxigenic Escherichia coli
EAEC	Enteroaggregative Escherichia coli
EIEC	Enteroinvasive Escherichia coli
E. ludwigii	Enterobacter ludwigii (a bacterial species identified through sequencing)
EMB	Eosin Methylene Blue (agar)
EPA `	Environmental Protection Agency
HO	Hydrogen Peroxide
H2S	Hydrogen Sulfide (a biochemical test result)
KB	Kilobase (a unit of DNA length, in this case, 1.5KB refers to the amplified DNA target region length)
KIA	Kligler Iron Agar
KH_2 PO_4	Potassium Dihydrogen Phosphate
L. nimipressuralis	*Lelliottia nimipressuralis*
MDR	Multidrug-Resistant
MG/L	Milligrams per liter
MPN	Most Probable Number

m-PCR	Multiplex Polymerase Chain Reaction
Na_2 HPO_4	Disodium Phosphate
NGS	Next-Generation Sequencing
ONPG	Ortho-Nitrophenol Galactoside
PA	Presence/Absence
POPi Act	Protection of Personal Information Act
pH	Potential of Hydrogen
SS	Salmonella-Shigella agar (a selective medium for bacterial growth)
TDS	Total Dissolved Solids
TDS	Total Dissolved Solids
UJ	University of Johannesburg
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
VITEK-2	Automated Microbial Identification and Antimicrobial Susceptibility Testing System
WHO	World Health Organization
CFU	Colony-forming units
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria

S - Susceptible (in antibiotic testing)

I - Intermediate (in antibiotic testing)

R - Resistant (in antibiotic testing)

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Onions, although generally resilient, are susceptible to various diseases and pathogen infections. If onions are harvested and packed before they have fully dried, they are prone to rapid deterioration. Onions (*Allium cepa*) are hardy crops but are vulnerable to post-harvest diseases if not properly cured. For example, neck rot occurs when *Enterobacter* species infect onions through moist, undried neck wounds. The *Enterobacter cloacae* complex is commonly implicated in onion bulb rot (Liu 2016). Outbreaks of bulb decay have been reported in onion-growing regions worldwide, causing significant losses (Zaid *et al.* 2011).

Water quality is critical for safe agriculture. Contaminated irrigation water can transmit pathogens to crops, leading to outbreaks of *Salmonella*, *E. coli*, and other bacteria (Liu 2018; CDC 2016). Pathogens may enter the farm environment via contaminated water, manure, or pesticides. For example, *Salmonella* can be introduced through animal manure or irrigation with polluted water (Liu 2018). Farmers are therefore advised to protect their water sources (e.g. covering wells) to reduce contamination (CDC 2016; WHO 2017). The presence of *E. coli* in water indicates fecal pollution (Price & Wildeboer 2017). International guidelines (WHO 2017; EPA 2012; EU 2015) set strict limits on coliform counts to ensure safe irrigation water.

The presence of *E. coli* in water is often a sign of fecal contamination, as this bacterium is a well-established indicator of such pollution in groundwater, surface water, and recreational water (Price & Wildeboer, 2017). E. coli naturally resides in the intestines of humans and animals and is excreted into the environment through fecal matter (Price & Wildeboer, 2017). The contamination of environmental water with pathogenic bacteria poses significant risks to water safety and the quality of crops grown in affected areas. To address these risks, regulatory bodies such as the World Health Organization (WHO), the US Environmental Protection Agency (EPA), and the European Union have set limits for bacterial contamination in water to ensure proper water quality management (Price & Wildeboer, 2017).

1.2 STUDY RATIONALE

The genus *Enterobacter* includes several species of clinical significance and is commonly associated with plants, food products, and environmental sources. The presence of *Enterobacter* species in onions can lead to bulb decay, resulting in significant crop losses for farmers (Liu et al., 2016). Prior studies have identified a high incidence of *Enterobacter cloacae* in diseased onion bulbs (*Allium cepa L.*), particularly during an outbreak in the muck-land regions of New York, affecting multiple onion cultivars (Zaid et al., 2011).

In South Africa, onion farming is a critical component of agricultural production, and bacterial outbreaks can cause substantial economic losses. The transmission of *Enterobacter* species from soil and water to onions is influenced by various factors, including soil composition, the quality of irrigation water, the use of manure, and farming practices surrounding the agricultural lands.

Environmental factors in Vhembe District, Limpopo Province such as contaminated rivers and soils may facilitate the spread of *Enterobacter* to crops. Previous studies in this region found high levels of *E. coli* and other Enterobacteriaceae in water sources (Alfasane *et al.* 2021; Alfinete *et al.* 2022). Given reports of *Enterobacter* in vegetables and water, this study focuses on identifying the specific *Enterobacter* species causing onion rot in Vhembe and evaluating their antibiotic resistance. The findings will help understand disease pathways and inform management practices.

1.3 AIM AND OBJECTIVE OF THE STUDY

1.3.1 AIM

To identify *Enterobacter* species associated with onion bulb decay and to determine their antimicrobial susceptibility patterns.

1.3.2 objectives

- ✓ To isolate Enterobacter species in irrigation water, river water, soil and decaying onions using standard culture methods,
- ✓ To characterize the Enterobacter species found using Vitek-2 system,
- ✓ To assess the antimicrobial susceptibility of isolate using Vitek-2 system,
- ✓ To assess the close relatedness of the different species of Enterobacter using Sequencing.

1.4 RESEARCH QUESTIONS

- Which Enterobacter species are associated with onion bulb decay in the studied farms?
- What are the antimicrobial resistance profiles of these Enterobacter isolates?
- Is the contamination of irrigation water or soil linked to the presence of pathogenic Enterobacter species?
- What are the factors associated with the infections of onions?

1.5 SIGNIFICANCE OF THE STUDY

This study was conducted in the Vhembe District of Limpopo Province, focusing on onion farming and the factors contributing to onion bulb decay. It investigated potential sources of contamination, including irrigation water quality, fertilizer application, and soil conditions. The research aimed to enhance understanding of these factors and provide novel insights, with a specific emphasis on identifying and characterizing *Enterobacter* species associated with onion deterioration.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The *Enterobacteriaceae* family of Gram-negative bacteria includes several important pathogens, such as *Escherichia coli*, *Enterobacter*, *Erwinia*, *Dickeya*, *Citrobacter*, *Klebsiella*, *Pantoea*, *Proteus*, *Shigella*, *Salmonella*, and *Serratia*. *Escherichia coli*, the type species of *Enterobacteriaceae*, is well-known for causing gastrointestinal infections in humans and is often referred to as enterobacteria or "enteric bacteria" (Gordon et al., 2019).

Since its classification as a genus in 1960, *Enterobacter* has become one of the largest genera within *Enterobacteriaceae*. These bacteria are commonly found in various environments, including the intestinal microbiota of humans and animals, animal feces, plants, water, insects, and food sources. Certain species, such as *Enterobacter cloacae* and *Enterobacter mori*, have been recognized as phytopathogens, causing bacterial wilt in mulberry trees in China. *E. cloacae* has also been associated with several post-harvest plant diseases, including papaya necrosis, ginger rhizome rot in Brazil, and onion bulb rot in Washington's Columbia Basin (Liu et al., 2016).

Additional *Enterobacter* species have been linked to plant diseases, such as *Enterobacter cancerogenus*, which is associated with canker in poplar trees, and *Lelliottia nimipressuralis* (formerly *Enterobacter nimipressuralis*), found in damp elm wood. Moreover, *Enterobacter dissolvens* has been identified as a pathogen causing maceration rot in corn. The taxonomy of *Enterobacter* has been revised: for example, *E. agglomerans* is now *Pantoea*, and *E. sakazakii* is *Cronobacter*. Recent reclassifications include moves of *E. nimipressuralis* and *E. amnigenus* to *Lelliottia*, and some species into *Pluralibacter* and *Kosakonia* (Bushon, Brady & Lindsey 2015). Due to horizontal gene transfer and genetic diversity, 16S rRNA alone is often insufficient for definitive identification (Bushon *et al.*, 2015).

and *Cronobacter*, respectively. Due to the polyphyletic nature of the 16S rRNA gene sequence, it is no longer considered a reliable tool for accurately classifying newly discovered species (Bushon, Brady, & Lindsey, 2015).

2.2 ENTEROBACTERIACEAE

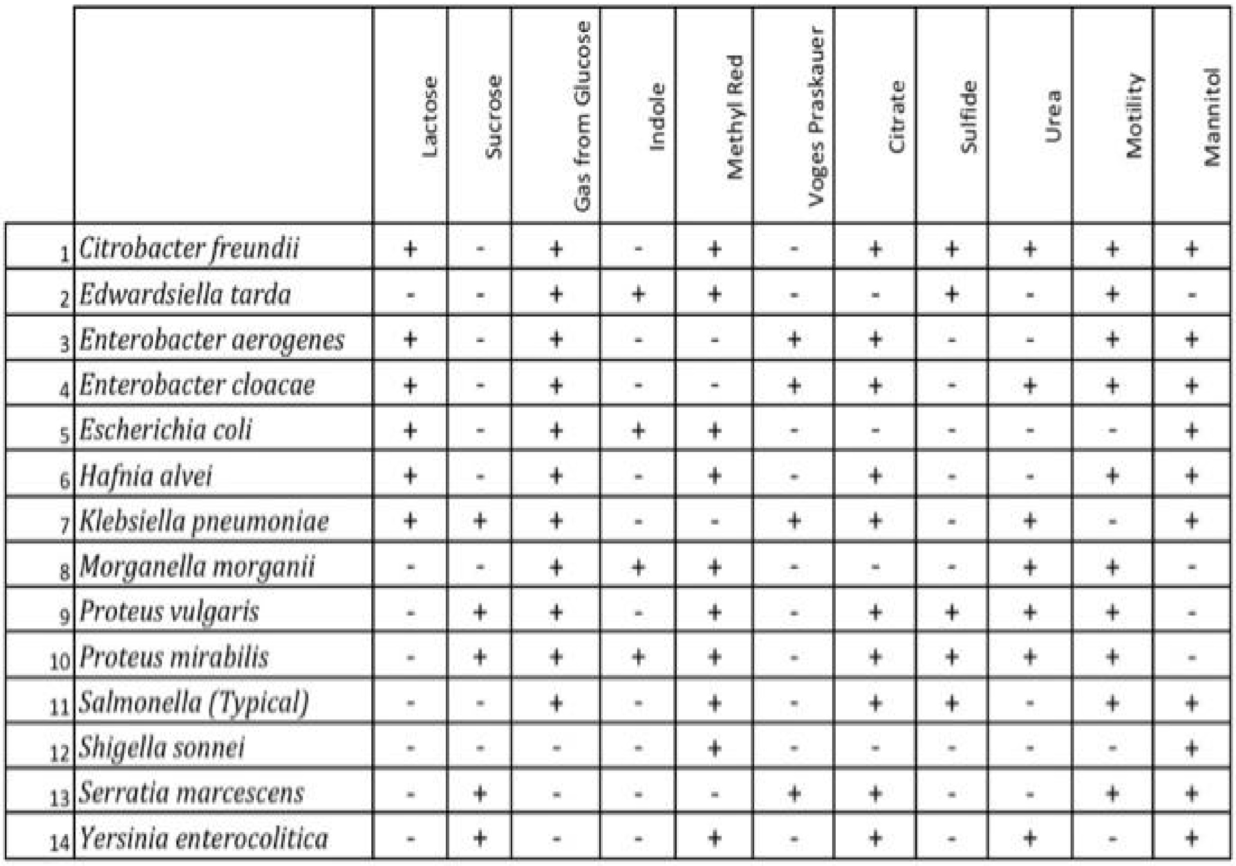
Enterobacteria, members of the *Enterobacteriaceae* family, are primarily known for causing gastrointestinal disorders. These bacteria are responsible for various human infections, such as urinary tract infections, wound infections, gastroenteritis, meningitis, septicemia, and pneumonia. While some enterobacteria act as true intestinal pathogens, others are opportunistic, particularly in individuals with compromised immune systems (Ramirez & Giron, 2023).

Many enterobacteria naturally reside in the large intestine, while others are introduced through contaminated or improperly handled food and beverages. Diseases caused by enterobacteria are often transmitted through the fecal- oral route and are closely associated with poor hygiene and sanitation. Regions with inadequate water purification systems see higher rates of illness and mortality due to enterobacterial infections. Even non-pathogenic strains can cause diarrhea in travelers not accustomed to local bacterial populations. Enterobacterial gastroenteritis can lead to significant fluid loss through vomiting and diarrhea, resulting in dehydration (Sandle T, 2014)

Structurally, enterobacteria are rod-shaped, facultatively anaerobic bacteria, meaning they can survive in both oxygenated and anaerobic conditions, although they prefer anaerobic environments (Janda & Abbott, 2021). The *Enterobacteriaceae* family is divided into eight tribes: *Escherichieae*, *Edwardsielleae*, *Salmonelleae*, *Citrobactereae*, *Klebsielleae*, *Proteeae*, *Yersineae*, and *Erwineae*. These tribes are further categorized into genera, each containing several species (Figure 2.1).

Enterobacteria can be divided into lactose fermenters and non-lactose fermenters (Table 2.1). The 1st group includes *Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, and Citrobacter freundii*. These bacteria are able to ferment lactose can grow on media containing lactose as a carbon source (Resendiz-Nava et al., 2021). Some *Enterobacteriaceae* species have flagella allowing them to move around (Colin et al., 2021). These species include *Escherichia coli and Salmonella spp*. The table below summarizes the unique biological characteristics, including their metabolic activities.

Table 2.1: Biochemical test results for differentiation of enterobacteriaceae species(Cheesbrough, 2006).



		Lactose	Sucrose	Gas from Glucose	Indole	Methyl Red	Voges Praskauer	Citrate	Sulfide	Urea	Motility	Mannitol
1	*Citrobacter freundii*	+	-	+	-	+	-	+	+	+	+	+
2	*Edwardsiella tarda*	-	-	+	+	+	-	-	+	-	+	-
3	*Enterobacter aerogenes*	+	-	+	-	-	+	+	-	-	+	+
4	*Enterobacter cloacae*	+	-	+	-	-	+	+	-	+	+	+
5	*Escherichia coli*	+	-	+	+	+	-	-	-	-	-	+
6	*Hafnia alvei*	+	-	+	-	+	-	+	-	-	+	+
7	*Klebsiella pneumoniae*	+	+	+	-	-	+	+	-	+	-	+
8	*Morganella morganii*	-	-	+	+	+	-	-	-	+	+	-
9	*Proteus vulgaris*	-	+	+	-	+	-	+	+	+	+	-
10	*Proteus mirabilis*	-	+	+	+	+	-	+	+	+	+	-
11	*Salmonella (Typical)*	-	-	+	-	+	-	+	+	-	+	+
12	*Shigella sonnei*	-	-	-	-	+	-	-	-	-	-	+
13	*Serratia marcescens*	-	+	-	-	-	+	+	-	-	+	+
14	*Yersinia enterocolitica*	-	+	-	-	+	-	+	-	+	-	+

Enterobacteria cause disease through several mechanisms, including motility, colonization, endotoxin production, and enterotoxin release. Motile strains possess peritrichous flagella, which allow them to move effectively within the host. Colonization is enhanced by fimbriae, filamentous structures that enable the bacteria to adhere to host tissues. Endotoxins, which are components of the bacterial cell wall, cause high fever in infected individuals, while enterotoxins target the small intestine, resulting in excessive fluid loss through vomiting and diarrhea (Ramirez & Giron, 2023).

Rapid diagnostic tests are available to identify enterobacteria based on their metabolic properties. Most species ferment glucose to produce acid, reduce nitrate to nitrite, and test negative for cytochrome oxidase. These biochemical tests help differentiate specific intestinal pathogens, including *Escherichia coli*, *Shigella* species, *Salmonella*, and certain *Yersinia* strains (Bruins et al., 2004).

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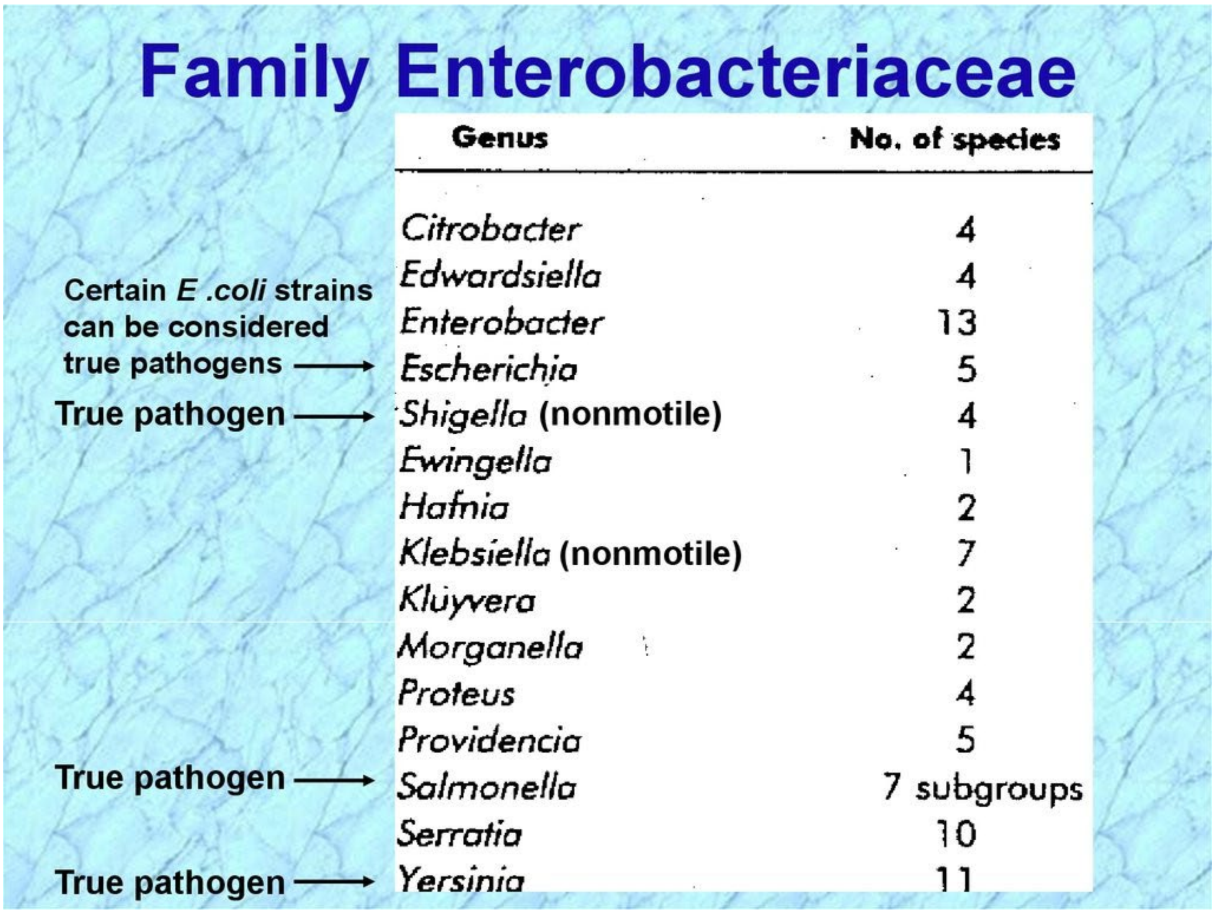


Family Enterobacteriaceae

	Genus	No. of species
	Citrobacter	4
	Edwardsiella	4
	Enterobacter	13
Certain *E .coli* strains can be considered true pathogens →	*Escherichia*	5
True pathogen →	*Shigella* (nonmotile)	4
	Ewingella	1
	Hafnia	2
	Klebsiella (nonmotile)	7
	Kluyvera	2
	Morganella	2
	Proteus	4
	Providencia	5
True pathogen →	*Salmonella*	7 subgroups
	Serratia	10
True pathogen →	*Yersinia*	11

Figure 2.1: Family of enterobacteriaceae. (Pelczar et al., 1993)

2.3 GRAM-NEGATIVE BACTERIA

Gram-negative bacteria are identified based on their response to Gram staining, a laboratory technique used to differentiate bacterial species. When subjected to this staining process, Gram-negative bacteria appear red due to their distinct cell wall composition. The structural differences between Gram-negative and Gram-positive bacteria influence not only their staining properties but also the types of infections they cause and the antibiotics used for treatment (Tripathi & Sapra, 2023) .

Gram-negative bacteria possess a protective capsule that shields them from host immune responses, particularly from white blood cells responsible for combating infections. Beneath this capsule lies an outer membrane that serves as a barrier against certain antibiotics, such as penicillin. When this membrane is disrupted, it releases toxic substances known as endotoxins, which contribute to the severity of symptoms associated with Gram-negative bacterial infections (Bush, 2020).

Infections caused by Gram-negative bacteria include *Escherichia coli* infections, brucellosis, *Pseudomonas* infections, *Campylobacter* infections, salmonellosis, cholera, shigellosis, plague, Legionnaires' disease, typhoid fever, and pertussis, among others (Oliveira & Raygaert, 2021).

2.4 WATER QUALITY

Safe irrigation water is vital for crop health. Guidelines by WHO, EPA, and the EU stipulate that irrigation water should contain zero *E. coli* per 100 mL (WHO 2017; EPA 2012; EU 2015). Water sources in Vhembe (rivers, wells) have been shown to contain fecal coliforms and pathogenic *E. coli* (Alfasane *et al.* 2021). Poor water quality has been linked to diarrheal diseases (Aijuka *et al.* 2018). Water contaminants like nitrates and high total dissolved solids (TDS) can also stress plants and favor microbial growth (Alfinete *et al.* 2022).

Physical parameters of water (pH, TDS, EC, temperature) influence microbial survival. Recommended pH for irrigation is 6.5–8.5; more acidic or alkaline water can inhibit crops or alter microbial communities. Temperatures above 25 °C can increase microbial metabolism in water, potentially boosting pathogen survival (An *et al.* 2002). High TDS (> 450 mg/L) can stress plants and indicate salt or organic contamination. Farmers in Vhembe primarily use surface water and groundwater, which may be subject to contamination from runoff and livestock (Castro-Rosas *et al.* 2012).

Water sources can be contaminated by various pollutants, including pathogenic microorganisms, fertilizers, and decomposing organic waste. Additionally, human activities that produce domestic waste contribute significantly to water pollution (Jerry, 2022). Contaminated water increases the risk of waterborne diseases (Jeffrey, 2017). An estimated 2 billion people worldwide lack access to clean water systems (WHO, 2021), and over 500 known pathogens are associated with waterborne diseases (Nicholas, 2015). Common waterborne pathogens include *Escherichia coli, Salmonella,* and *Shigella* (Joao, 2010).

To ensure access to clean water, proper water quality management practices must be implemented. These include monitoring wastewater discharges into surface water, enforcing pollution control measures, and adhering to established water quality guidelines (Michalak & Chojnacka, 2014).

2.4.1 Water quality standards

Water quality is measured against standards set up by WHO and national regulations as shown in Table 2.1 below.

Table 2.1: Water quality standards set by WHO (2011) and DWAF (1998)

VARIABLE	STANDARD
Total coliforms and *E. coli*	0-10 MPN/100ml and 0 MPN/100ml
Conductivity	70-350µs/cm
pH	6.50-8.50
Total dissolved solids	1000mg/L
Temperature	18-24ºC

2.5 PHYSICAL PARAMETERS

Water intended for consumption, as well as for municipal and industrial applications, must comply with established quality standards. For instance, the Environmental Protection Agency (EPA) has set regulatory limits for over 90 contaminants commonly found in water. These regulations are essential for maintaining drinking water safety by preventing contamination that could result in health issues or the transmission of waterborne diseases (Sensorex, 2021).

In industrial settings, water often undergoes treatment to ensure its suitability for critical operational processes. Water quality is assessed using three primary categories of parameters: **physical, chemical, and biological**.

- **Physical parameters** include attributes such as color, taste, odor, temperature, turbidity, total solids, and electrical conductivity.

- **Chemical parameters** encompass factors such as pH, acidity, alkalinity, chlorine concentration, hardness, dissolved oxygen levels, and biological oxygen demand.
- **Biological parameters** refer to the presence of microorganisms such as bacteria, algae, and viruses.

Given the diverse requirements of different applications, adherence to water quality standards is crucial to ensuring both public health and industrial efficiency (Omer, 2019).

2.5.1 pH

One of the key indicators of water quality is pH, which is defined as the negative logarithm of the hydrogen ion concentration. It is a dimensionless value that determines the acidity or alkalinity of a solution. Water with a lower pH contains a higher concentration of hydrogen ions (H^+), making it acidic, while water with a higher pH has more hydroxyl ions (OH^-), making it basic (Omer, 2019).

The pH scale ranges from 0 to 14, where 7 represents a neutral solution. A pH value below 7 indicates acidity, whereas a value above 7 indicates alkalinity. Pure water is considered neutral, with a pH of approximately 7.0 at 25°C. However, due to the presence of atmospheric carbon dioxide, normal rainfall tends to be slightly acidic, with a pH of around 5.6. For safe domestic consumption, drinking water should maintain a pH range of 6.5 to 8.5, which is also the optimal range required to support most living organisms (Omer, 2019).

2.5.2 Total Dissolved Solids

Total Dissolved Solids (TDS) refers to the inorganic and trace organic substances dissolved in water. The **National Drinking Water Quality Standards (NDWQS)** set an acceptable limit for TDS at **1000 mg/L** (Rahmanian et al., 2015). TDS is measured by assessing the remaining solids in water after the removal of suspended particles. When the concentration of dissolved solids exceeds **300 mg/L**, it can adversely affect both living organisms and industrial processes (Karki, 2018).

2.5.3 Electrical conductivity

Conductivity is a measure of the presence of ions in water, which is primarily influenced by saltwater and, to a lesser extent, by leaching processes. High conductivity levels can also indicate industrial pollution (U.S. EPA, 2025). The removal of vegetation and the shift to monoculture farming can lead to increased runoff, which reduces water recharge during the dry season. This reduced recharge can, in turn, contribute to higher salinity in the water (Kaushal et al., 2021; U.S. EPA, 2025)

2.5.4 Temperature

Temperature plays a significant role in various water quality aspects, affecting properties such as palatability, viscosity, solubility, odor, and the rate of chemical reactions. It also influences processes like sedimentation, chlorination, and biological oxygen demand (BOD). Additionally, temperature impacts the absorption of dissolved heavy metals in the body. Water temperatures between 10-15°C are generally considered most appealing to humans (Omer, 2019).

While temperature is not a direct indicator of water potability, it is an important physical factor in the overall quality of natural water systems, including rivers and lakes. As temperature increases, the solubility of oxygen in water decreases, which can affect aquatic life. Higher temperatures also accelerate the growth of aquatic microorganisms, leading to an increased consumption of dissolved oxygen, thereby reducing oxygen levels. Moreover, temperature can influence the effectiveness of water disinfection, with disinfection efficiency typically declining at lower temperatures (Karki, 2018). Increased conductivity can indicate intrusion in upstream water sources, which may be influenced by temperature changes (Tiwari, 2015).

2.6 MICROBIAL CONTAMINANTS

Microbiological contamination refers to the unintended or accidental introduction of microorganisms, including bacteria, yeast, mold, fungi, viruses, prions, protozoa, or their toxins and byproducts. In water, microbial contamination is often linked to fecal contamination, which typically arises from human activities (such as wastewater treatment plants, combined sewage overflows (CSOs), and non-collective sewage systems), as well as from domesticated animals (due to manure application or pit stock overflow) and wildlife (Jung et al., 2017).

2.6.1 Total coliforms (TC)

The **total coliform bacteria test** is the primary method for detecting bacterial contamination in water supplies. Total coliform counts serve as a standard indicator of the sanitary quality of water. These bacteria are commonly found in the soil, surface water, and in the waste of humans or animals (Bassett, 2017).

2.6.2 Fecal Coliforms (FC)

Fecal coliforms are a subgroup of total coliforms that are typically found only in the intestines and feces of warm-blooded mammals. They are considered a more precise indicator of animal or human waste compared to total coliforms because their sources are more specific (Oram, 2020).

Among the fecal coliform group, *E. coli* is the most prevalent species. As one of the five main groups of bacteria within the total coliforms, *E. coli* is rarely found thriving and multiplying in the environment. Therefore, *E. coli* is considered the most reliable indicator of fecal contamination and the potential presence of pathogens among coliform bacteria (Bassett, 2017).

2.7 METHODS USED IN THE DETECTION, ISOLATION AND IDENTIFICATION OF WATER MICROORGANISMS

Various methods are used to detect microorganisms in water. In the early 1900s, methods were developed to evaluate water quality in response to public health concerns, with a focus on quantifying *E. coli* and coliform bacteria as indicators of water purity (Eckner, 1998). Culture-based techniques are frequently employed for the detection and enumeration of fecal coliforms (Douterelo et al., 2014).

2.7.1 Colilert® Quanti-Tray®

As stated by Afnor (2001), the Colilert system utilizes defined substrate technology to enable the simultaneous detection of both total coliforms (TCs) and *E. coli* directly from water samples. This system is available in two formats: the most probable number (MPN) method and the presence/absence (PA) method. The MPN method is carried out using a specially designed disposable incubation tray known as the Quanti-Tray®. manufactured by IDEXX Laboratories (IDEXX, 2020).

Principle

Colilert® uses two nutrient indicators, Ortho-nitrophenol galactoside (ONPG) and 4-methyl-umbelliferyl-β-D-glucuronide (MUG), to detect the presence of coliforms and *E. coli* in water. Coliforms metabolize ONPG through the enzyme β-D-galactosidase, resulting in a color change from colorless to yellow. In contrast, *E. coli* metabolizes MUG using the enzyme β-D- glucuronidase, producing fluorescence when exposed to ultraviolet (UV) light (Sundram et al., 2000).

2.7.2 Multiple Tube fermentation technique

This detection method utilizes multiple tubes, and the results are determined by examining replicate tubes and dilutions, which are reported as the Most Probable Number (MPN) of organisms present. The MPN is an estimate of the average concentration of coliforms in the water sample, calculated using specific probability formulas (APHA, 1998).

For over 80 years, this technique has been a reliable method for monitoring water quality. The process involves inoculating a series of tubes with appropriate decimal dilutions of water samples. A positive presumptive reaction is indicated by gas production, acid formation, or substantial growth in the test tubes after 48 hours of incubation at 35°C.

2.7.3 Analytical Profiling Index (API) (bioMérieux, 2018).

The API 20 E method is a diagnostic tool used for identifying non-fastidious, gram-negative rods through 21 biochemical tests, supported by a database for bacterial identification. The system consists of a strip with 20 microtubes, each containing dehydrated substrates. A bacterial suspension is added to rehydrate the media, and then the bacterial sample is inoculated. Throughout the incubation period, various color changes occur, either spontaneously or after reagent addition, indicating metabolic activity. The results are interpreted using a reference table, which compares the reaction profile to a database for bacterial identification (Bio Merieux®sa).

2.7.4 Multiplex Polymerase Chain Reaction (m-PCR)

Multiplex Polymerase Chain Reaction (m-PCR) is a technique that allows for the simultaneous amplification of multiple DNA sequences within a single PCR reaction. This is accomplished by using multiple primers and a heat- activated DNA polymerase in a thermal cycler. The primers must be precisely designed to function effectively at the same annealing temperature during the PCR process. This method enables the efficient amplification of different target DNA regions in one reaction, making it a valuable tool for genetic analysis (SA Biosciences and Qiagen).

2.8 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) has become a pressing global concern, not only in clinical settings but also in agricultural environments, where the use of antibiotics in crop production contributes to the emergence and spread of resistant bacteria. *Enterobacter* species, which are commonly associated with both environmental and clinical sources, have been increasingly implicated in the post-harvest spoilage of onions and other vegetables. These bacteria are capable of acquiring resistance genes through horizontal gene transfer, making them potential reservoirs of antimicrobial resistance within the plant microbiome (Kumar et al., 2022). Studies have shown that *Enterobacter* isolates from fresh produce can carry extended-spectrum β-lactamase (ESBL) genes, posing a potential risk to human health when consumed raw (Zurfluh et al., 2020). The presence of AMR in phytopathogenic and opportunistic bacterial species such as *Enterobacter* highlights the importance of monitoring resistance in agricultural systems, as these organisms can act as a bridge for resistance gene transmission between the environment and clinical pathogens (Mabeya et al., 2023). Therefore, assessing the antimicrobial susceptibility of *Enterobacter* species involved in onion decay is essential for understanding their role in both crop loss and the broader AMR landscape.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY SITE

The study was carried out in the Vhembe district (Figure 3.1), located in the Limpopo province of South Africa. It primarily examined farms within various municipalities, encompassing both commercial and subsistence farming, along with the rivers that flow through the area.

Figure 3.1: Map showing the study site of Vhembe district.Picture taken from https://municipalities.co.za/map/129/vhembe-district-municipality.

3.2 SAMPLE COLLECTION

A total of thirty six samples (Table 3.1), including water, soil, and onions, were collected from the farms and their surrounding areas. Water samples were collected from various sources, including irrigation water used on the farms and nearby rivers, using sterile 500ml bottles (LASEC®, Johannesburg; GA, RSA). Soil samples were taken from around the onions on the farms, and onions were gathered from four different farms, both commercial and subsistence. All sample collections were conducted using sterile plastic containers (LASEC®). Upon collection, physical parameters such as pH, temperature, total dissolved solids (TDS), and electrical conductivity were measured using a portable multi-parameter reader (PC 70 Vio Portable Multimeter, LASEC®). The samples were then placed in a cooler box and transported immediately to the University of Venda's microbiology laboratory for further analysis.

3.2.1 TYPES OF SAMPLES COLLECTED

Table 3.1 lists the sources of the samples (onions, soil, and water) collected from three farms (Farm 1, Farm 2, and Farm 3). Each sample is identified by a unique ID and categorized as coming from either commercial or substantial farming. Farm 1 was a commercial while farm 2 and 3 were substantial.

Table 3.1: Table showing the sample type and their codes.

Univen ID	Sources	Type farming
Onion A2	Onion Farm 1 Site A2	Commercial Farming
Onion A1	Onion Farm 1 Site A1	Commercial Farming
Onion A3	Onion Farm 1 Site A3	Commercial Farming
Onion A4	Onion Farm 1 Site A4	Commercial Farming
Onion A5	Onion Farm 1 Site A5	Commercial Farming
Onion B2	Onion Farm 1 Site B2	Commercial Farming
Onion B3	Onion Farm 1 Site B3	Commercial Farming
Onion B4	Onion farm 1 Site B4	Commercial Farming
Onion B5	Onion Farm 1 Site B5	Commercial Farming
OBM	Onion Farm 2	Substantial farming
OBE	Onion Farm 2	Substantial farming
OCM	Onion Farm 3	Substantial farming
OCE	Onion Farm 3	Substantial farming
S2 IWA	Soil farm 1	Commercial farming
IWA S3	Soil Farm 1	Commercial farming
IWA S1	Soil Farm 1	Commercial farming
S3 IWA	Soil Farm 1	Commercial farming
SI IWA	Soil Farm 1	Commercial farming
SBE	Soil Farm 2	Substantial farming
SBM	Soil Farm 2	Substantial farming
SCM	Soil Farm 3	Substantial farming
SCE	Soil Farm 3	Substantial farming
BHW	Borhole water Farm1	Commercial farming

IWA W1	Water Farm 1	Commercial farming
IWC 3W	Water Farm 1	Commercial farming
IWB W2	Water Farm 1	Commercial farming
FB3M water	Water Farm 2	Substantial Farming
FBME water	Water Farm 2	Substantial Farming
FB2M water	Water Farm 2	Substantial Farming
FB2E water	Water Farm 2	Substantial Farming
FB1E	Water Farm 2	Substantial Farming
FC2M water	Water Farm 3	Substantial Farming
FCMM water	Water Farm 3	Substantial Farming
FCME water	Water Farm 3	Substantial Farming
FC3E water	Water Farm 3	Substantial Farming
FC1E water	Water Farm 3	Substantial Farming

3.3 ISOLATION OF *ENTEROBACTERIACEAE* FROM THE SAMPLES

3.3.1 Water samples

The Colilert Quanti-Tray system (IDEXX, Westbrook, Maine, USA), was employed to detect Enterobacter spp. in water samples, with the most probable number (MPN) per 100mL being determined. The process adhered to the manufacturer's protocol. A 100mL water sample was placed into a sterile conical flask (IDEXX), and Colilert medium was added and thoroughly mixed until fully dissolved. The mixture was then transferred into a Quanti- Tray, sealed with a Quanti-Tray sealer (Model 2020, IDEXX), and incubated at 35°C for 18 hours. Positive controls, including *E. coli* Quanti-Cult reference strains, were included in the procedure, and blank water samples were processed in parallel with the test samples (Traore et al., 2016).

Upon completion of incubation, wells that showed no color change, appearing less yellow than the comparator, were deemed negative for total coliforms. Wells that turned yellow or exhibited a more intense yellow than the comparator were considered positive for total coliforms. Additionally, wells that fluoresced under UV light (Spectroline CM UV; Sigma-Aldrich, St Louis, MI, USA) at 365nm were identified as positive for *E. coli*, in accordance with the manufacturer's guidelines.

3.3.2 Membrane filtration

A 100 mL sample was taken from each water source and filtered using a sterile membrane with a 0.45 µm pore size (Sartorius Stedim Biotech GmbH, 37070 Gottingen, Germany). The filter was then carefully placed onto EMB (Eosin Methylene Blue) and MacConkey agar plates (Davies Diagnostics, Johannesburg, GA, RSA) using sterile forceps and incubated (Inco Therm digital, Labotec; Midrand, GA, RSA) at 37°C for 24 hours . After incubation, purified colonies were separated by sub-culturing them onto the same agar media (Tankeshwar, 2022).

3.3.3 From soil samples

Before vortexing (Vortex-Genie 2 mixer, LASEC), all soil samples were sieved and combined with phosphate-buffered saline (PBS; LASEC) for 5 minutes. The resulting liquid mixture was then poured onto EMB (Eosin Methylene Blue) agar and MacConkey agar plates (Davies Diagnostics) using the pour plate method, followed by streaking. The plates were incubated (Inco Therm digital, Labotec) at 37°C for 24 hours. After incubation, isolated colonies were obtained by sub-culturing onto new plates of the same media using quadrant streaking.

3.3.4 From Onion samples

The scales from diseased bulbs and fresh onions were surface-sterilized by soaking them in 70% ethanol (Merck; Dormstadt, Germany) for 30 seconds, followed by three rinses in sterilized water. The onions were then sliced into small pieces, ranging from 1 to 5 mm in length. These tissue pieces were macerated in a PBS solution (Merck) for 5 minutes. The resulting vortexed solution was streaked and poured onto EMB (Eosin Methylene Blue) and MacConkey agar plates (Davies Diagnostics), which were incubated (Inco Therm digital, Labotec) at 37°C for 24 hours. After incubation, isolated colonies were obtained by sub-culturing onto fresh plates of the same media using quadrant streaking.

3.4 IDENTIFICATION AND CHARACTERIZATION OF SPECIES USING VITEK-2® SYSTEM

At the University of Johannesburg (UJ), the stock culture strains were subcultured onto nutrient agar plates (Davies Diagnostics) to ensure their purity. A densitometer (1100 NTU; Sigma-Aldrich) was used to adjust the turbidity of the bacterial suspensions to match a McFarland 0.5 standard using a 0.45% sterile sodium chloride solution. To maintain consistent turbidity, the time between suspension preparation and card loading was kept under 30 minutes. The VITEK® 2 system (bio-Merieux; Marcy-l'Étoile, Isére, France) was then manually loaded with ID-GNB cards, AST-No. 12 cards, and the bacterial suspension. The bacterial suspension was automatically added to each test card, which was then sealed and incubated for three hours. During incubation, the cards were read every 15 minutes using kinetic fluorescence measurement. The VITEK® 2 system software analyzed the data and automatically reported the results (Ling et al., 2001).

3.5 MOLECULAR CHARACTERIZATION OF *E. COLI*

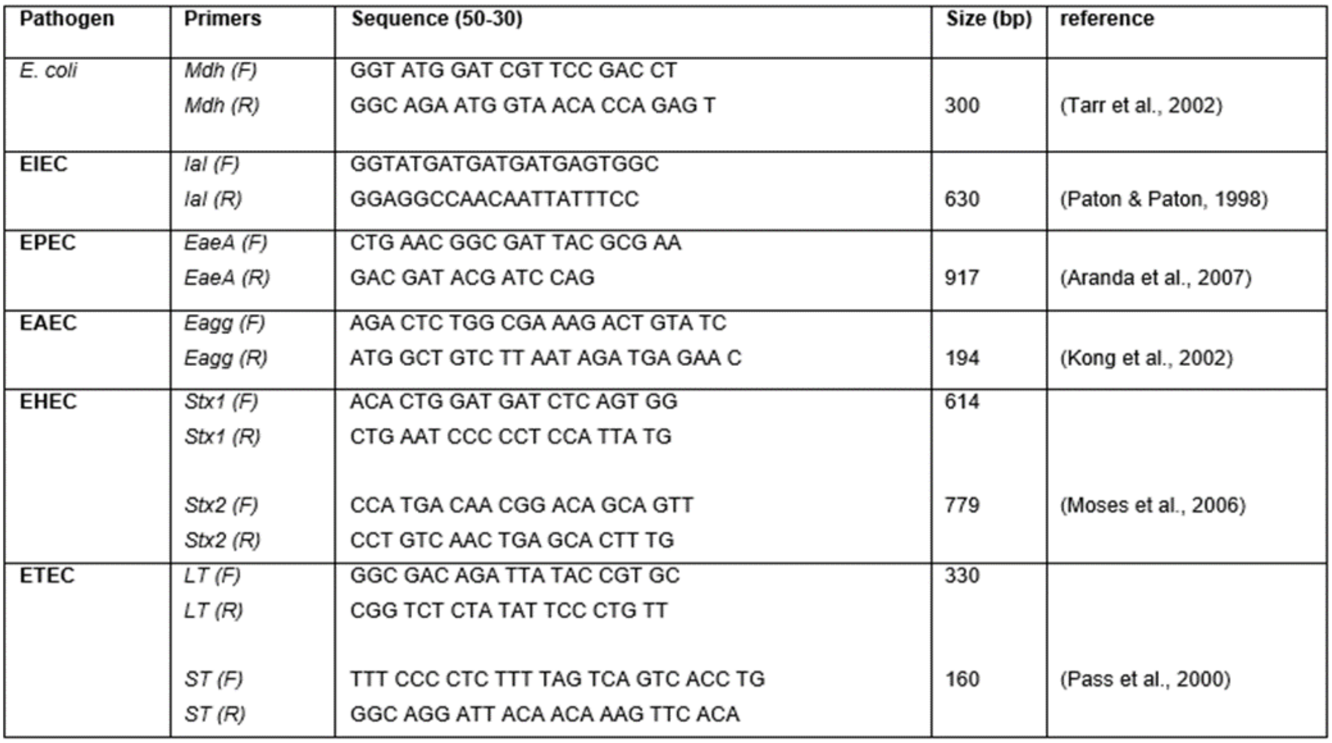
3.5.1 DNA Extraction

The cells from a 2 mL sample of a single enriched colony were collected by centrifugation (Eppendorf Centrifuge 5420; Sigma-Aldrich) at 13,000 x g for two minutes, after which the supernatant was discarded. DNA extraction from the bacterial cells was performed using the silica thiocyanate method with an adapted spin method (Omar and Barnard, 2014). The modification included adding 250 µL of 100% ethanol (Merck) to the lysis buffer to improve DNA binding to the Celite. In the spin method, the Celite with the bound DNA was transferred to a DNA binding membrane (Corning CoStar Spin-X; Sigma-Aldrich). The DNA was then eluted using 100 µL of Qiagen elution buffer, as outlined by Omar et al. (2010).

3.5.2 m-PCR

The isolates' genomic DNA was extracted using the Qiagen kit based on the manufacturer's instructions (Qiagen, Hilden, Germany). To detect the six Enterobacter pathotypes, an m-PCR protocol was used as described by Omar and Barnard et al (2014). m-PCR reactions were carried out in a total volume of 20µl, containing 1µl Quigen PCR multiplex mix (including m-PCR buffer, HotStart Taq DNA polymerase, and dNTP mix), 2µl of primer mix (Table 3.2), 5µl of PCR grade water, and all reactions were carried out using a BIORAD M cycler

Table 3.2: Table showing the specific primers used in m-PCR reaction (Tarr et al., 2002; Paton & Paton, 1998; Aranda et al., 2007; Kong et al., 2002; Moses et al., 2006; Pass et al., 2000).



Pathogen	Primers	Sequence (50-30)	Size (bp)	reference
E. coli	*Mdh (F)* *Mdh (R)*	GGT ATG GAT CGT TCC GAC CT GGC AGA ATG GTA ACA CCA GAG T	300	(Tarr et al., 2002)
EIEC	*Ial (F)* *Ial (R)*	GGTATGATGATGATGAGTGGC GGAGGCCAACAATTATTTCC	630	(Paton & Paton, 1998)
EPEC	*EaeA (F)* *EaeA (R)*	CTG AAC GGC GAT TAC GCG AA GAC GAT ACG ATC CAG	917	(Aranda et al., 2007)
EAEC	*Eagg (F)* *Eagg (R)*	AGA CTC TGG CGA AAG ACT GTA TC ATG GCT GTC TT AAT AGA TGA GAA C	194	(Kong et al., 2002)
EHEC	*Stx1 (F)* *Stx1 (R)*	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	
	Stx2 (F) *Stx2 (R)*	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA CTT TG	779	(Moses et al., 2006)
ETEC	*LT (F)* *LT (R)*	GGC GAC AGA TTA TAC CGT GC CGG TCT CTA TAT TCC CTG TT	330	
	ST (F) *ST (R)*	TTT CCC CTC TTT TAG TCA GTC ACC TG GGC AGG ATT ACA ACA AAG TTC ACA	160	(Pass et al., 2000)

3.6 ANTIMICROBIAL TESTING

Antimicrobial testing provides a quantitative sensitivity result via minimal and highly controlled handling of antibiotics, which inhibits the visible growth of cultures. Thus, antimicrobial resistance can be detected accurately at the earliest possible stage, The VITEK® 2 system, an automated instrument used for microbial identification was used for antimicrobial susceptibility testing (AST). It provided rapid and accurate results The instrument measures bacterial response to different antibiotics and determines Minimum Inhibitory Concentrations (MICs). This was done at the University of Johannesburg using an established method.

3.7 MITOCHONDRIAL 16S SEQUENCING

Mitochondrial 16S rRNA sequencing is commonly used for species identification, phylogenetic studies, and evolutionary analysis. The 16S rRNA gene in the mitochondrial genome is highly conserved and provides valuable genetic information for differentiating species (Palumbi et al., 1991).

Briefly, PCR amplification of the 16S rRNA gene was done using specific primers (Table 3.3) targeting variable regions of Enterobacter. The amplicons were purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050), and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific). This was done at Inqaba Biotec in Pretoria.

Table 3.3: Primers used to characterize Enterobacter

Target Gene	Primer Name	Sequence (5' → 3')	Reference
16S rDNA	Ent-F	AGAGTTTGATCCTGGCTCAG	Wajid et al., 2013
16S rDNA	Ent-R	GGTTACCTTGTTACGACTT	Wajid et al., 2013
rpoB	rpoB-F	TCAAGGARCAYCARGAAGG	Wajid et al., 2013
rpoB	rpoB-R	TGTGGTGTTGGCGGTCRCA	Wajid et al., 2013
Enterobacter cloacae	Enc-F	CGCAGAGTTGAGCCTGC	Smith et al., 2020
Enterobacter cloacae	Enc-R	CGTTGCTTCTTCTGCGT	Smith et al., 2020

Data Analysis:

FinchTV (https://finchtv.software.informer.com/1.4/) is used to view the raw chromatogram files (.abi). CLC Bio Main Workbench was used to assemble the forward and reverse sequencing reads to form a consensus sequence for each sample. BLASTn analysis (with default parameters) (Altschul et al., 1997) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matches the query sequence above a certain threshold (99% query coverage; 99% identity)..

CHAPTER 4

RESULTS AND DISCUSION

4.1 DESCRIPTION OF STUDY SITES

The study was conducted in the Vhembe district, which is located in the north part of the Limpopo Province in South Africa. It covers approximately 25,000 square kilometers. The district is predominantly rural, with a population of approximately 1.2 million people. Agriculture is an important sector in the local economy, where crops such as maize, beans, spinach, cabbage and onions are grown. In most irrigation systems, the irrigation water sources are mainly limited to private ponds or reservoirs, as well as direct river water. Owing to this limitation of other water sources, samples were taken from three locations, namely, Alldays-Commercial Farm 1, Vhufulwi Village-Subsistence Farm 2, and Thengwe Village-Subsistence Farm 3.

Only the commercial farm (Figure 4.1) gave us permission to take photos and measure their size. The highly productive farms were reluctant and due to popi act, we had to abide to their decision.

Samples were collected from June 2023 to April 2024. The area is arid and hot; the topsoil is coarse, of sandy texture. The crop depends mainly on irrigation water from a borehole. The irrigation is done automatically by a rotating pivot sprayer. Three samples were collected from the sprinklers on the pivot, and three soil samples were taken under each sprayer. Field was divided in 2 sides across a central road. The onions had a short time before harvest. Since the water from the borehole could not be fetched from a tap, water dripping was collected nto the sampling bottles via a leak. The area around the borehole was relatively wet due to the leak. Birds like finches were seen drinking and bathing in the small puddle below the borehole pump. A viscous soil sample was subsequently collected from the area surrounding the borehole. Faeces of small animals (species unknown) could also be observed surrounding the pump.

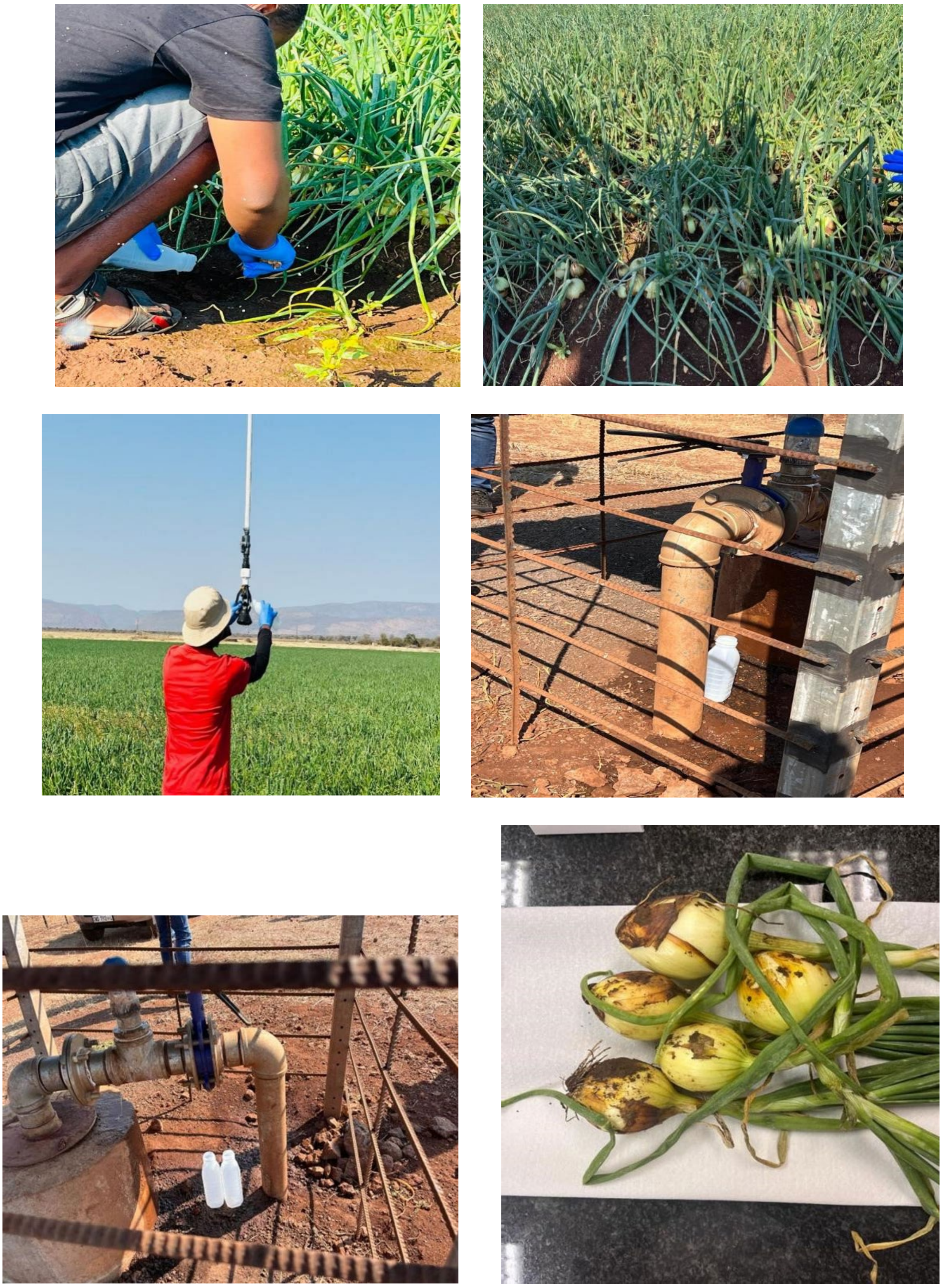


Figure 4.1: Pictures taken at the commercial farm

4.2 ASSESSING THE PHYSICAL PARAMETERS OF WATER SAMPLES

Physical parameters of irrigation water measured from the three farms are presented in Table 4.1, including temperature (°C), electrical conductivity (EC, µS/cm), pH, and total dissolved solids (TDS, mg/L).

Temperature is an important physical parameter that might impact water quality and the survival of aquatic ecosystems. However, there is no guideline to be referred to. The temperatures at the farms recorded ranged between 19.8-25°C, mostly within the optimum range for bacterial proliferation. This is quite typical for some pathogens such as *Escherichia coli* and Enterobacteriaceae, among others (Dai et al., 2018). Such temperatures may enhance microbial growth and increase the likelihood of irrigation water being contaminated. The slight temperature elevation at Farm 2 could be attributed to environmental factors, such as exposure to sunlight, soil composition, and microbial activity in water sources (Skeie et al., 2021).

Table 4.1: Physical parameters of water samples

Sampling sites	Temperature	Electrucal Conductivity	pH	TDS
FARM 1	21,9	263	6,26	150,5
	21,8	227	6,3	145,2
	21,5	196.6	6,17	128,9
	21,73333333	***245***	***6,243333333***	***141,5333333***
FARM 2	22,2	201	6,13	126,6
	25	220	6,32	140,7
	24,5	222	6,43	142,3
	23,9	***214,3333333***	***6,293333333***	***136,5333333***
FARM 3	19,8	358	5,9	229
	20	363	5,99	232
	20,2	400	5	450
	20	***373,6666667***	***5,63***	***303,6666667***
DWAF Standard*	N/A	< 540 µS/cm	[6,5 – 8,5]	< 0,02 mg/l

Electrical conductivity (EC) determines the ability of water to conduct an electric current and is influenced by the presence of dissolved ions. The values obtained for electrical conductivity across the farms ranged between 214.3-373,6 and were within the recommended threshold set by the Department of Water Affairs and Forestry ≤540 µS/cm, which indicates an acceptable level of mineral ions in water (DWAF, 1996). Electrical conductivity within the recommended limit may indicate that water is unlikely to contain excess salinity that could affect crop growth. Slight increases in EC noted at Farm 3 might be due to mineral leaching from fertilizers or natural soil constituents, and this could act over time to degrade water quality (Nielsen et al., 2016).

pH indicates the acidity or alkalinity of water and plays a major role in determining the overall water quality and the health of aquatic ecosystems. The pH values for all farms ranged between 5.0-6.4, which is lower than the recommended pH range of 6.5-8.5 for agricultural water by DWAF (1996). This slight acidity could be from fertilizer runoff, decaying organic matter, or natural soil properties, each influencing microbial survival and growth differently (Zhang et al., 2020). Acidic conditions can promote the growth of certain microbial species and may be one of the reasons for pathogen survival and contamination.

Total Dissolved Solids (TDS) measures the total amount of dissolved solids in water and can be an indicator of the presence of various contaminants or substances in the water. The values obtained ranged between 136.5-303.7. In Farm 3, water samples showed a significant rise in TDS, with a peak of 450 mg/L. High TDS indicates mineral buildup, likely from fertilizer application and runoff from the environment, which could lead to soil salinity and reduce nutrient absorption in crops (Dai et al., 2018). TDS levels higher than the acceptable limits can be harmful to plant health and water quality, hence the need for monitoring dissolved solids in irrigation water (Nielsen et al., 2016).

The deviations from recommended DWAF standards in pH and TDS indicate potential chemical imbalances that can impact plant growth and facilitate microbial contamination in agricultural settings. Such imbalances may lead to unfavorable crop conditions and increase contamination risk through microbial proliferation (DWAF, 1996).

4.3 MICROBIOLOGICAL ANALYSIS

Detection of Total coliform and E. Coli: Colilert Q-Tray

There were high total coliform concentrations (Figure 4.3) reaching the threshold limit of 2419.6MPN / 100ml (Table 4.2); and obtained for all 3 farms, denoting significant microbial contamination. Generally, coliforms indicate organic contamination introduced through animal waste, decaying vegetation, or even human activities around water sources (Skeie et al., 2021). Detection of coliforms indicates that these water sources have been compromised, thus threatening possible risks to the crops irrigated with this water.

The detection of *E. coli* (Figure 4.3) was significant, especially in Farms 2 and 3 (Table 4.2). The values ranged between 0-200.5 MPN. The presence of *E. coli* in irrigation water indicates fecal contamination, originally from human, livestock, or wildlife sources near water systems (Xu et al., 2019). It serves as an indicator for potential health risk due to the capabilities of *E. coli* to cause foodborne illness upon consumption.

Figure 4.2: Detection of Total coliform and E. Coli: Colilert Q-Tray. Positive wells for total coliform (Left picture) and positive blue wells fluorescence for *E. coli* (right picture)

Table 4.2 shows the microbial contamination levels in onion and irrigation water samples, using the Colilert Q-Tray method. Results are given in Most Probable Number (MPN/100 mL).

Table 4.2: Detection of total coliforms and *E.coli*: Colilert Q-tray

Sample ID Farm 1	**Total Coliforms (MPN/100ml)**	**E. Coli (MPN/100ml)**	**Conclusion**
Onions site A	2419.6	1.00	Contaminated
Onions site B	2419.6	0	No *E. Coli*
Irrigation water	2419.6	0	No *E. Coli*
Sample ID Farm 2	**Total Coliforms (MPN/100ml)**	**E. Coli (MPN/100ml)**	**Conclusion**
Onions 1	2419.6	100.5	Contaminated
Onions 2	2419.6	100.5	Contaminated
Irrigation water	2419.6	200.5	Contaminated
Sample ID Farm 3	**Total Coliforms (MPN/100ml)**	**E. Coli (MPN/100ml)**	**Conclusion**
Onions 1	2419.6	100.5	Contaminated
Onions 2	2419.6	100.5	Contaminated
Irrigation water	2419.6	200.5	Contaminated

The contamination likely comes from the farms or from human-related activities. This emphasizes the need for more efficient management of water from the catchment areas. High levels of total coliforms and E. coli reveal poor water quality due to contamination sources external to the water. Treatment of the water and regular monitoring will therefore be necessary for irrigation safety since untreated water may transmit harmful bacteria to crops (Skeie et al., 2021).

Detection of Pathogens by Membrane filtration

The presence of Enterobacter (Table 4.3) in water samples were detected using the membrane filtration. A membrane filters containing colonies of presumptive microorganisms were placed on EMB and MAC media for Enterobacter species to grow. Few water samples were presumptive positive for Enterobacter species when grown in MAC media, as shown by the colonies appearing as pink due to lactose fermentation. (Rijal, 2022).

Figures 4.3 and 4.4 illustrate the pathogens isolated from the samples. The isolates were presumptively identified based on their physical characteristics such colour, the texture, shape, size, and odour. (Table 4.3).

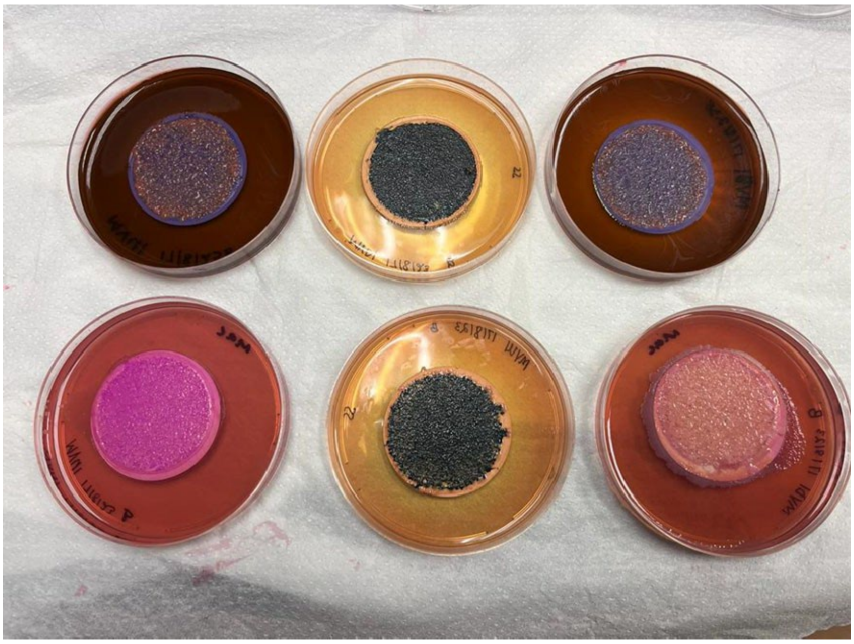


Figure 4.3: Growth of bacteria on selective media after membrane filtration. *Plates showing colonies on (a) Eosin Methylene Blue (EMB) agar, (b) MacConkey agar, and (c) Salmonella-Shigella (SS) agar following incubation.*

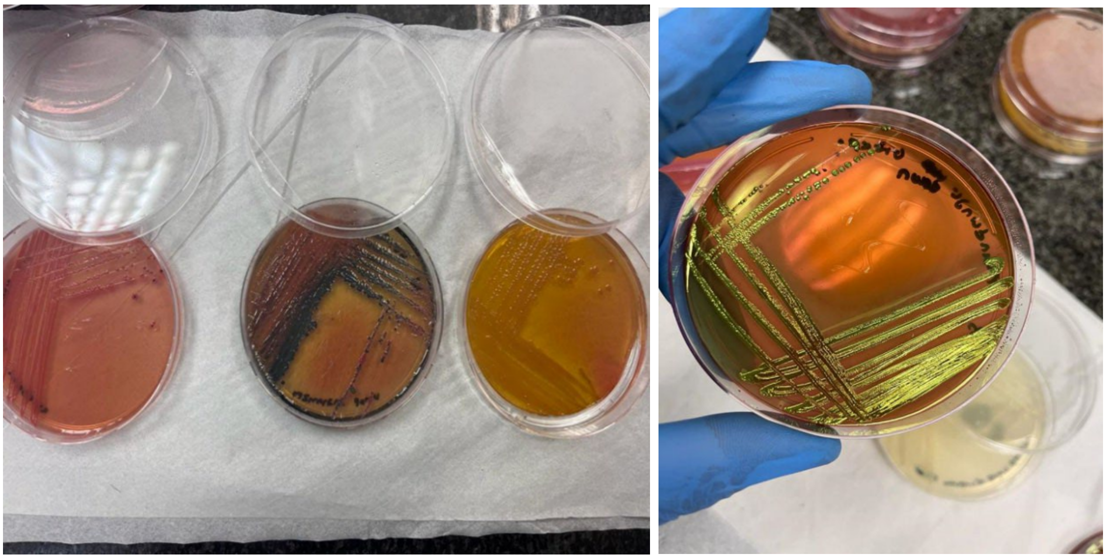


Figure 4.4: Colony morphology after subculturing on selective media. *EMB agar (left) displaying metallic-green sheen colonies characteristic of E. coli, MacConkey agar (center) showing lactose-fermenting pink colonies, and SS agar (right) showing black colonies of Salmonella*

Table 4.3: Identification by morphology on the plates

Sample	Colour and Morphology	Presumptive
1	Pink, mucoid, and moist	*Citrobacter, Klebsiella spp, Enterobacter spp*
2	Pink, mucoid, and moist	*Citrobacter, Klebsiella spp, Enterobacter spp*
3	Pink with a dot and slight rough	*Enterobacter spp*
4	Pink mucoid, and moist	*Enterobacter, Klebsiella spp., Citrobacter spp*
5	Pink mucoid, and moist	*Enterobacter, Klebsiella spp., Citrobacter spp*
	Pink with mucous texture	*Klebsiella spp*
6	Pink and mucoid	*Citrobacter, Klebsiella spp*
7	Yellow	*Proteus spp*
8	Pink, smooth,	*Enterobacter spp, Klebsiella spp*
9	Pink-red with a dot, smooth-moist	*Citrobacter, spp, E. coli, Enterobacter spp*
10	Pink	*E.coli*
11	Pink	*E. coli*
12	clear with a dot	*hafnia*
13	Pink, mucoid, and moist	*Klebsiella spp, Enterobacter spp, Citrobacter spp*
14	Metallic green sheen	*E.coli*
15	Pink with a dot and slight rough	*Enterobacter spp, klebsiella, Citrobacter spp*
16	Pink, smooth and mucoid	*Klebsiella spp, Citrobacter spp*
17	Brown with mucous texture	*Enterobacter spp, klebsiella*
18	Metallic green sheen	*E. coli*
19	Pink with a dot	*E. coli,*
20	Pink mucoid, and moist	*Enterobacter spp Klebsiella spp., Citrobacter spp*
21	Pink-red with a dot, smooth-moist	*Citrobacter, spp, E. coli, Enterobacter spp*
22	Brown with mucous texture	*Enterobacter spp, klebsiella*
23	Dark pink	*Klebsiella spp, Enterobacter spp, E. coli*
24	Pink, smooth and mucoid	*Enterobacter spp, klebsiella*
25	Pink with a dot	*E. coli*
26	Yellow	*Proteus spp*
27	Pink and mucoid	*Citrobacter, Klebsiella spp*
28	Pink with a dot and slight mucoid	*Klebsiella spp, Enterobacter spp*
29	Black	*Salmonella*
30	Yellowish pink	*Proteus spp*
29	Purple	*Enterobacter spp, E. coli*
30	Metallic Green sheen	*E. coli*

31	Clear-White	*Proteus spp, Providencia spp*
32	Pink, large, mucoid and with red pigment	*E. coli, Citrobacter spp, klebsiella spp*
33	Pink with white dot, smooth- moist	*Klebsiella spp, Enterobacter spp*
34	Brown with mucous texture	*Enterobacter spp, klebsiella*
35	Brown with mucous texture	*Enterobacter spp, klebsiella*
36	Brown with mucous texture	*Enterobacter spp, klebsiella*

Following subculture, the colonies were stained with Gram stain and categorized. Table 4.4 shows the pathogens stained characteristics and the analysis demonstrated the presence of both Gram-negative bacteria (GNB) and Gram-positive bacteria (GPB). From the onion samples, all were categorized as GNP (100%) while from the soil samples only 4/9 samples were GNP (44%) whereas for water samples, 4/14 (29%) were found to be GNP.

Table 4.4: Gram staining results of isolates. Univen ID (sample code), Gram reaction (GNB/GPB), with GNB abbreviations defined in the caption (GPB = Gram-positive bacteria, GNB = Gram-negative bacteria)

Univen ID	**Gram rxn**
Onion A2	GNB
Onion A1	GNB
Onion A3	GNB
Onion A4	GNB
Onion A5	GNB
Onion B2	GNB
Onion B3	GNB
Onion B4	GNB
Onion B5	GNB
OBM	GNB
OBE	GNB
OCM	GNB
OCE	GNB
S2 IWA	GPB
IWA S3	GPB
IWA S1	GPB
S3 IWA	GNB
SI IWA	GPB
SBE	GNB
SBM	GPB
SCM	GNB
SCE	GNB

BHW	GPB
IWA W1	GNB
IWC 3W	GNB
IWB W2	GPB
FB3M water	GPB
FBME water	GPB
FB2M water	GPB
FB2E water	GPB
FB1E	GNB
FC2M water	GPB
FCMM water	GPB
FCME water	GNB
FC3E water	GPB
FC1E water	GPB

4.4 CHARACTERIZAYION OF ENTEROBACTER USING VITEK-2

After the presumptive isolates were sub-cultured and categorized, Vitek®-2 was run and Table 4.5 shows the different organisms detected. In this study we focused only on gram negatives.

The internal biochemical test results for *Klebsiella oxytoca* and the *Enterobacter cloacae* complex provide insight into their metabolic functions and antibiotic resistance. The biochemical profile of *Klebsiella oxytoca* closely resembles that of other *Klebsiella* species, with most tests yielding positive results that confirm its broad metabolic capabilities, including sugar fermentation and other enzymatic functions, which are characteristic of *Enterobacterales* (Janda & Abbott, 2006; bioMérieux, 2018). However, certain negative results, such as for hydrogen sulfide (H_2S) and beta-glucuronidase (BGLU), help differentiate it from related species (bioMérieux, 2018; Wang et al., 2022).The detected pathogens included Enterobacter cloacae, Klebsiella *spp*, Pseudomonas aeruginosa, Bacillus *spp* and Citrobacter. Cumulatively, 10 isolates were found to be Enterobacter Cloacae Complex.

TABLE4.5: VITEK 2 identification of isolates. *Univen ID, Gram reaction, and species identified by VITEK 2 (e.g. Enterobacter cloacae complex). Abbreviations: A2 = Onion sample Farm A2, OBM = onion bulb (maconkey agar), IWA S3 = irrigation water sample S3.*

Univen ID	**Gram rxn**	**Detected Pathogens**
Onion A2	GNB	Enterobacter Cloacae Complex
Onion A1	GNB	Enterobacter Cloacae Complex
Onion A3	GNB	Klebsiella oxytoca
Onion A4	GNB	Enterobacter Cloacae Complex
Onion A5	GNB	Enterobacter Cloacae Complex
Onion B2	GNB	Enterobacter Cloacae Complex
Onion B3	GNB	Enterobacter Cloacae Complex
Onion B4	GNB	Enterobacter Cloacae Complex
Onion B5	GNB	Klebsiella oxytoca
OBM	GNB	Klebsiella oxytoca
OBE	GNB	Klebsiella oxytoca
OCM	GNB	Raoultella Ornithinolytica
OCE	GNB	Enterobacter Cloacae Complex
S2 IWA	GPB	Bacillus Cereus
IWA S3	GPB	Bacillus Cereus
IWA S1	GPB	Bacillus Cereus
S3 IWA	GNB	Enterobacter Cloacae Complex
SI IWA	GPB	Bacillus subtilis
SBE	GNB	Klebsiella pneumonia
SBM	GPB	Lysinibacillus sphaericus
SCM	GNB	Pseudomonas aeruginosa
SCE	GNB	Pseudomonas aeruginosa
BHW	GPB	Bacillus Cereus
IWA W1	GNB	Citrobacter amalonaticus
IWC 3W	GNB	Enterobacter Cloacae Complex
IWB W2	GPB	Bacillus Cereus
FB3M water	GPB	Bacillus Cereus
FBME water	GPB	Bacillus licheniformis
FB2M water	GPB	Bacillus Cereus
FB2E water	GPB	Bacillus Pumilus
FB1E water	GNB	Bacillus megaterium
FC2M water	GPB	Bacillus Cereus
FCMM water	GPB	Bacillus Cereus
FCME water	GNB	Rhizobium radiobacter
FC3E water	GPB	Bacillus licheniformis
FC1E water	GPB	Bacillus megaterium

Continuous isolation of Enterobacter cloacae and Klebsiella oxytoca from the farms is a serious indication of extensive contamination due to either water or handling activities. Such bacteria can be related to waterborne and environmental contamination, and these are often present in natural or less well-managed water systems (Bandyopadhyay et al., 2018). The presence of Pseudomonas aeruginosa in the soil samples from Farm 3 further points toward environmental risks, since this bacterium thrives in moist soil and is resistant to many commonly used disinfectants. The presence of these pathogens reflects water contamination that could arise either from the use of untreated water or poor hygiene practices on the farm. These findings put a focus on the need for safe water practices and treatment as a way of reducing the chance of pathogen transfer to crops (Bandyopadhyay et al., 2018).

Generally, the observation of Enterobacter cloacae in every sample of onion points to great and ubiquitous contamination across the farm environment, notably in irrigation water and probably stretching to the soil. Enterobacter cloacae is a gram-negative bacterium that is ubiquitously found in water, soil, plants, and even the gastrointestinal tracts of humans; therefore, it has the ability to live in very different environments. According to Sanders et al (2018),

The main source of Enterobacter could be traced from water. Indeed, the irrigation water itself is likely to be the vector for cloacae contamination in onions, facilitating the introduction of environmental and waterborne pathogens into crops. In assition, previous works have pointed out that water with fecal material, organic matter, or even untreated wastewater may introduce Enterobacter cloacae and other species of Enterobacteriaceae into crops (Skeie et al., 2021).

It may also be present in soil, which serves as a reservoir of E. cloacae under conditions favorable for its survival, either from decaying plant material or organic fertilizers. The contamination of soil to onion may occur at the time of growth, when the bacteria may be attached to the roots or bulb surface. Thus, E. cloacae may be a contaminant of soil that can persist with repeated use of the same water for irrigation (Li et al., 2019).

Poor postharvest handling, packaging, and storage practices may also be plausible means for contamination. In the case of hygiene conditions not being followed, it may also lead to contamination of by E. cloacae. Equipment, surface, and/or hand contaminations during these steps may cause cross-contamination. However, the homogeneity in the presence of E. cloacae throughout the samples suggests preharvest sources, such as irrigation water and soil,being major contributors.

4.5 CHARACTERIZATION OF *E. COLI* BY m-PCR

Table 4.6 shows the detection of different Escherichia coli pathotypes in samples obtained from onions and irrigation water in Farm 1, Farm 2, and Farm 3, respectively.

The findings of this study shows that 4 strains of *E. coli* were detected. In Farm 1, only COM EC was detected; in Farm 2, water and onions had EPEC, ETEC, EAEC, EIEC pathotypes. While Farm 3 had EPEC, EAEC, and ETEC pathotypes. The prevalent pathotypes were found to be in Farm 2 and Farm 3. The detected pathotypes occurred on substantial farms only with a consistent presence of enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC).

Farm 2, which exhibited greater diversity of E. coli strains, may be exposed to different contamination sources or environmental stressors (Xu et al., 2019). Pathotypes such as EPEC and EAEC are associated with diarrheal diseases, emphasizing the health risks posed by irrigation water contaminated with pathogenic E. coli (Bandyopadhyay et al., 2018).

A study by Aijuka et al (2018) reported that EAEC was the predominant pathotype isolated from various sources including irrigation water in South Africa. Furthermore, a study conducted in the Vhembe district, demonstrated the identification of EPEC and EAEC in river water used for irrigation (Traoré et al., 2016). In particular, EAEC has been reported as a pathotype associated with the contamination of food and irrigation water in developing countries such as Vietnam (Prayoga et al., 2021), Mexico (Castro-Rosas et al., 2012) and South Africa (Chidozie et al., 2021).

Table 4.6: Identification of *E. Coli* pathotypes

Sample ID Farm 1	***E. Coli* Pathotypes**
Onions site A	E. COM
Onions site B	No *E. Coli*
Irrigation water	No *E. Coli*
Sample ID Farm 2	***E. Coli* Pathotypes**
Onions 1	EPEC/ETEC
Onions 2	EPEC/ETEC/EAEC
Irrigation water	EPEC/ETEC/EIEC/EAEC
Sample ID Farm 3	**E. Coli Pathotypes**
Onions 1	EPEC
Onions 2	EPEC/ETEC/EAEC
Irrigation water	EPEC/ETEC

The presence of multiple E. coli pathotypes suggests diverse and sustained contamination, likely from wastewater discharge or poor sanitation practices in areas surrounding these farms. These results highlight the importance of controlling contamination sources in irrigation water to prevent outbreaks of foodborne diseases due to contaminated vegetables and fruits carrying pathogens as reported by Xu et al (2019).

4.6 ANTIMICROBIAL TESTING ON ISOLATES USING VITEK-2

Antimicrobial testing was done to determine the effectiveness of antibiotics against different enterobacter organisms, which will help in selecting the most effective treatment for infections and in monitoring the development of antimicrobial resistance.This was done using the Automated Systems which provide rapid and precise antimicrobial susceptibility results (Table 4.7).

Various antimicrobial agents were tested, with many of the commonly used ones showing resistance, indicating that these drugs are no longer effective in killing or inhibiting the growth of *Enterobacter* species.

The Antimicrobial Susceptibility Testing was done on Enterobacter cloacae, Klebsiella oxytoca, and Pseudomonas aeruginosa, all showing multidrug resistance. Such bacteria showed resistance against a wide array of antimicrobials, including ampicillin and colistin, commonly applied in livestock and agriculture (Nielsen et al., 2016).

Resistance may be due to continuous exposure to antimicrobial agents through agricultural runoff or animal farms nearby. The existence of antibiotic-resistant bacteria in agricultural settings poses serious risks to human health as the bacteria can transfer resistance genes to other pathogens. The resistance shown by these Gram-negative bacterial strains is a very serious cause for concern, as this points to untreatable infections in the case of entry via the food chain. Antimicrobial resistance in agriculture requires stringent monitoring and responsible use of antibiotics to prevent the spread of resistant strains (Xu et al., 2019). It is reported that E. cloacae often carries antimicrobial resistance genes, which may have been acquired from agricultural or medical environments where antibiotics are frequently used (Kim et al., 2020). Resistance to many classes of antibiotics is a concern for public health because of its potential to increase and spread its resistant genes horizontally in an agricultural environment like soil and water via gene transfer (Nielsen et al., 2016)

Table 4.7 depicts the resistance and susceptibility patterns of the isolated pathogens to various antibiotics.

TABLE 4.7: Antibiotic susceptibility results of isolates (VITEK 2). *resistance (R), intermediate (I), sensitive (S) for each antibiotic tested per species.*

Antimicrobial Agents	Pseudomonas aeruginosa	Klebsiella oxytoca	Enterobacter cloacae Complex	Raoultella ornithinolytica	Citrobacter amalonaticus	Klebsiella pneumonia
Ampicillin	-	R>=32	-	R >=32	-	R >=32
Imipenem	-	I2	I2	I 2	I 2	I 2
Amoxicillin/Clavulanic Acid	-	I16	R >=32	R>=32	R >=32	R16
Piperacillin/Tazobactam	R>=128	S<=4	R>=128	S 8	R64	R <=4
Cefuroxime	-	I16	R >=64	R >=64	R >=64	R >=64
Cefuroxime Axetil	-	I16	R >=64	R >=64	R >=64	R >=64
Cefoxitin	-	S<=4	R >=64	R >=64	S <=4	S <=4
Cefotaxime	R>=64	-	R8	I 2	R >=64	R <=1
Ceftazidime	R>=64	S2	I16	I 8	R16	R <=1
Cefepime	R>=64	S <=1	I 4	I 4	I 8	R <=1

Ertapenem	-	S <=0.5	I1	I 1	I 1	I 1
Meropenem	I4	S1	R<1	S 1	R >4	S 1
Amikacin	S4	S 8	I8	S 4	I 32	S 8
Gentamicin	S<=1	S2	S2	S <=1	S 4	S <=1
Tobramycin	S<=1	S2	R<=2	S <=1	S 4	S <=1
Ciprofloxacin	S<=0.25	S <=0.25	S<=0.25	S<=0.25	S <=0.25	S <=0.25
Tigecycline	R>=8	S <=0.5	S<=0.5	S<=0.5	S <=0.5	S <=0.5
Colistin	R>=8	R>=64	R>=64	R>=64	R>=64	R>=64
Trimethoprim/Sulfamethoxazole	-	S<=20	S<=20	S <=20	S<=20	S <=20

4.7 CHARACTERIZATION OF ENTEROBACTER SPECIES BY SEQUENCING

All Isolates were sequenced by Sanger and found to be *Enterobacter ludwigii* as shown in Table 4.8 and the gel of amplicons is shown in Figure 4.8.

Table 4.8: Sequencing results of *Enterobacter* isolates. *Shows sample ID, closest match species by 16S (with GenBank accession), and percent identity.*

Name of sample	Predicted Organism
N/A B2 003 24/6/24	Enterobacter ludwigii
NA As 004 24/6	Enterobacter ludwigii
005 NA 24/6	Enterobacter ludwigii
017 NA 24/6	Enterobacter ludwigii
020 NA 24/6	Enterobacter ludwigii
022 24/6 NA	Enterobacter ludwigii
023 NA 24/6	Enterobacter ludwigii
033 NA 24/6	Enterobacter ludwigii
035 NA 24/6	Enterobacter ludwigii
036 NA 24/6	Enterobacter ludwigii

The fact that all the isolates were sequenced by Sanger sequencing and identified as Enterobacter ludwigii suggests a high degree of microbial consistency across samples. Such evidence indicates that this bacterium, belonging to the Enterobacter cloacae complex, is indeed the dominant bacterial contaminant in these agricultural samples. Sanger sequencing has become one of the most conventionally applied methods for microbial identification due to its accuracy in defining certain DNA sequences. (Shendure et al., 2017). Focusing on evolutionarily conserved gene regions, such as 16S rRNA, Sanger sequencing precisely identified bacterial species, which may support the identification of all the isolates specifically to E. ludwigii.

E. ludwigii is indeed a versatile and adaptable bacterium in the Enterobacter cloacae complex, often found in different environments, including soil, water, and plants (Davin-Regli et al., 2019). It is highly resistant to environmental stressors such as pH, temperature, and nutrient availability, making it especially well-suited to agricultural environments where these factors are frequently changing. This could be one reason it is so common in irrigation water and soil.

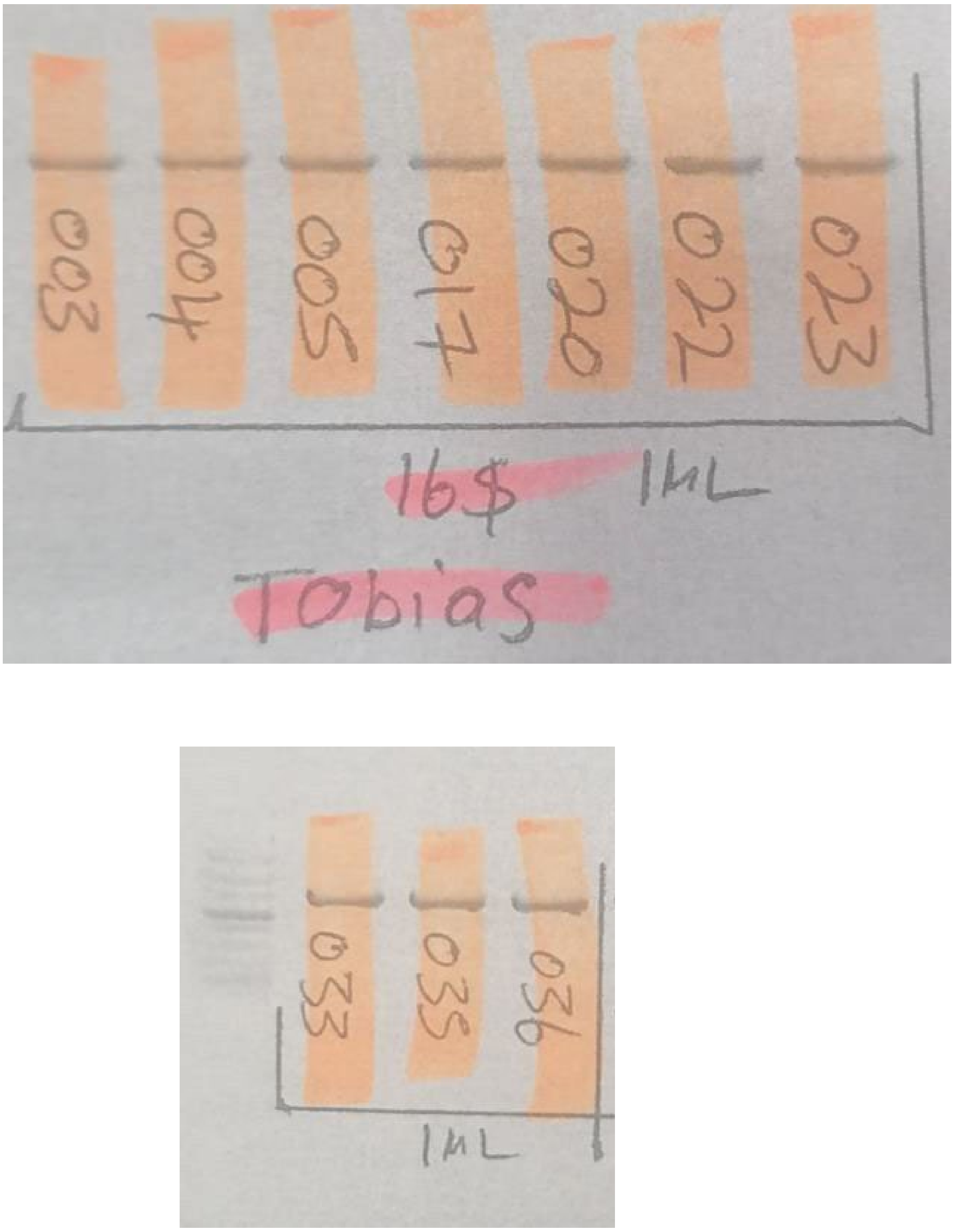


Figure 4.5: Agarose gel electrophoresis of 16S rRNA PCR products. An image of a 1.5% agarose gel stained with ethidium bromide. M: 100 bp DNA ladder; lanes 1–5: Enterobacter isolates (1.5 kb band indicated); P: positive control (known Enterobacter 16S amplicon); N: negative control (no template).

4.8 GENETIC TREE DEMONSTRATING THE RELATEDNESS OF ISOLATES

The genetic family tree of Enterobacter ludwigii provides insight into its phylogenetic positioning within the genus Enterobacter, belonging to the Enterobacteriaceae family. This family comprises a large number of medically important bacteria, such as Escherichia coli, Salmonella, and Shigella, many of which are opportunistic pathogens. Understanding the genetic relationships within this genus supports the identification of the evolutionary history and pathogenic potential of E. ludwigii (Figure 4.6)..

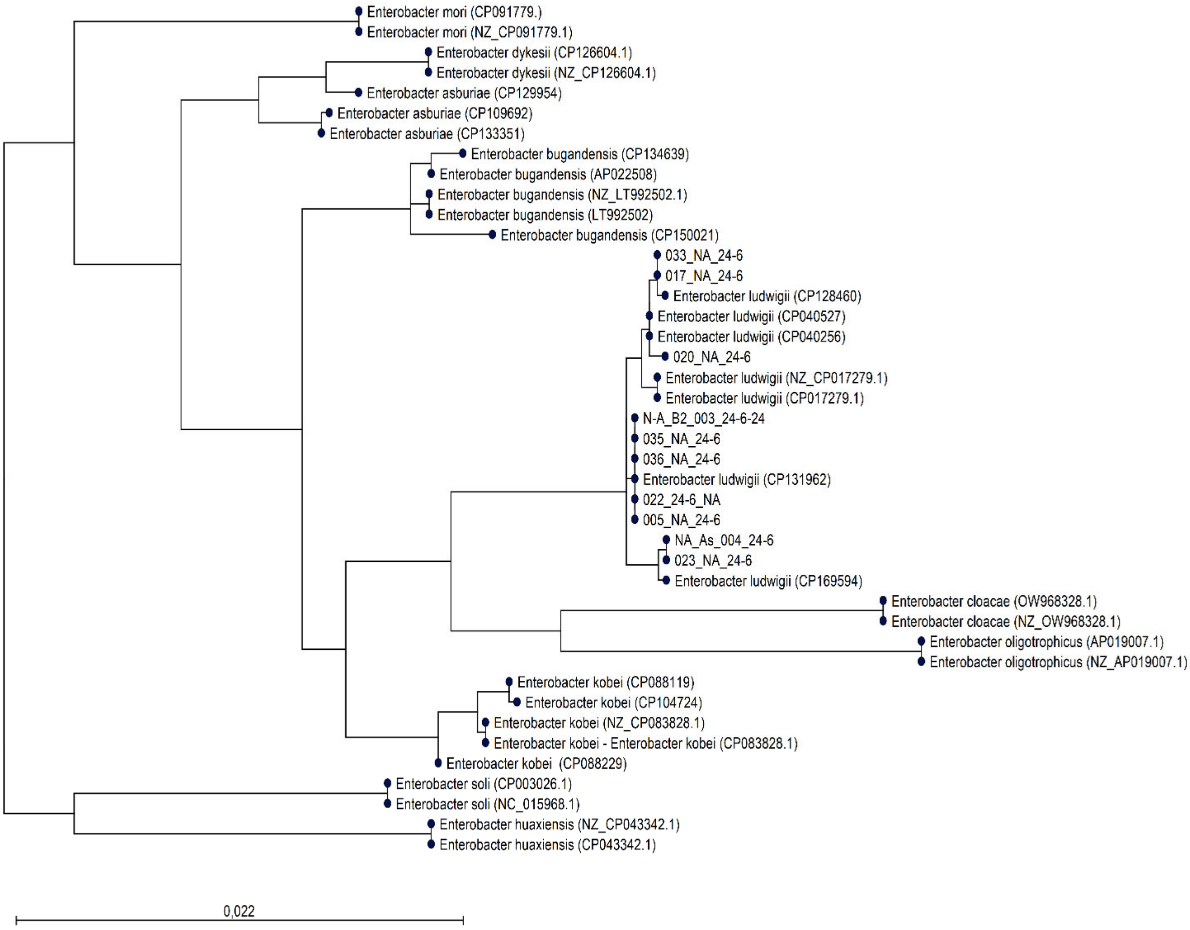


Figure 4.6: Neighbor-joining phylogenetic tree of Enterobacter isolates based on partial 16S rRNA sequences. The tree (constructed with Kimura 2-parameter model) shows relationships among the study isolates and reference sequences (GenBank accession numbers prefixed NZ or CP). Bootstrap values (>50%) are indicated at branch nodes.

Enterobacter ludwigii is a Gram-negative bacterium belonging to the family Enterobacteriaceae. Other members of the genus Enterobacter include Enterobacter cloacae, Enterobacter aerogenes, and Enterobacter sakazakii. These species have exhibited some biochemical and genetic similarities; however, their virulence and antimicrobial resistance vary (Bergey et al., 2012).

Phylogenetic analysis has revealed that E. ludwigii is genetically closest to E. cloacae and E. aerogenes, as all three species belong to the same clade within the genus Enterobacter (Rosenblueth et al., 2004). This relationship underscores their shared ancestry, though each species has distinct genetic markers and metabolic pathways that differentiate them (Bergey et al., 2012).

Some distinctive genomic features make Enterobacter ludwigii very different from its sister complexes. Having an inheritance core with the gene in common, such as with E. cloacae and E. aerogenes, this genetic basis presents different aspects concerning the metabolic ability of the bacteria and the capability for being pathogenic. E. ludwigii displays singularities on its virulence factors due to its enzymes production and potential ability to colonize various ecotopes such as that of the human host (Bergey et al., 2012).

This differentiation is also reflected in the biochemical characteristics of E. ludwigii from its close relatives, such as its specific reactions in various biochemical tests like catalase, citrate, and urease tests (Bergey et al., 2012). This genetic divergence has implications for the adaptability of the bacterium to different environmental and host conditions.

Horizontal gene transfer (HGT) is one of the major contributors to the genetic evolution of Enterobacter species, including E. ludwigii. HGT enables the bacterium to acquire genetic material from other organisms, which might contribute to acquiring new traits, such as antibiotic resistance and pathogenicity (Ochman et al., 2000). This process is important in the evolution of E. ludwigii, especially in acquiring resistance to beta-lactam antibiotics and other antimicrobial agents (D'Andrea et al., 2013).

The transfer of virulence factors via plasmids, transposons, and other mobile genetic elements has been documented within the Enterobacteriaceae family, leading to increased virulence in some strains of E. ludwigii (Carattoli, 2009). Such genetic plasticity allows E. ludwigii to adapt rapidly to changing environmental pressures and evade host immune responses.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The result of this study has shown high levels of microbial and physical contamination in irrigation water, soil, and onion samples, with the persistent occurrence of Enterobacter ludwigii and other pathogens, hence raising critical concerns about food safety, public health, and agricultural sustainability.

Since pH and TDS deviate from the set DWAF standards, there are chemical imbalances within irrigation waters, which could affect crop health negatively and at the same time provide avenues for microbial proliferation. This therefore, calls for a more careful water quality management practice to ensure the maintenance of safe and productive agricultural environments.

High counts of coliforms and Escherichia coli from all irrigation sources suggest a high level of microbial contamination from nearby human, animal, or agricultural activities. The high prevalence of E. coli, along with multiple diarrheagenic E. coli pathotypes, presents serious foodborne disease risks if contaminated produce enters the food supply. These results emphasize that regular monitoring and irrigation water treatment are fundamental to reducing levels of contamination.

That the same Enterobacter ludwigii was present in all samples of onions, as obtained by Sanger sequencing, infers that it is a common contaminant within this agricultural setup. E. ludwigii is an opportunistic pathogen with serious health risks among immunocompromised individuals; it may even facilitate the spread of antibiotic-resistant genes, particularly in settings that have minimal regulation of microbial life. The presence of E. ludwigii indicates lapses in proper sanitation and a treated water source in this setting of agricultural production.

The presence of antibiotic-resistant pathogens, such as multidrug-resistant Enterobacter ludwigii, is a serious public health concern. Potential implications for the spread of antimicrobial resistance genes in the agricultural environment are important reasons for prudent use of antibiotics in local livestock and enhanced management of water quality to prevent the introduction of resistant bacteria to crops.

5.2 RECOMMENDATIONS

In order to prevent these situations, a mix of water treatment, periodic microbial testing, soil management, and strict post-harvest hygiene is required. Buffer zones between livestock and areas of produce, treatment of water before irrigation, besides training on sanitation for agricultural workers, will reduce contamination and improve results in food safety. It emphasizes the importance of valid water management and hygiene practices in agriculture, produces safety, and public health protection. Continued research and investment in water treatment and stricter regulations around antibiotic use in agriculture are necessary to minimize contamination and support sustainable food production practices in contaminated environments.

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