# FIT-FOR-PURPOSE GUIDELINES FOR MANAGING MICROBIOLOGICAL WATER QUALITY RISKS IN SMALLHOLDER FARMS AND INFORMAL FRESH PRODUCE SUPPLY CHAINS

**Report**
to the Water Research Commission

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## EXECUTIVE SUMMARY

Smallholder agriculture plays a crucial role in South Africa's rural economy, contributing to poverty alleviation, employment creation, and inequality reduction. The smallholder farms are typically family-operated farms with the family managing less than 10 hectares and frequently integrating both crop and livestock production. This sector complements the commercial agricultural industry and contributes meaningfully to national food and nutrition security. Irrigation underpins the productivity of these systems, accounting for approximately 53–61% of South Africa's total water use and supporting 25–30% of agricultural output. However, irrigation water has been highlighted as a dominant pathway for microbial and antimicrobial resistance contamination of fresh produce, particularly within informal supply chains where regulatory oversight is limited.

Building on previous WRC-funded projects (K5/1773/4; K5/1875/4; K5/2706/1/21), this project addressed a crucial gap: the absence of fit-for-purpose guidelines for managing risk-based microbiological and decision-support tools tailored to the realities of smallholder systems. Across three case studies spanning diverse agro-ecological contexts, more than 265 samples from irrigation water, soil, produce, and associated environments were analysed using physicochemical testing, quantification of indicator organisms, pathogen detection, antimicrobial susceptibility testing, genetic fingerprinting, and whole-genome sequencing (WGS). Complementary data from integrated systems were incorporated through matched external funding, enabling a One Health interpretation of contamination pathways.

## RESEARCH FINDINGS

A comprehensive literature review examined microbial contamination indicators and foodborne pathogens in South African smallholder fresh-produce supply chains, focusing on faecal indicator bacteria, multidrug-resistant (MDR) Enterobacterales, global irrigation water guidelines, and mitigation strategies. The review highlighted contamination sources in the water-soil-plant nexus and the implications for food safety.

Faecal indicator bacteria (e.g., generic *E. coli*, faecal coliforms, intestinal enterococci) are commonly used to assess contamination levels; however, they do not consistently predict the presence of specific pathogens such as *Salmonella* spp. or pathogenic *E. coli* due to differences in survival rates. Therefore, direct pathogen testing is recommended in high-risk situations. The results of the assessment of microbiological quality, contamination points, prevalence, and characteristics of human pathogenic bacteria in fresh-produce production systems from smallholder farmers were presented through three case studies. The solicited project specifically excluded sampling and analysis of animal feed and faeces in one of the case studies. However, the cost of the analysis of animal feed and faeces from the vegetable-pig production scenarios was covered by funding from the South African Pork Producers' Organisation (SAPPO) under the project titled: *Evaluation of the microbiome, isolation, and characterisation of zoonotic and multidrug-resistant bacteria of interest to the pork industry in*

*fresh produce and waste food consumed by humans and animals*. Selected results are included in this integrated report, since consideration of the broader food-producing environment is crucial for the identification of contamination points in integrated fresh-produce supply chains.

The findings demonstrate persistent faecal contamination of surface waters, frequent exceedance of international guideline values for *E. coli* and faecal indicators in irrigation water, and widespread MDR among environmental *E. coli* and Enterobacterales. Although classical foodborne pathogens were not as prevalent, WGS revealed clinically significant resistance (e.g., $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *tet*(A), *sul2*) and virulence determinants associated with extraintestinal pathogenic *E. coli* (ExPEC), with highly similar genomic resistome profiles for *E. coli* detected in irrigation water and harvested crops, confirming direct water-to-produce transmission. Integrated vegetable-pig systems evidently amplified contamination and the dissemination of antimicrobial resistance across the water-soil-produce continuum, while river systems receiving wastewater inputs showed severe microbiological deterioration, reinforcing irrigation water as the dominant exposure pathway.

## AIMS

All project aims were fully achieved through comprehensive reviews, collaborative site selection, rigorous microbiological and genomic analyses, pathogen characterisation, cross-sectoral data linkages, and development of a practical guide.

**Aim 1: Determine guidelines for smallholders.** This objective was fully accomplished through the synthesis of new empirical data obtained from three large-scale case studies across South Africa, complemented by a comprehensive review of international guidelines and additional behavioural insights derived from the FS4A study involving 106 farmers in Gauteng. The resulting guidelines outline a structured, risk-based framework that should categorise water sources by complexity, incorporate crop-specific risk assessments, and use empirically established microbial thresholds, including *Escherichia coli* levels and antimicrobial resistance parameters, relevant to smallholder agricultural systems. The guidelines propose the inclusion of decision trees and multi-barrier intervention strategies, with a focus on cost-effective microbial testing methods to ensure compliance with international good agricultural practice and food safety standards. Additionally, the framework acknowledges local challenges, including limited laboratory capacity, outdated local standards, economic constraints, and diverse farming practices.

**Aim 2: Hold a consultative workshop.** A consultative workshop was held with key experts from the University of Pretoria and the University of KwaZulu-Natal. This session resulted in the strategic selection of nine representative smallholder sites across Gauteng, KwaZulu-Natal, Limpopo, and the North West, covering diverse water sources (rivers, dams, boreholes, municipal), irrigation methods, and production systems (vegetable-only, vegetable-pig, aquaculture-integrated). Standardised sampling protocols for water, soil, produce, animal

faeces, and feed were agreed upon, together with physicochemical and microbiological methods that ensured comparability across sites and seasons.

**Aim 3: Identify microbial hazards.** This aim was achieved through a comprehensive literature review of over 120 peer-reviewed publications that summarised microbial indicators and risks in South African irrigation water, produce, and supply chains across formal and informal sectors. It identified *E. coli*, faecal coliforms, MDR Enterobacterales, pathogenic *E. coli*, and *Salmonella* as key microbiological hazards in the water-soil-plant nexus. The review traced contamination sources to polluted water, manure-amended soils, and vulnerable produce, especially leafy green vegetables. The findings of the review were further supported by the complementary study on farmers' knowledge, attitudes, and practices, which found that smallholder farming practices often involve untreated water, extensive manure use, and poor hygiene during harvesting and handling. It highlighted low awareness of wastewater hazards and limited regulations in informal markets, emphasising the socio-economic factors influencing contamination. This foundation supports targeted guideline development.

**Aims 4–6: Conduct case studies.** Across the three case studies, more than 265 irrigation water, soil, and fresh-produce samples were analysed, complemented by 37 river-water monitoring events. Results revealed persistently elevated faecal coliform and *E. coli* levels in river and dam sources, with produce from smallholder farms routinely exceeding the proposed South African and WHO microbiological thresholds for crops consumed raw. Contamination was directly traced to upstream sewage discharges in the river, livestock runoff, and on-farm manure application. Although classical foodborne pathogens were not frequently isolated (only a small number of *Salmonella* spp. were isolated from water, and no Shiga toxin-producing *E. coli* was detected on produce), diarrhoeagenic *E. coli* pathotypes were present in animal-derived samples. Moreover, most *E. coli* isolates from both water and produce exhibited MDR, predominantly to ampicillin, tetracycline, and, crucially, third-generation cephalosporins. Whole-genome sequencing of over 80 high-quality *E. coli* genomes uncovered widespread resistance determinants ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *tet(A)*, *sul2*) and virulence genes (*pap*, *sfa*, *iutA*) associated with extraintestinal pathogenic *E. coli* (ExPEC). The near-identical genomic resistome profiles observed between isolates from the same farm's irrigation water and harvested crops provide compelling evidence of direct water-to-produce transmission of MDR, potentially pathogenic strains. Through collaboration with the National Institute for Communicable Diseases (NICD) and the US-FDA GenomeTrakr network, these South African smallholder isolates were assigned to globally significant sequence types (ST10, ST69, ST131) and integrated into the National Center for Biotechnology Information (NCBI) databases for international comparability, firmly establishing the One Health implications of contaminated irrigation water in informal fresh-produce systems.

**Aim 7: Produce a practical guide.** This aim was fully achieved by providing evidence-based guidance to inform a stand-alone, farmer-friendly guide (approved by the WRC) that translates the scientific results into practical tools: risk-based water categories, crop-specific microbial limits, decision trees for water-source selection, low-cost testing and treatment options, multi-

barrier checklists, manure-management protocols, and gender-sensitive training modules. Future initiatives should leverage the insights from this comprehensive report to develop a user-friendly guide tailored for direct application by extension officers, AgriParks, and farmer cooperatives.

**Aim 8: Knowledge dissemination.** Knowledge was disseminated through a multi-layered strategy targeting science, policy, and practice. Three peer-reviewed original articles have been prepared and published during 2024 and 2025 (*Journal of Applied Microbiology, Journal of Food Protection, Microbiology Resource Announcements*), with genomic data integrated into the US-FDA GenomeTrakr and NICD databases. A fourth paper is being prepared as part of a doctoral student's output. Four posters were presented at national and international conferences, including the 8th World One Health Congress held in Cape Town in 2024. Stakeholder engagement included a One Health workshop (2022–2023), World Food Safety Day dialogues, and a 2025 radio interview. Direct farmer outreach included two training sessions that reached ~60 smallholder producers, as well as a dedicated farmer study-group dialogue (June 2025).

## CONCLUSIONS AND PRIORITY RECOMMENDATIONS

This study confirms that irrigation water is the primary driver of microbial and antimicrobial-resistant contamination in South Africa's smallholder fresh-produce systems. Risks are heightened by declining surface water quality due to wastewater contamination, integrated crop-livestock production practices, and ongoing gaps between food safety knowledge and its practical application on farms. The findings indicate that strict, one-size-fits-all water quality standards are not suitable for resource-constrained smallholder settings. Instead, adopting flexible, risk-based, and multi-barrier management strategies is essential to achieve meaningful improvements in food safety while ensuring operational practicality.

To translate these findings into action, the following priority recommendations are proposed:

### Implement fit-for-purpose, tiered irrigation water quality classifications

Establish simplified, risk-based tiers linked to water source, crop susceptibility (especially leafy greens consumed raw), and irrigation method. These should be operationalised through decision-tree tools and affordable, field-appropriate testing options to enable on-farm risk assessment without reliance on advanced laboratory infrastructure.

### Adopt integrated, multi-barrier risk management across the water-soil-produce continuum

Combine water protection and basic treatment options with safer irrigation practices (e.g., drip irrigation where feasible), crop restrictions in high-risk settings, improved animal management, and post-harvest interventions to achieve cumulative pathogen reductions along the farm-to-market pathway.

**Strengthen gender-responsive capacity building to close knowledge-practice gaps**

Deliver targeted training and extension services that address contamination pathways, manure and water management, and antimicrobial resistance risks, with specific support for women farmers who face disproportionate structural barriers to implementing food safety practices.

**Institutionalise national, evidence-based guidelines linked to market and policy frameworks**

Embed fit-for-purpose guidelines for irrigation water quality within national food safety policies, certification schemes, and AgriPark support frameworks, using local surveillance data to ensure relevance, feasibility, and long-term regulatory coherence.

**Prioritise continuous surveillance of microbial and antimicrobial resistance hazards**

Strengthen routine monitoring of hygiene indicators alongside targeted surveillance of emerging antimicrobial resistance to enable adaptive, site-specific interventions and support One Health-aligned risk management across agricultural environments.

**Disseminate guidelines and training materials in locally relevant languages and accessible formats**

Ensure that fit-for-purpose guidelines for managing microbiological water quality risks are translated into local South African languages and distributed using plain-language booklets, pictorial guides, and mobile-friendly formats to improve comprehension, inclusivity, and practical uptake among smallholder farmers with diverse literacy levels.

Together, these measures establish a scientifically sound and contextually relevant approach to minimising contamination risks, reducing environmental transmission of antimicrobial resistance, protecting consumer health, and strengthening the resilience and sustainability of South Africa’s smallholder fresh-produce systems within a One Health framework.

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## ACRONYMS and ABBREVIATIONS

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|---|---|
| **AIEC** | adherent-invasive *Escherichia coli* |
| **AMR** | antimicrobial resistance |
| **BOD** | biological oxygen demand |
| **CFU** | colony-forming units |
| **COD** | chemical oxygen demand |
| **DAEC** | Diffusely adherent *Escherichia coli* |
| **DSS** | Decision-support system |
| **EAEC** | enteroaggregative *Escherichia coli* |
| **EHEC** | enterohaemorrhagic *Escherichia coli* |
| **EIEC** | enteroinvasive *Escherichia coli* |
| **EPEC** | enteropathogenic *Escherichia coli* |
| **ETEC** | enterotoxigenic *Escherichia coli* |
| **ERIC-PCR** | enterobacterial repetitive intergenic consensus polymerase chain reaction |
| **ESBL** | extended-spectrum β-lactamase |
| ***E. coli*** | *Escherichia coli* |
| **ExPEC** | extraintestinal pathogenic *Escherichia coli* |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **FSMA** | FDA Food Safety Modernization Act |
| **GAPs** | good agricultural practices |
| **IFAD** | International Fund for Agricultural Development |
| **LMICs** | low- and middle-income countries |
| **MALDI-ToF MS** | Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Mass Spectrometry |
| **MDR** | multidrug resistant/resistance |
| **NICD** | National Institute for Communicable Diseases (South Africa) |
| **NMEC** | neonatal meningitis *Escherichia coli* |
| **StatsSA** | Statistics South Africa |
| **STEC** | Shiga toxin-producing *Escherichia coli* |
| **US-FDA** | United States Food and Drug Administration |
| **WGS** | whole genome sequencing |
| **WRC** | Water Research Commission |
| **WWTPs** | wastewater treatment plants |
| **qPCR** | quantitative polymerase chain reaction |

## GLOSSARY

- **AmpC β-lactamase**: A type of enzyme produced by certain bacteria that confers resistance to β-lactam antibiotics, including some cephalosporins, by hydrolysing the antibiotic molecules.
- **Aquaponics:** An integrated farming system that combines aquaculture (raising fish) with hydroponics (growing plants in water), where nutrient-rich water from fish tanks is used to fertilise plants.
- **BOD (biological oxygen demand):** A measure of the amount of oxygen consumed by microorganisms in decomposing organic matter in water, indicating pollution levels.
- **CFU (colony-forming units):** A unit used to estimate the number of viable bacteria or fungal cells in a sample, based on the number of colonies formed on a growth medium.
- **Coliforms:** A group of gram-negative, rod-shaped bacteria commonly found in the environment, used as indicators of sanitary quality in water and food, with faecal coliforms specifically suggesting contamination from animal or human waste.
- **Drip irrigation:** A method of watering plants where water is delivered slowly and directly to the root zone through a network of tubes or emitters, minimising evaporation and runoff.
- **Enterobacterales:** An order of gram-negative bacteria that includes many pathogens such as *Escherichia coli*, *Salmonella*, and *Klebsiella*, often associated with gastrointestinal infections.
- **ERIC-PCR (enterobacterial repetitive intergenic consensus polymerase chain reaction):** A molecular fingerprinting technique used to assess genetic relatedness among bacterial isolates by amplifying repetitive DNA sequences.
- **ESBL (extended-spectrum beta-lactamase):** Enzymes produced by bacteria that break down a broad range of β-lactam antibiotics, including penicillins and cephalosporins, leading to multidrug resistance (MDR).
- ***Escherichia coli* (*E. coli*):** A bacterium commonly found in the intestines of humans and animals, used as an indicator of faecal contamination; certain strains (pathovars) can cause foodborne illness.
- **Eutrophication:** The process by which a body of water becomes enriched with nutrients, leading to excessive algal growth, oxygen depletion, and degraded water quality.
- **Faecal coliforms:** A subset of coliform bacteria typically originating from the faeces of warm-blooded animals, serving as indicators of potential pathogenic contamination in water sources.
- **Faecal indicator bacteria:** Microorganisms like *E. coli* or faecal coliforms whose presence in water suggests contamination by faecal matter and potential health risks from pathogens.
- **Fit-for-purpose:** A concept referring to water quality standards tailored to specific uses, such as irrigation, ensuring safety without requiring potable-level treatment.
- **MDH (malate dehydrogenase):** An enzyme involved in bacterial metabolism; in *E. coli*, the *mdh* gene is used for molecular identification and characterisation.

- **MDR (multidrug-resistant):** Describes bacteria resistant to at least three classes of antimicrobial agents, complicating treatment of infections.
- **Microbiological quality:** The assessment of water or food based on the presence, absence, or levels of microorganisms, including bacteria, viruses, and parasites, to determine safety for use or consumption.
- **MPN (most probable number):** A statistical method used to estimate the concentration of viable microorganisms in a sample, particularly when direct counting is impractical.
- **Multidrug-resistant human pathogenic Enterobacterales:** Members of the Enterobacterales order that cause human infections and exhibit resistance to multiple antibiotics, posing significant public health challenges.
- **Overhead irrigation:** A watering method where water is sprayed over the top of crops using sprinklers, which can potentially increase the risk of microbial contamination on edible plant parts.
- **Pathovars:** Variants of a bacterial species that are distinguished by their ability to cause disease in specific hosts or tissues, such as diarrhoeagenic *E. coli* pathovars.
- **Pseudomonas aeruginosa:** A qram-negative bacterium commonly found in soil and water, capable of causing infections in humans, especially in immunocompromised individuals, and often resistant to multiple antibiotics.
- ***Salmonella* spp.:** A genus of rod-shaped bacteria that can cause foodborne illnesses like salmonellosis, commonly transmitted through contaminated water, food, or animal products.
- **Shiga toxin-producing *E. coli* (STEC):** A pathogenic subgroup of *E. coli* that produces Shiga toxins (encoded by genes like *stx1*), leading to severe illnesses such as haemolytic uremic syndrome.
- **Smallholder farmers:** Family-operated agricultural enterprises typically on less than 10 hectares of land, practising mixed farming (crops and livestock) with limited mechanisation and focus on local markets.
- **Water-soil-plant nexus:** The interconnected system where contaminants in irrigation water can transfer to soil and subsequently to plants, affecting food safety along the agricultural chain.
- **Whole genome sequencing (WGS):** A laboratory process that determines the complete DNA sequence of an organism's genome, enabling detailed analysis of genes, including those for virulence and antibiotic resistance.

# CHAPTER 1: BACKGROUND

## 1.1 Introduction

It is widely recognised worldwide that smallholder agriculture plays a crucial role in alleviating poverty, reducing inequality, and promoting employment opportunities. In South Africa, smallholder farming systems are characterised by a variety of agricultural practices, diverse farm sizes, and varying levels of market engagement. These operations are typically family-operated, often with female household members overseeing daily farming activities (Kamara et al., 2019; Thabane et al., 2025). Smallholder farmers generally practice mixed farming methods on land sizes of less than 10 hectares (Viviers et al., 2024; Thabane et al., 2025). This diversity amongst farming operations and practices complicates the process of formalising an overarching definition of smallholder farmers in South Africa (Carelsen, Ncube and Fanadzo, 2021). Unlike commercial agricultural enterprises, smallholder farmers emphasise diversified production, including crop and livestock, to mitigate risk and boost income, with limited mechanisation and a focus on local markets. However, it is well known that the dual structure of the South African agricultural sector, consisting of a well-established commercial farming industry and a smaller-scale smallholder farming sector (Carelsen, Ncube and Fanadzo, 2021), significantly contributes towards food security. The quality and safety of the available water sources used for irrigation are thus essential for all agricultural activities across both sectors.

Irrigated agriculture in South Africa accounts for an estimated 53–61% of all available water sources, including both surface and groundwater resources (Bonthuys, 2018). Moreover, irrigation accounts for approximately 25–30% of total agricultural production in South Africa (Bonthuys, 2018). The horticultural industry's demand for clean water far exceeds the available supply of potable sources. Typically, smallholder farmers have limited resources and limited access to formal irrigation systems and thus rely on readily available water sources for crop production (Van Koppen et al., 2017; Nephawe et al., 2021). Therefore, river water is frequently used to irrigate fresh produce; however, due to the high treatment costs, it is often used without prior purification. Elevated pollution levels in most South African rivers increase the potential for crop contamination, posing a concern for food safety. Furthermore, as smallholder farmers often use overhead irrigation, microbial pathogens in the water can contaminate plant surfaces (EC, 2017), a worst-case scenario that could lead to foodborne illnesses.

It has been extensively documented that irrigation water can serve as a source of pathogens on fresh produce, potentially affecting food safety and public health (Uyttendaele et al., 2015; Banach and Van Der Fels-Klerx, 2020; Richter et al., 2022). Consequently, maintaining the microbiological quality of water used during primary production is crucial. Although several preventive and mitigation strategies exist to assist growers in managing irrigation water quality, there is an ongoing need for clear, practical guidance on the most effective measures to

minimise microbial contamination. Generally, irrigation water microbiological standards require evaluation using quantitative methods, with an acceptable threshold of fewer than 1 000 *Escherichia coli* per 100mL (DWAF, 1996; WHO, 2006; Rock et al., 2018).

The 1996 Department of Water Affairs (DWAF) guidelines, which are part of the comprehensive South African Water Quality Guidelines, established specific thresholds and concentration-based limits for various water quality parameters such as faecal coliforms and *E. coli* to ensure the safety and quality of water designated for crop irrigation, particularly for crops for raw consumption. Subsequently, updated guidelines have been published that present a more advanced, risk-based, and site-specific decision-support system (DSS), marking a significant shift in how microbiological water quality is assessed (du Plessis et al., 2017; du Plessis, Annandale, and Benade, 2021). Concurrently, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) highlight that reliance solely on *E. coli* levels is inadequate for ensuring food safety (FAO and WHO, 2021), as *E. coli* concentrations do not fully reflect the range of potential pathogens. Additionally, research has indicated that the presence of non-pathogenic *E. coli* does not reliably indicate contamination with Shiga-toxin-producing *E. coli* (STEC) (Haymaker et al., 2019).

Incorporating other testing methodologies, such as physicochemical analyses, can also provide important insights into various parameters essential for comprehensive water quality assessment (Iwu, Korsten and Okoh, 2020; Alegbeleye and Sant'Ana, 2023). Moreover, the increasing use of antimicrobials in the healthcare system and intensive livestock farming has contributed to increased levels of antibiotic resistance among environmental microbial populations. This situation exerts selective pressures that facilitate the transfer of antibiotic resistance genes to potential human pathogenic bacteria (Manyi-Loh et al., 2018). Contaminated environmental resources, especially irrigation water, are known to play an important role in the increased prevalence and dissemination of potentially antibiotic-resistant human pathogenic bacteria (Larsson et al., 2018). Bacterial pathogens such as *Klebsiella pneumoniae* have been identified in the healthcare system and the agroecosystem, including surface and wastewater, irrigation water, soil, vegetable crops, and animal husbandry (Ebomah and Okoh, 2020; Richter et al., 2020a; Iwu et al., 2021). Determining microbial water quality for the irrigation of fresh produce involves selecting key parameters and criteria for the evaluation process.

Britz et al. (2012) reported on the microbiological quality of irrigation water sources, including rivers and canals, across multiple South African provinces, including the Western Cape, Mpumalanga, Limpopo, North West, Gauteng, and KwaZulu-Natal. The findings indicated widespread contamination of irrigation water with faecal matter in many regions. The study emphasised the need for further research to establish a direct connection between water quality and produce contamination, as well as to develop effective mitigation strategies. Building on this, du Plessis et al. (2015) reported on the potential link between irrigation water and the microbial contamination of fresh produce, from farm to market, including both pre- and post-harvest stages. Results demonstrated a clear link between water quality and microbial

presence in fruits and vegetables, identifying irrigation water as a significant pathway for pathogens such as *E. coli*, *Salmonella*, and *Listeria*. The study also identified ongoing contamination risks along the supply chain, compounded by inadequate hygiene practices and water management. Additionally, antibiotic-resistant bacteria emerged, with *E. coli* isolates from water and produce exhibiting resistance to multiple antibiotics.

Despite these insights, additional data were necessary to fully understand the microbiological safety of fresh produce, including the impact of processing, the prevalence of MDR pathogenic bacteria, and their characteristics across various produce types (whole, minimally processed, and ready-to-eat). To address this gap, a study was undertaken in the Tshwane and Cape Town metropolitan areas. The primary objective was to develop a crop-specific knowledge base to inform microbiological guidelines tailored for both commercially produced and small-scale farm produce, including at the point of sale (both formal and informal). du Plessis et al. (2021) concluded that microbial contamination is a widespread concern in South Africa's fresh vegetable supply chains, with water quality being a crucial factor. Both formal and informal sectors face challenges, though sources and contamination levels may differ. Leafy green vegetables were identified as particularly vulnerable, and the presence of antibiotic-resistant pathogens increased the associated health risks. A key deliverable from this project was a policy brief outlining water-quality criteria and parameters to enhance food safety across both formal and informal food systems. Further, the project identified knowledge gaps to inform the development of practical, fit-for-purpose guidelines for managing microbiological water quality risks suitable for smallholder farmers and informal food traders. The current project focused on creating these microbiological water quality guidelines that are achievable and relevant in these contexts, complementing existing risk-based, site-specific irrigation water quality guidelines, such as those detailed in the *Risk-Based, Site-Specific Irrigation Water Quality Guideline*, comprising 'Volume 1: Decision Support System (DSS) Description' and 'Volume 2: Technical Support Guideline' (du Plessis et al., 2017, 2021). Given the limitations of applying broad, hypothetical water quality standards across diverse scenarios, particularly in the informal sector, there is an urgent need for practical, fit-for-purpose guidelines for managing microbiological water quality standards that safeguard public health, protect the environment, and ensure the microbiological safety of fresh produce throughout the supply chain.

### 1.2 Project Aims

The following were the aims of the project:

1 To develop fit-for-purpose guidelines for managing microbiological water quality criteria for irrigation water used in informal (smallholder) fresh-produce farming systems, aligned with international norms and standards, but specific for local needs based on scientific evidence.
2 Workshop with experienced collaborators to establish sampling strategies and methodologies and to select appropriate sites in South Africa regarding sources of

environmental microbial contamination, including irrigation water, irrigated vegetable crops on informal farming systems (smallholder farmers).

3 To review the status of research on microbial indicators/index of contamination/risk in irrigation water, fresh produce (whole and minimally processed) from farm to retail and in informal supply chains (smallholder farmers), as well as formal supply chains in South Africa. Moreover, to summarise available information on farming practices, irrigation and soil amendment sources, food safety knowledge and practices, and retail outlets in the formal and informal supply chains.

4 To evaluate the microbiological quality of irrigation water and irrigated vegetable crops and to determine the sources of contamination in informal supply chains.

5 To identify and determine the occurrence, dissemination, and characteristics of potential human pathogenic bacteria in the irrigation water and associated fresh produce from the selected smallholder farms.

6 To compare and link information from whole genome sequencing (WGS) of isolates from the environment and produce, which allows identification and genetic characterisation (virulence genes, antibiotic resistance genes, and serotype, among others) of potential human pathogenic bacteria. In addition, to link information from WGS of isolates in the water-plant-animal-food-human health nexus with various sectors or stakeholders, or academic or reference laboratories. This link will include the National Institute of Communicable Diseases (NICD) and the United States Federal Drug Administration (US-FDA), with whom collaborations have already been established.

7 To establish fit-for-purpose guidelines for managing microbiological water quality criteria for vegetable crop production in informal (smallholder farmers) supply chains.

8 To disseminate the knowledge generated by publishing in peer-reviewed journals, via conference presentations and workshops with collaborators and farmers as a support function regarding water and food microbiological quality and safety.

### 1.3 Scope

This research focused on evidence-based studies to inform microbiological quality standards for irrigation water used in smallholder fresh-produce farming systems in South Africa, ensuring alignment with international guidelines while addressing local contextual realities. To achieve this, three complementary case studies were undertaken to assess microbial contamination and the presence of antimicrobial-resistant foodborne pathogens across key stages of the production environment, irrigation water, soil, and fresh vegetables, in four provinces.

The first case study involved single visits to six diverse smallholder farms in Gauteng, North West, and Limpopo between June and November 2022, representing a range of production systems (e.g., aquaculture, mixed farming, organic and conventional practices), irrigation methods, and water sources. The second case study consisted of three integrated vegetable-pig farms, each visited once, in Gauteng, from March 2023 to April 2024, using culture-based and molecular methods to characterise contamination pathways and antimicrobial resistance

profiles. The third case study included a longitudinal assessment of river water over all seasons through analysis of the microbial quality of water drawn from the uMsunduzi River by local small-scale farmers, as well as leafy green vegetables sold by informal street vendors in Sobantu, Pietermaritzburg, KwaZulu-Natal.

Across the three case studies, a multi-matrix sampling approach was used to assess microbial contamination and potential antimicrobial resistance (AMR) risks in irrigation water, soil, and fresh produce from smallholder farming environments. The samples (water, soil, produce) were aseptically collected, transported at 4°C, and analysed within 24h using accredited laboratory techniques. Microbiological analyses included aerobic plate counts and quantification of hygiene indicator organisms (faecal coliforms, *E. coli*, and enterococci) using MPN and culture-based methods. Target pathogens, including *Salmonella* spp., *Listeria monocytogenes* and ESBL-producing Enterobacterales, were detected using selective enrichment and plating onto chromogenic media, followed by MALDI-TOF identification. Irrigation water quality was evaluated using physicochemical parameters, including temperature, pH, turbidity, COD, electrical conductivity, and dissolved oxygen, to contextualise microbial risk within local farming practices and environmental conditions. The Kirby-Bauer disk diffusion method was used to screen for phenotypic antimicrobial resistance and evaluate susceptibility to multiple clinically relevant antibiotics. Selected potential pathogenic isolates were further characterised using molecular assays for virulence genes. High-resolution typing and genomic comparisons of resistant isolates were further performed using WGS to detect specific ESBL/AmpC genetic markers, sequence types, virulence determinants, and complete resistance gene profiles.

# CHAPTER 2: REVIEW OF THE MICROBIAL CONTAMINATION INDICATORS AND FOOD-BORNE PATHOGENS

## 2.1 Introduction

The importance of food safety and water microbiological quality in safeguarding public health cannot be overstated, as they underpin preventive health strategies worldwide (Elbehiry et al., 2025). The complex relationship among environmental quality, agricultural practices, and human health necessitates a comprehensive understanding of how microbial contaminants spread across ecosystems, ultimately affecting the safety of food and water. Microbial contamination can occur at any point in the fresh-produce production process, including on the farm, in processing facilities, during transport or storage, and at the retail level (Althaus et al., 2012). However, because many microorganisms found in environmental settings are harmless commensals, it is necessary to identify and distinguish pathogenic bacteria that contribute to global diseases, including foodborne illnesses (Nüesch-Inderbinen et al., 2015).

Diverse bacterial communities are found on vegetables, including *Actinobacteria*, *Bacteroidetes*, *Bacillota,* and *Pseudomonadota,* with compositions that differ significantly between vegetable types (Berg et al., 2014). For example, Berg et al. (2014) reported that produce types, including spinach, lettuce, tomatoes, sprouts, and peppers, all had high relative loads of taxa belonging to Enterobacterales and tended to share more similar bacterial communities than other vegetable types. Enterobacterales form part of the normal epiphytic microflora of vegetables and include members ubiquitous in terrestrial and aquatic environments, as well as human foodborne pathogens. Enteric bacterial pathogens such as pathogenic *Escherichia coli* and *Salmonella* spp. are commonly linked to foodborne illness outbreaks that can result in serious consequences, including severe illness and, in some cases, fatalities (Elolu et al., 2025). These pathogens typically harbour virulence genes that primarily encode proteins essential for host attachment, growth, invasion, and colonisation, thereby contributing to disease development (Alegbeleye, Singleton and Sant'Ana, 2018; Elolu et al., 2025). The presence of species-specific virulence genes serves as an indicator of pathogenic potential and is valuable for assessing food safety risks (Menck-Costa et al., 2023).

In fresh-produce production, agricultural water has been identified as one of the most important risk factors for microbial contamination (Truchado et al., 2018). Microbial transport from contaminated soil to the produce may also occur via splashing from water droplets, from both rain and irrigation water (Machado-Moreira et al., 2019). A diverse range of water sources is used for irrigation and is typically characterised by differences in microbiological quality (Pachepsky et al., 2011). Furthermore, the microbiological quality of irrigation water can be impacted by various environmental factors, with weather conditions being particularly significant (Truchado et al., 2018). Globally, studies have linked high *E. coli* levels in irrigation water to a greater likelihood of detecting pathogens, yet others have found *E. coli* unreliable as an indicator of pathogen presence in water sources (Kim et al., 2023). These inconsistencies underscore the need for direct quantification of bacterial foodborne pathogens

in irrigation water. Furthermore, the emergence and dissemination of AMR complicate the selection of suitable indicators, as resistance genes can be horizontally transferred among diverse bacterial taxa, thereby amplifying public health risks (Manaia, 2017).

### 2.2 Faecal Indicator Bacteria (*E. Coli*/Faecal Coliforms)

Faecal indicator bacteria, such as generic *Escherichia coli* and faecal coliforms, serve as cost-effective, practical indicators of faecal contamination in irrigation and produce processing water, aiding in microbiological safety assessments (FAO and WHO, 2021). Although faecal indicator bacteria provide valuable information for risk assessment and source tracking, their presence does not reliably indicate specific pathogens such as *Salmonella*, pathogenic *E. coli*, or *Listeria monocytogenes*, due to differences in survival, die-off, and persistence rates (FAO and WHO, 2023; Elolu et al., 2025). Generic *E. coli* is considered a robust indicator of recent faecal pollution from warm-blooded animals, including humans, and its detection in irrigation or washing water signals the potential introduction of pathogenic microorganisms that may adhere to or become internalised in produce (Luna-Guevara et al., 2019; Alegbeleye and Sant'Ana, 2023).

Regulatory guidelines, therefore, recommend strict thresholds: water intended for direct contact with edible portions of crops during primary production or final rinsing should contain <1CFU/100mL of *E. coli* for potable-quality uses, whereas higher tolerable limits may apply to irrigation water not making contact with edible parts, depending on crop type and time to harvest (FAO and WHO, 2019; 2021). The absence of a perfect correlation between faecal indicator bacterial levels and pathogen presence underscores the need for risk-based approaches and, in high-risk scenarios, supplementary pathogen-specific testing rather than sole reliance on indicator organisms (FAO and WHO, 2023; Mengesha et al., 2023). The presence of antimicrobial-resistant bacteria in irrigation water cannot be reliably predicted or inferred from faecal indicator bacteria concentrations alone. Monitoring antimicrobial resistance in freshwater ecosystems requires site-specific strategies alongside standardised protocols, as the presence and spread of resistant bacteria depend on factors beyond faecal contamination levels (Beltrán de Heredia et al., 2025).

### 2.3 Multidrug-Resistant Human Pathogenic Enterobacterales

Multidrug-resistant (MDR) human pathogenic Enterobacterales, including extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Serratia* spp., represent a potential threat to the quality and safety of irrigation water. These organisms are no longer restricted to clinical settings, serving as reservoirs for antimicrobial resistance genes that can disseminate through agricultural environments and contaminate fresh produce, thereby potentially increasing the risk of infections caused by antibiotic-resistant bacterial pathogens in humans (Richter et al., 2024; Viviers et al., 2025). Pathogens from faecal pollution in untreated wastewater, manure, or runoff are prevalent in irrigation water and often exhibit MDR to three or more antibiotic classes, including clinically significant β-

lactams, aminoglycosides, tetracyclines, and colistin (Igbinosa et al., 2023; Richter et al., 2024).

### 2.3.1 Pathovars of *Escherichia coli*

*Escherichia coli* pathovars represent a diverse group of bacterial strains that can transition from commensal to pathogenic roles, posing significant risks to the microbiological quality of irrigation water in fresh-produce production. These pathovars are classified primarily into intestinal pathogenic or diarrhoeagenic *E. coli* (DEC), which cause gastrointestinal illnesses, and extraintestinal pathogenic *E. coli* (ExPEC), which lead to infections outside the gut (Riley, 2020). In agricultural contexts, irrigation water contaminated with faecal matter from human, animal, or environmental sources serves as a key vector for these pathovars, facilitating their transfer to fresh produce such as leafy greens, sprouts, and other vegetables consumed raw (Riley, 2020). The presence of pathogenic *E. coli* in irrigation water undermines water quality standards, often exceeding regulatory thresholds like those set by the FAO and WHO (e.g., <1CFU/100mL for *E. coli* in irrigation water contacting edible crop parts to be consumed raw), and contributes to foodborne outbreaks by enabling adhesion, internalisation, and persistence in produce (Iwu et al., 2022). Unlike generic *E. coli* used as faecal indicators, pathovars exhibit variable correlations with contamination levels, necessitating pathogen-specific monitoring to assess true risks in dynamic freshwater ecosystems used for irrigation (Pakbin et al., 2021; Beltrán de Heredia et al., 2025). These pathovars are distinguished by serotyping (O, H, K antigens), virulence gene profiling and assignment to phylogroups (e.g., A, B1, B2, D). In irrigation water, environmental persistence favours DEC pathovars like enterohaemorrhagic *E. coli* (EHEC) and enterotoxigenic *E. coli* (ETEC), while ExPEC, such as uropathogenic *E. coli* UPEC emerge as opportunistic contaminants (Riley, 2020).

#### Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *Escherichia coli* (EHEC) is often synonymous with Shiga toxin-producing *E. coli* (STEC), like serotype O157:H7, and is a significant concern for irrigation water quality due to its low infectious dose (10–100 cells) and association with severe outbreaks in fresh produce (Gambushe, Zishiri and El Zowalaty, 2022). Virulence factors include Shiga toxins (*stx1* and *stx2* genes, phage-encoded) causing cytotoxicity and haemolytic uremic syndrome (HUS), the locus of enterocyte effacement (LEE) pathogenicity island with *eae* (intimin) and *tir* (translocated intimin receptor) for attaching/effacing lesions, and adhesins like long polar fimbriae (*lpf*) and curli (*csg*) for biofilm formation on produce surfaces (Croxen et al., 2013; Pakbin et al., 2021). Clinically, EHEC manifests as haemorrhagic colitis, bloody diarrhoea, and HUS, with children and the elderly at highest risk. Epidemiologically, EHEC reservoirs in cattle and water sources drive produce contamination, with the 2011 German O104:H4 outbreak linked to imported, irrigated fenugreek sprouts, highlighting the role of irrigation water in amplifying transmission (Frank et al., 2011). Multidrug-resistant (MDR) EHEC in water exacerbates risks, with resistance to β-lactams and tetracyclines commonly reported (Iwu et al., 2022).

### Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) is an important diarrhoeagenic pathogen in infants and can contaminate irrigation water through human faecal pollution. Its pathogenicity is driven by virulence factors encoded on the locus of enterocyte effacement (LEE), including *espA*, *espB*, and *espD* for type III secretion system translocation structures and *eae*/*tir* for the formation of attaching and effacing lesions. Additional non-LEE effectors, such as *nleA* and *nleF*, facilitate immune evasion and disruption of epithelial tight junctions (Croxen et al., 2013; Pakbin et al., 2021). A South African study reported an approximate 2.9% prevalence of EPEC in water samples from produce farms, with EPEC persisting in biofilms and transferring to vegetables during irrigation (Iwu et al., 2022).

### Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC), a significant cause of traveller's diarrhoea, can enter irrigation water through sewage or animal waste. Its virulence is driven by plasmid-encoded colonisation factors that mediate intestinal adhesion and by heat-labile (LT) and heat-stable (ST) enterotoxins, which elevate cAMP or cGMP to induce secretory diarrhoea, with EAST1 further contributing to enterotoxic activity (Croxen et al., 2013; Pakbin et al., 2021).

### Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *Escherichia coli* (EIEC) exhibits invasive behaviour similar to that of *Shigella*, contaminating water via human faecal sources (Pakbin et al., 2021). Pathogenicity is driven by plasmid-borne virulence determinants, including ipaH/B/C/D, which enable type III secretion, mediate host invasion, and promote cytoskeletal rearrangement (Croxen et al., 2013; Pakbin et al., 2021). Detection rates up to 10% in irrigation water from soil-adjacent sampling have been reported. Therefore, EIEC poses a tangible risk to root crops and food safety (Iwu et al., 2022). Clinically, EIEC infection produces dysentery-like disease with bloody diarrhoea.

### Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *Escherichia coli* (EAEC) causes persistent diarrhoea and persists in water via biofilms. Virulence includes aggregative adherence fimbriae (*aaf*/*agg*) and toxins like EAST1, Pet, and Pic (Croxen et al., 2013; Pakbin et al., 2021).

## 2.3.2 Other pathovars: DAEC, AIEC and ExPEC

Diffusely adherent *Escherichia coli* (DAEC) uses Afa/Dr adhesins (*afa*/*dra*) for attachment, while adherent-invasive *Escherichia coli* (AIEC) invades via *fimH* and *ibeA*, both of which are relevant in water-contaminated produce (Pakbin et al., 2021). Extraintestinal pathogenic *E. coli* (ExPEC), including UPEC and neonatal meningitis *E. coli* (NMEC), usually carry papC (P fimbriae) and ibeA, and MDR strains in irrigation water indicate zoonotic risks from animal manure (Riley, 2020; Iwu et al., 2022).

### *Salmonella* spp.

*Salmonella* spp. is classified as a pathogenic microorganism and does not form part of the faecal indicator bacteria, yet it may be present in water contaminated with faecal material. *Salmonella* spp. is regarded as a major foodborne pathogen and a leading cause of gastroenteritis in humans and animals (Lamichhane et al., 2024). In food safety, *Salmonella enterica* subspecies *enterica* is the key species of concern, with over 2 500 serovars identified, but a small subset causes most human infections globally. Prominent serovars include *Salmonella* Enteritidis, often linked to eggs and poultry; *Salmonella* Typhimurium, common in various animal-derived foods and a significant cause of outbreaks; and *Salmonella infantis*, increasingly reported for antimicrobial resistance in poultry products (Lamichhane et al., 2024).

The linkage between water quality and food safety underscores how *Salmonella* spp. in contaminated water can enter the food chain, for example through irrigation, and how faecal indicator bacteria guidelines help mitigate risks by indicating potential pathogen presence (Holcomb and Stewart, 2020). Therefore, global and national surveillance organisations such as the WHO, FAO, and CDC emphasised the importance of monitoring these key serovars throughout the entire farm-to-fork process to prevent transmission (FAO and WHO, 2019).

## 2.4 Global Comparative Irrigation Water Microbiological Quality Guidelines and Mitigation Strategies

Several international organisations and countries have developed microbiological quality standards for irrigation water used in agriculture. These guidelines aim to establish safety benchmarks; however, significant regional variations exist and are often tailored to local climatic, economic, and social contexts (Banach and Van Der Fels-Klerx, 2020). In South Africa, for example, the irrigation water recommendations of the Department of Water Affairs and Forestry (DWAF, 1996) have set a generic *E. coli* limit of ≤1 000CFU/100mL, a threshold comparable to that in the World Health Organization (WHO, 2006) guidelines. Importantly, while the South African guidelines have evolved to incorporate risk-based and site-specific approaches, gaps persist in addressing smallholder-specific realities, such as microbial indicator thresholds tailored to informal supply chains and integrated farming systems. A comparison of international guidelines for water quality is shown in Table 1. Recognising the need to address local water-use challenges and move beyond the limitations of the 1996 DWAF guidance, South Africa revised its irrigation water framework in 2017 (du Plessis et al., 2017; du Plessis, Annandale and Benade, 2021). The *Risk-Based, Site-Specific Irrigation Water Quality Guideline*, comprising ‘Volume 1: Decision Support System (DSS) Description’ and ‘Volume 2: Technical Support Guideline’, represents a fundamental shift from the earlier generic approach. The updated system is (i) explicitly risk-based, (ii) customised to site-specific conditions, and (iii) primarily operated through a software-enabled decision support system (DSS) (du Plessis et al., 2017). However, for microbiological quality parameters, these guidelines present practical challenges, and enhanced on-the-ground operational realities should be acknowledged. Importantly, precise prediction of soil, crop, and

equipment responses depends on the use of locally relevant input data to effectively simulate a range of climate and irrigation scenarios.

**Table 1:** Guidelines and legislation for the microbiological quality of irrigation water, drinking water, treated wastewater, and recreational water.

| Country/region | Criteria (CFU/100mL)[a] | Document type | Reference(s) |
|---|---|---|---|
| **IRRIGATION WATER (for all water types)** | | | |
| **Canada** | <100 faecal coliforms | Guideline | Canadian EMC, 2008 |
| | 1 000 total coliforms | | |
| **Canada (British Columbia)** | 200 faecal coliforms | Guideline | BCMAL, 2003. Treating Irrigation and Crop Wash Water for Pathogens. Factsheet #512.000-3 |
| | 77 *E. coli* | | |
| | <20 faecal streptococci | | |
| **European Union** | Between 10 *E. coli* | Guideline | EC, 2020 |
| Fresh fruit and vegetables (FFV) (likely to be eaten uncooked), direct contact of irrigation water with the edible portion. | 100 *E. coli* | | |
| FFV is likely to be eaten uncooked, with no irrigation water contact with the edible portion. | 1 000 *E. coli* | | |
| FFV is likely to be eaten cooked, with direct contact of irrigation water with the edible portion. | 1 000 *E. coli* | | |
| FFV is likely to be eaten cooked, with no direct contact of irrigation water with the edible portion. | 10 000 *E. coli* | | |
| **POST-HARVEST** | | | |
| Post-harvest cooling and transport of non-ready-to-eat. | 100 *E. coli* | | |
| Water is used for washing in the case of ready-to-eat products. | | | |
| Cleaning equipment and surfaces used to handle the products. | | | |
| Water used for washing produce that is likely to be eaten cooked. | 1 000 *E. coli* | | |
| Final washing and ice/cooling of ready-to-eat FFV | Potable water microbiological quality | | |
| **South Africa** | | | |
| Any crop[a] | <1 *E. coli* | Guideline | du Plessis et al., 2017 |
| Vegetables and crops[b] | <1 000 *E. coli* | | |
| Fodder, tree plantations, nurseries, parks, etc.[c] | >1 000 *E. coli* | | |
| **FDA Food Safety Modernization Act (FSMA) United States** | <126 MPN *E. coli* | | FDA (FSMA), 2019 |

| | | | |
|---|---|---|---|
| **DRINKING/POTABLE WATER** | | | |
| **WHO** | <1 *E. coli* | Guideline | WHO, 2011 |
| | 0 *E. coli* | Directive | EC, 1998 |
| | 0 enterococci | | |
| | *For water in bottles or containers*[a] | | |
| | 0 *E. coli*/250mL | | |
| | 0 enterococci/250mL | | |
| | 0 *Pseudomonas aeruginosa*/250mL<br>Colony count 22°C: 100CFU/mL<br>Colony count 37°C: 100CFU/mL | | |
| **European Union** | 0 *E. coli* | Decree | EU, 2020 |
| | 0 enterococci | | |
| **United States** | 0 enterococci | | |
| | 0 for total coliforms, faecal coliforms and *E. coli* | Regulation | EPA, 2017 |
| **South Africa** | 0 *E. coli* | Regulation | SANS 241–1 (2015); DWS 2017 |
| **(TREATED) WASTEWATER** | | | |
| **WHO** | Guideline | WHO, 2006 | Guideline |
| Unrestricted irrigation crops eaten raw | <1 000 *E. coli* | | |
| | ≤1 helminth eggs (no./L) | | |
| High-growing leaf crops or drip irrigation | $\leq 10^4$ *E. coli* | | |
| Restricted irrigation | $\leq 10^5$ *E. coli* | | |
| **Italy** | <10 *E. coli* and absence of *Salmonella* | Regulation | Cirelli et al., 2008; Uyttendaele et al., 2015 |
| **Spain** | *<100 E. coli* | Regulation | BOE, 2007, Uyttendaele et al., 2015 |
| **South Africa** | | | |
| Unrestricted use | <1 *E. coli* | Guideline | Rodda et al., 2010[a] |
| Increasing risk | <1-1 000 *E. coli* | | |
| Excessive risk | <10 000–100 000 *E. coli* | | |
| **RECREATIONAL WATERS (coastal and fresh waters)** | | | |
| **WHO** | <500 enterococci | Guideline | WHO, 2003 |
| **EU** | Inland waters:<br>*<330* enterococci or<br>*<900 E. coli* | Guideline | EC, 2006 |
| | Coastal and transitional waters: <185 enterococci | | |
| **United States** | *<35* enterococci or<br>*<126 E. coli* | Guideline | EPA, 2012 |

| | | | |
|---|---|---|---|
| **South Africa** | < faecal streptococci;<br><200 *E. coli* | Guideline | DWAF, 1996 |

[A] Any crop with any irrigation method

[B] Likelihood of contamination from crops and other vegetables eaten raw

[C] Any irrigation, for production of fodder, tree plantations, nurseries, parks, etc.

Despite these developments, implementation remains limited, as the guidelines cannot be universally applied as default water quality standards across South Africa, especially within the informal agricultural sector. Other parameters also impact the overall microbiological quality, such as physicochemical parameters including pH (typically 6.5–8.5), temperature (usually below 25°C according to WHO but affected by local climate conditions), electrical conductivity, turbidity, total dissolved solids, and nutrient content (Mudinga Daniel et al., 2025).

As climate change and water scarcity affect the availability of water sources across regions, agricultural water reuse is also gaining attention. A comprehensive review of agricultural water reuse regulations and guidelines worldwide reported that global standards often appear homogeneous at the international level, yet practical implementation reveals substantial regional heterogeneity (Shoushtarian and Negahban-Azar, 2020). Moreover, water reuse guidelines are primarily human health-centred but insufficient in addressing potentially dangerous pollutants, including emerging contaminants, with significant discrepancies between the guidelines. Important parameters like pathogens, heavy metals, and salinity are often included in only a small subset of guidelines (Shoushtarian and Negahban-Azar, 2020).

Regarding fit-for-purpose guidelines for managing microbiological water quality risks, water scarcity indicates an important need for reform to strengthen resilience against climate-induced shortages. The gaps and discrepancies identified by Shoushtarian and Negahban-Azar (2020) in water reuse guidelines could lead to the underestimation of risks in agricultural settings, where contaminated irrigation water is a significant vector for pathogens such as *Salmonella* spp. in produce, potentially amplifying food safety outbreaks in vulnerable regions. Moreover, thresholds are often set that cannot be met, given the quality of the available water sources. For small-scale growers in Kentucky, United States, as an example, agricultural water must comply with FSMA standards focusing on *E. coli* presence, while accounting for localised contamination patterns (Tope, Thomas and London, 2025). By emphasising human health while neglecting integrated pollutant management, current frameworks fall short of true fit-for-purpose adaptability, hindering sustainable reuse and public acceptance.

## 2.5 Microbial Contamination Sources Within the Water-Soil-Plant Nexus

The water-soil-plant nexus forms a dynamic, interconnected system in which microbial contaminants, such as bacteria, viruses, fungi, and protozoa, can migrate between compartments, posing significant risks to ecosystem health, agricultural productivity, and food safety (Pandey et al., 2014; Richter et al., 2022). Primary contamination sources include irrigation water containing sewage runoff, manure-amended soils, livestock or wildlife faeces,

and effluents from industrial or healthcare facilities, which contaminate water, soil, and plants through runoff, deposition, or root uptake (Alegbeleye, Singleton and Sant'Ana, 2018).

### 2.5.1 Overview in Africa

There are significant geographical disparities in research on microbial contamination across Africa, with some countries receiving considerably more attention than others. South Africa remains the most extensively studied African nation to date, where robust infrastructure and research networks enable more detailed assessments of groundwater and irrigation systems, fresh-produce contamination, antibiotic resistance, and faecal source tracking (Bain et al., 2014; Lapworth et al., 2017; Richter et al., 2023; Adugna et al., 2024). Ethiopia, Nigeria, and Uganda follow, with research addressing microbial and chemical contamination risks to drinking water, irrigation systems, and household exposure pathways (Fuhrimann et al., 2015; Gizaw et al., 2022; Berihun et al., 2023; Igbinosa et al., 2023; Adugna et al., 2024; Kipsang, Kibet and Adongo, 2024; Abdulkadir et al., 2025). Moderate research coverage is evident in Kenya and Ghana, where studies have evaluated faecal contamination in drinking water, the environmental effects of livestock husbandry, and irrigation water quality in food production areas (Douti et al., 2021). In contrast, Chad and Cameroon are among the least studied of the identified countries, with limited but valuable reports documenting pathogenic bacteria, including *Salmonella* spp. and *E. coli*, in water systems (Mounpor et al., 2023; Assomo et al., 2024).

In some African countries, the use of untreated or partially treated wastewater for crop irrigation, coupled with limited sanitation infrastructure, insufficient water treatment facilities, and reliance on animal manure as fertiliser, contributes to conditions that facilitate widespread microbial contamination (Obaideen et al., 2022). This contamination occurs throughout the entire continuum, from contaminated irrigation water and soil to cultivated crops, establishing a complex transmission pathway for foodborne pathogens such as bacteria, parasites, and viruses. Understanding the sources, extent, and epidemiology of microbial contamination in this nexus is essential for developing effective interventions to protect vulnerable populations and ensure food safety.

### 2.5.2 Water sources and contamination patterns

Irrigation water represents the primary vehicle for introducing pathogens into agricultural systems across Africa. Studies from multiple regions demonstrate consistently high microbial contamination levels; however, various methodologies and reporting structures are used in the published literature, making direct comparisons difficult. In Uganda, the Nakivubo wetland, a significant source of irrigation water, showed mean concentrations of thermotolerant coliforms of $2.9 \times 10^5$ CFU/100mL and *E. coli* of $9.9 \times 10^4$ CFU/100mL, with hookworm eggs detected in 13.5% of water samples (Fuhrimann et al., 2015). Household water was analysed in the Dessie Zuria district, Ethiopia, where 25% of households (n=412) used river water as their primary drinking-water source (Berihun et al., 2023). Of the total water samples,

approximately 66% were positive for faecal coliform bacteria, with water storage duration exceeding three days, dipping methods for water withdrawal, and uncovered storage containers being significant risk factors for contamination (Berihun et al., 2023b). In Ghana, irrigation water quality at the Vea Irrigation Dam exceeded WHO bacterial load standards, with *E. coli* counts significantly above international thresholds (Douti et al., 2021).

Additionally, agricultural soils act as significant reservoirs for pathogenic bacteria, which become contaminated through wastewater, contaminated irrigation water, organic amendments, and animal contact. Various studies have reported on the application of animal manure, which increases microbial loads (Chan et al., 2023; Viviers et al., 2024). This contamination, in turn, leads to contamination in leafy green vegetables and other fresh produce, posing a risk when the produce is consumed raw or without proper washing with safe water. In Uganda, wastewater-irrigated lettuce (2.89 log CFU/g) and Swiss chard (2.65 log CFU/g) were linked to *E. coli* in water and soil (Ali et al., 2023). Similarly, on Nigerian farms, MDR ESBL-producing *E. coli* was isolated from 68% of soil samples, 84% of manure samples, and 24.4% of harvested vegetables, while ready-to-eat salads showed contamination rates of 20–36.6% (Igbinosa et al., 2023).

### 2.5.3 South Africa

The complex interactions within the water-soil-plant system in South Africa pose significant public health challenges, as microbial pathogens from various sources can infiltrate agricultural environments, impacting food safety. Emerging economies such as South Africa continue to experience significant challenges in providing reliable access to safe drinking and domestic water. This often results in resource-constrained communities relying on microbiologically contaminated river water, thereby increasing public health risks (Mbanga et al., 2020). Moreover, the contamination pathway is intensified by insufficient or failing wastewater management infrastructure, the widespread practice of applying untreated or minimally treated wastewater for irrigation, and the use of animal manure as fertiliser, all of which contribute to the transfer of pathogenic microorganisms from environmental sources to cultivated crops (Naidoo and Olaniran, 2013; Iloms et al., 2020). The consequences are especially significant in rural areas, where communities frequently store water in containers for prolonged periods without sufficient treatment, potentially facilitating microbial growth and increasing the risk of human exposure (Potgieter, van der Loo and Barnard, 2021). Understanding the specific sources, types of pathogens, and the geographical distribution of this contamination in South Africa is vital for developing targeted, effective interventions to safeguard both food security and public health.

#### Irrigation water sources

Water sources throughout South Africa, including rivers, dams, boreholes, and even stored household water, consistently exhibit significant microbial contamination, serving as primary vehicles for pathogen dissemination within the agricultural environment. Studies quantifying

bacterial contamination in wastewater treatment plants (WWTPs) and their receiving surface water bodies have revealed alarmingly high concentrations of indicator bacteria. For instance, mean *E. coli* concentrations ranged from 2.41–6.68 log CFU/100mL, while enterococci ranged from 2.41–6.5 log CFU/mL across various sampling sites in KwaZulu-Natal (Mbanga et al., 2020). This research further identified a substantial proportion of these isolates as intestinal (89.1%) and extraintestinal (7.6%) pathogenic *E. coli*, directly linking water contamination to potential health hazards for communities using river water both upstream and downstream of WWTPs (Mbanga et al., 2020). The occupational risk for WWTP workers was also found to be significant, with infection probabilities reaching 100% for those exposed to raw influent multiple times weekly over a year (Mbanga et al., 2020).

Beyond WWTPs, general river catchments in provinces such as the North West also demonstrate widespread bacterial contamination. As an example, Constance and Moses (2012) reported significantly high counts of heterotrophic bacteria, faecal coliforms, *E. coli*, and *Klebsiella* spp. in water samples from the Marico, Molopo, Crocodile, Elands, and Hex catchments, with *E. coli* prevalence ranging from 63–75% and *Klebsiella* from 76–95%. These levels frequently exceeded the guidelines for domestic, recreational, and irrigation water use of the South African Department of Water Affairs, underscoring the pervasive nature of surface water pollution (Constance and Mbewe, 2012). More recently, Duze et al. (2025) highlight the significant public health concerns associated with the Jukskei River in Johannesburg, Gauteng, noting that most sampling sites exceeded 2.6 and 3.3 log CFU/100mL for total coliforms (which is not a useful hygiene indicator) and *E. coli*.

Groundwater, often perceived as a safer alternative, is also highly susceptible to faecal contamination, particularly in rural areas and near landfill sites. In the Vhembe District Municipality in Limpopo, a study reported that 87.5% of boreholes were contaminated with *E. coli* during the wet season and 72.5% during the dry season (Sekgobela et al., 2023). This study utilised host-specific quantitative polymerase chain reaction (qPCR) assays to pinpoint contamination sources, identifying human (BacHum and HF183) and animal (Cytb, BacCan, and Pig2Bac) genetic markers in groundwater samples, indicating a mixed origin of faecal pollution (Sekgobela et al., 2023). Similarly, at the Roundhill municipal landfill in Berlin, Eastern Cape, groundwater samples from boreholes nearest the landfill showed critical contamination, with *E. coli* concentrations in one borehole reaching 4.08 log MPN/100mL, rendering the water unsuitable for drinking (Silwani et al., 2025).

AMR is also crucially linked to water contamination in South Africa. A recent meta-analysis study reported on the characterisation of extended-spectrum β-lactamase (ESBL)-producing *E. coli* from irrigation water and fresh produce, revealing that 95.1% of the isolates carried resistance genes from three or more antibiotic classes (Richter et al., 2024). The frequent detection of ESBL genes like $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-15}}$ associated with mobile genetic elements indicates that contaminated water sources serve as a significant reservoir for MDR strains, posing a severe threat to public health within a One Health context (Richter et al., 2024).

### Agricultural soils

Agricultural soils in South Africa become contaminated through various pathways, primarily through irrigation with microbiologically compromised water and the application of organic amendments, including raw manure. While direct studies on soil microbial loads in South Africa are less detailed than those on water, the presence of contaminated irrigation water directly implies soil contamination. The persistence of *E. coli*, both commensal and pathogenic, in various environments, including plants, underscores the role of soil as a crucial intermediary in the water-soil-plant contamination pathway (Richter et al., 2024).

### Fresh produce (vegetables)

The final crucial stage of microbial contamination in the water-soil-plant nexus in South Africa involves the transfer of pathogens to fresh produce, particularly leafy vegetables, which are often consumed raw. The detection of MDR ESBL-producing *E. coli* in fresh-produce samples highlights a direct link between contaminated irrigation water, agricultural environments, and the food supply (Richter et al., 2024). Through whole-genome sequence characterisation, a high likelihood that the isolates are human pathogens was reported, underscoring the potential public health risk posed by consuming contaminated produce. The study also noted that *E. coli* can persist on plants, suggesting that crops may serve as a direct source of exposure for consumers (Richter et al., 2024).

Complementing these findings, a study on commercial spinach supply chains in Gauteng revealed ESBL/AmpC-producing Enterobacteriaceae in 12.12% of harvested and processed spinach and 25% of retail samples, with contamination increasing post-harvest (Richter et al., 2020b). Dominant species included *Serratia fonticola* (45.86%), *E. coli* (20.83%), and *Klebsiella pneumoniae* (18.75%), nearly all (98%) exhibiting multidrug resistance to aminoglycosides (89.58%), chloramphenicol (79.17%), and β-lactams, alongside prevalent CTX-M Group 1, TEM, SHV, and CIT-type genes, and high integron carriage (79.17%, especially class 3 at 72.92%) (Richter et al., 2020). Similarly, bacterial profiling of irrigation water and *Brassica rapa* leafy green vegetables from small-scale farms in North West and Mpumalanga showed that flood-irrigated areas exhibited higher microbial diversity and faecal indicators, linked to contamination with MDR pathogens such as ESBL-producing *E. coli* and *Salmonella enterica* (Kgoale et al., 2023). In the Free State, fresh vegetables (spinach and cabbage) from farms and informal markets harboured resistant bacteria such as *E. coli* (18.4%), *Burkholderia cepacia* (15.8%), and *Pseudomonas luteola* (15.8%), with 84% resistant to tetracycline, 71% to penicillin, and 79% displaying multidrug resistance across β-lactams, chloramphenicol, tetracycline, aminoglycosides, and macrolides, highlighting leafy greens as reservoirs for antibiotic-resistant pathogens (Mohapi et al., 2025).

## 2.6 Significance and Implications of Contamination

The widespread microbial contamination present in South Africa's water, soil, and plant ecosystems has significant implications for public health, food security, and environmental

sustainability. Communities relying on contaminated river water for domestic and recreational purposes face a substantial health risk, with probabilities of infection from ingesting even small volumes of water reaching as high as 92.9% in upstream areas near WWTPs (Mbanga et al., 2020). Furthermore, certain studies have identified MDR ESBL-producing *E. coli* in irrigation water and in produce, highlighting their potential as a reservoir of antimicrobial resistance. This complicates treatment efforts and underscores the need for a comprehensive One Health approach to protect human and animal health (Richter et al., 2024). This contamination can compromise food safety by rendering fresh produce unsafe, potentially increasing health risks and nutritional challenges among vulnerable populations, such as children (Mbanga et al., 2020). Additionally, microbial contamination can negatively impact the agricultural sector by reducing productivity and creating potential trade barriers.

Addressing this complex issue requires a comprehensive and coordinated approach that goes beyond traditional sectoral boundaries. In the agricultural sector, implementing stringent water-quality standards for irrigation, responsible management of animal waste, and enhanced hygiene practices for fresh-produce cultivation and handling are crucial to breaking the transmission cycle of pathogens (Richter et al., 2024). A One Health approach, which recognises the interconnectedness of human, animal, and environmental health, is indispensable for developing sustainable solutions that can effectively mitigate the risks posed by microbial contamination within South Africa’s vital water-soil-plant nexus.

## CHAPTER 3: CASE STUDY 1: Diversified Fresh-Produce Small-Scale Farm Scenarios

*S.A Viviers, L. Richter-Mouton, and L. Korsten*

### 3.1 Introduction

In peri-urban areas of South Africa, subsistence farming has led to a high diversity of smallholder farmers who not only grow fresh produce for their own consumption (Mdluli et al., 2013) but also aim to sell surplus produce to earn income (Mugambiwa and Tirivangasi, 2017). This smallholder farming supply aligns with rising consumer demand for minimally processed fruits and vegetables (Castro-Ibáñez et al., 2017; Machado-Moreira et al., 2019). However, water scarcity limits access to formal irrigation, forcing reliance on available sources like surface water, boreholes, and municipal supplies (van Koppen et al., 2017; Nephawe et al., 2021; Bonthuys, 2018; Iwu and Okoh, 2019). Surface water is often polluted due to wastewater infrastructure failures and raw sewage discharge (Gemmell and Schmidt, 2012; Schreiner et al., 2018; Mnguni, 2022; Ngubane et al., 2022), posing risks via overhead irrigation, which can transfer microbial pathogens to crops (EC, 2017; Uyttendaele et al., 2015).

Irrigation water is a key contamination pathway for fresh produce, potentially introducing pathogens like Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* spp., especially in raw-consumed items such as leafy greens (Uyttendaele et al., 2015; IFSAC, 2021; Gambushe et al., 2022; Rocha et al., 2022). AMR in these bacteria exacerbates treatment challenges (Manyi-Loh et al., 2018; Larsson et al., 2018). Standards for irrigation water quality vary globally, often relying on *E. coli* limits (<1 000/100mL), though this indicator is insufficient for broader pathogen detection (DWAF, 1996; WHO, 2006; FAO and WHO, 2019; Haymaker et al., 2019; Rock et al., 2021). Recently, updated guidelines have also been published in South Africa that possibly present a more advanced, risk-based, and site-specific Decision Support System (DSS), marking a significant shift in how microbiological water quality is assessed (du Plessis et al., 2017; du Plessis, Annandale and Benade, 2021), but gaps remain in addressing smallholder contexts.

Physicochemical parameters (e.g., pH, BOD, COD, turbidity) further inform water quality, as deviations in these parameters affect soil and crop health (Iwu et al., 2020; Alegbeleye and Sant'Ana, 2023; Abbas et al., 2020; Maphuhla et al., 2021). Contaminated water, manure, and handling practices can introduce pathogens and AMR into the agroecosystem (Tope et al., 2016; Alegbeleye et al., 2018; Richter et al., 2020; Iwu et al., 2021). In low- and middle-income countries (LMICs) like South Africa, underreporting of foodborne illnesses and challenges with surveillance hinder risk management (Bisholo et al., 2018; Richter et al., 2023; Mafiz et al., 2018). A One Health approach is essential to address environmental transmission of AMR (WHO, 2015; Koutsoumanis et al., 2021).

This project aims to develop fit-for-purpose guidelines for managing microbiological water quality criteria for irrigation water in smallholder systems, aligned with international standards. Objectives include assessing water and crop quality, isolating pathogens, applying whole-

genome sequencing for health linkages, and disseminating knowledge. To achieve this, Case Study 1 sampled six diversified smallholder farms (Farms A–F) across Gauteng, North West, and Limpopo, representing varied water sources (borehole, river, dam, municipal), irrigation methods (overhead, drip, and hosepipe), crop types (leafy greens, roots, and fruits), and systems (aquaculture, integrated livestock, organic, and conventional).

### 3.2 Experimental Procedures

Six smallholder farms (Farms A–F) across Gauteng, North West, and Limpopo in South Africa were sampled, representing a range of water sources, irrigation methods, crop types, and production systems (aquaculture, integrated, organic, and conventional).

Farm A practises mixed farming by producing both fresh produce and fish through an aquaculture system (where production was for both household income and consumption). Borehole water is used as the source water, which is pumped to the farm's aquaculture system. The water then flows through the system, where it is enriched with nutrients via fish (Tilapia) waste and nitrogen-fixing bacteria. Subsequently, the water is pumped to a holding dam, where sediment settles to the bottom, and the top water is used for the irrigation of fresh produce. Irrigation areas include an aquaponics system where lettuce is produced, as well as a net-covered field is irrigated with a hosepipe (Figure 1). The farmer produces lettuce, which is sold to the Johannesburg fresh-produce market, as well as hemp, kale, tomatoes, okra, and spinach for local kitchens and consumers. Additionally, the fish (Tilapia) are sold to local restaurants. The farm was visited in the dry winter season (20 June 2022).

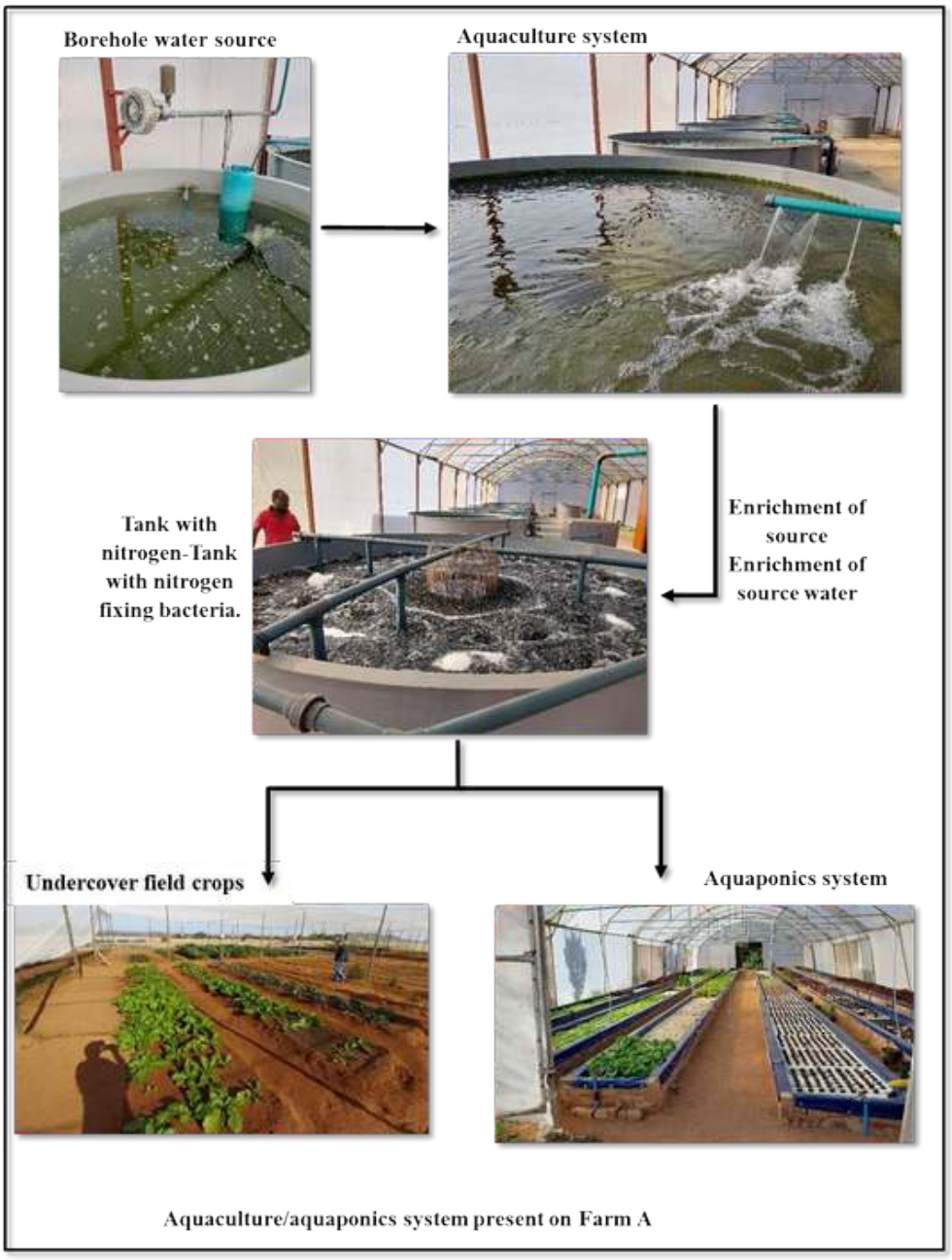



**Figure 1:** Visual representation of the production practices followed on Farm A, which is an aquaponics and fresh-produce production farm.

Farm B practises mixed farming, producing both fresh produce (Chinese spinach, rape, and kale) and raising pigs and chickens, with production for both household consumption and income. This farm was therefore classified as an integrated farm with livestock. The water source of the farm is a borehole used to irrigate fresh produce with overhead sprinklers (Figure 2). The farmer directly adds the generated pig and chicken manure onto the fields as fertiliser. The farm was also visited in the dry winter season (21 June 2022).




**Figure 2:** The fresh-produce sampling sites of Farm B, which is an integrated farm (animal husbandry and fresh production) and uses borehole water for irrigation.

Farm C practises organic farming and produces a variety of fresh produce, including spinach, kale, radishes, beetroot, cabbage, carrots, onions, rocket, and lettuce, using borehole water via overhead sprinklers (Figure 3). Organic fertiliser is used during production, while the

revenue generated is for both household consumption and income. This farm was visited in the dry winter season (22 August 2022).




**Figure 3:** Visual representation of the borehole water source, compost used, and irrigated fresh-produce fields on Farm C, which is an organic farm.

Farm D practises conventional farming and produces fresh produce using river water via overhead irrigation. The Magaliesberg water catchment area is the source of irrigation water,

with a canal running through various farms and settlements. The river water is pumped into water-holding dams, from which the water is then pumped to the fields for overhead irrigation (Figure 4). The farm supplies fresh produce (baby carrots, kale, turnips, beetroot, onions, rocket, herbs, etc.) to various local retailers, and also exports selected products to Europe. The farm was visited in the dry spring season (19 September 2022).

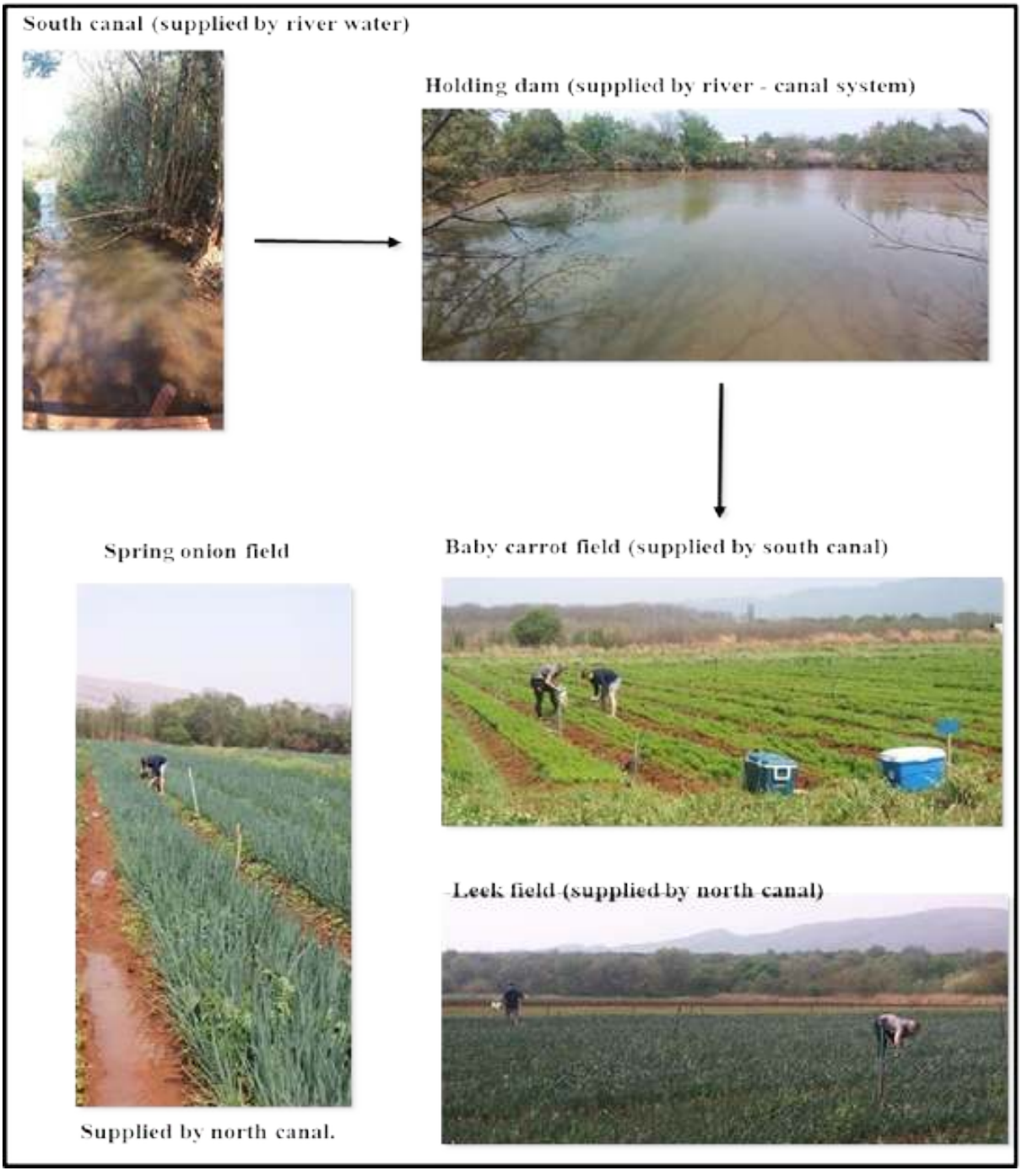



**Figure 4:** Visual representation of the fresh-produce fields irrigated with river water from the Skeerpoort River on Farm D, which is a conventional farm.

Farm E practises mixed farming by producing fresh produce and raising livestock such as goats and sheep. The Rust de Winter Dam is the main water source, which supplies farms in the area via a canal system. Water is stored in a dam until it is pumped onto the fields. The farm's fresh produce is predominantly overhead-irrigated, except for the green pepper fields, which are drip-irrigated (Figure 5). Tomatoes, green peppers, spring onions, watermelons, maize, cabbage, and *morogo* are typically produced. The farm was visited in the wet spring season (15 November 2022).

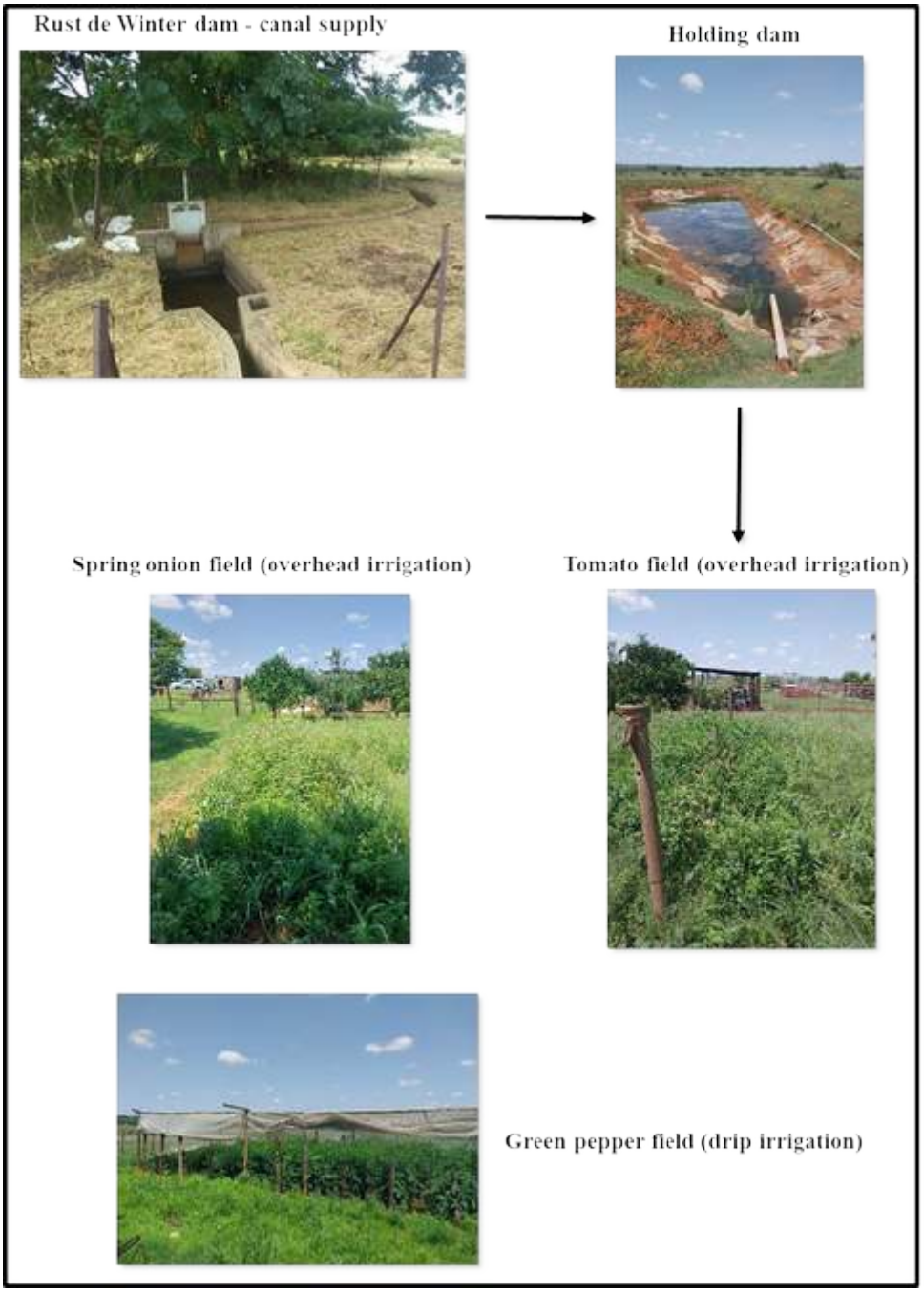



**Figure 5:** Farm E is a conventional farm using irrigation water supplied via a canal from the Rust de Winter Dam.

Farm F practises mixed farming by producing fresh produce and raising chickens. The main water supply is municipal water, used to irrigate the fresh produce via an extensive drip irrigation system. Occasionally, the municipal water is mixed with borehole water and liquid fertiliser in two 2 400L Jojo tanks before being pumped to the fields. The soil in the fields is amended with chicken manure and liquid fertiliser through the irrigation system (Figure 6). The farm produces a variety of fresh produce, including spinach, green peppers, and onions. The farm was visited in the wet spring season (25 November 2022).

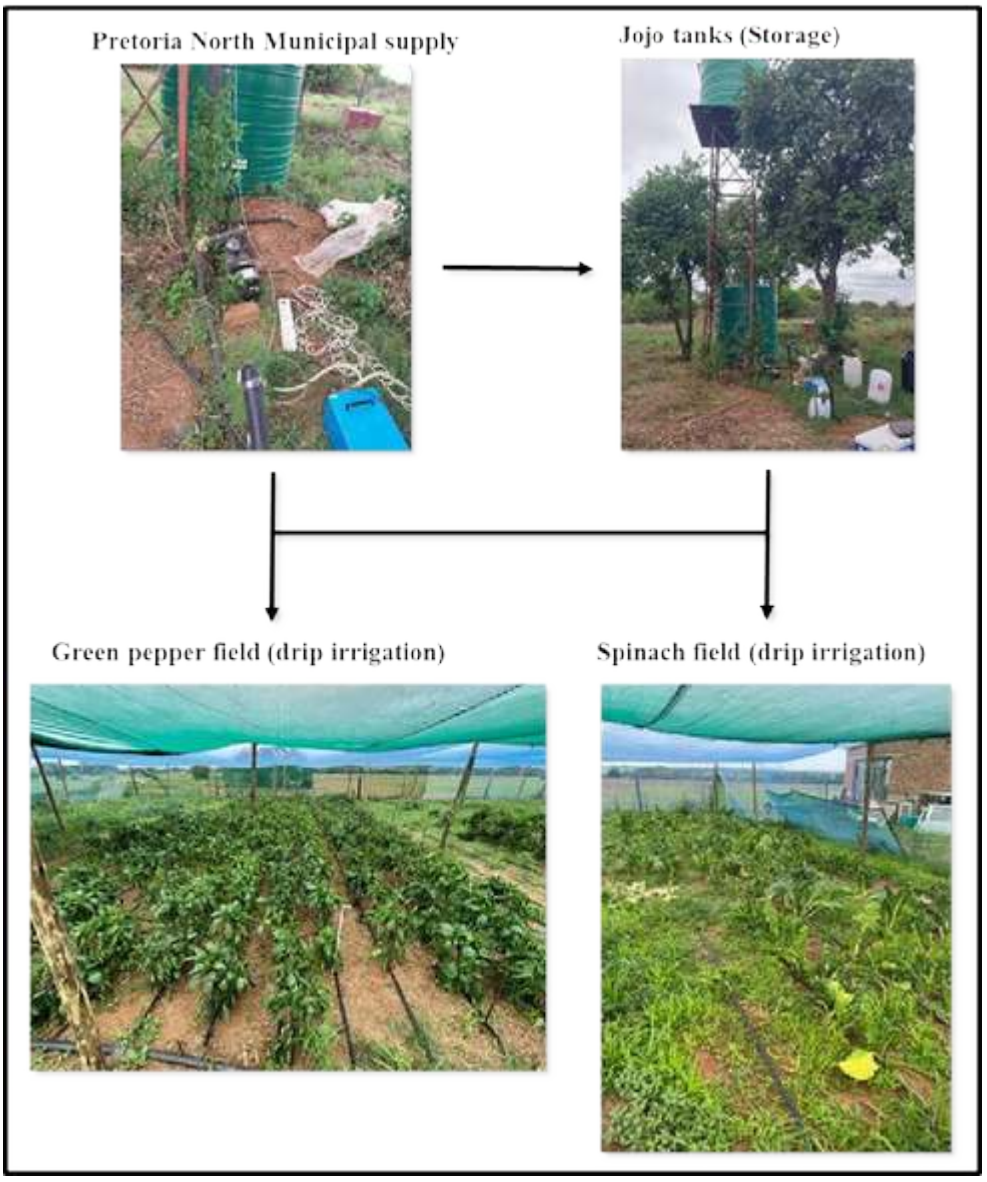



**Figure 6**: Farm F practices mixed farming, including chicken and fresh-produce production using municipal water and a drip irrigation system.

### 3.2.1 Sample collection

A total of 235 samples (water, soil, and fresh produce) were collected at selected sampling points from Farms A–F, depending on the production system and availability of fresh-produce

types. Of these, 224 samples were designated for microbiological analysis, and the remaining 11 samples for physicochemical analysis.

**Water**

A total of 55 water samples were collected from each farm at each point in the irrigation system: either borehole water samples (n=31), river water samples (n=12) or municipal water samples (n=12). The samples consisted of 100mL, 1L, and 100L samples collected from each point in the irrigation system on each farm: the water source, storage dams, and irrigation points, where available. The 100mL and 1L water samples were collected in ethanol-sterilised, air-dried plastic bottles at each site. The 100L water samples were collected by filtering water at each water source site through a kidney dialysis filter (FDA 2021).

**Soil**

Soil samples (n=85) comprised composite samples of 25g (5g from each replicate) where each fresh-produce type was sampled and stored in a cooler box at 4ºC until analysis within 24h.

**Fresh produce**

Based on availability, at least three types of fresh-produce samples were collected at harvest at each farm (n=95). Of each leafy fresh-produce type (lettuce, spinach, rape, kale, and rocket), five replicates were collected in an unbiased random way across the fields. Additionally, whole produce types, consisting of a composite of at least three tomatoes, baby carrots, radishes, green peppers, or onions, were collected using a 70% ethanol sterilised knife. All samples were stored and transported in a cooler box at 4ºC until analysis within 24h.

**Sample processing and enumeration for indicator (total coliforms/*E. coli*) bacteria**

Each of the 100mL water samples was used to enumerate coliforms and *E. coli* using the MPN-based Colilert-18 system. The contents of one Colilert-18 pack (IDEXX Laboratories Incorporated, Westbrook, Maine, United States) were subsequently added to a 100mL water sample, which was then shaken to mix well. The entire contents of the sample/reagent mixture were poured into a Quanti-Tray, sealed, and incubated at 37ºC for 24h, and the results were interpreted according to the manufacturer's instructions. The total coliforms and *E. coli* counts were recorded as log MPN/100mL. For the 1L water samples, the samples were first filtered through nitrocellulose membranes (0.45µm pore size, Sartorius, Göttingen, Germany). The membrane was subsequently placed into 50mL buffered peptone water (BPW) and incubated for 24h at 37°C, and used for further selective enrichment of *E. coli* (non-O157 and O157) and *Salmonella* spp. The kidney dialysis filter membranes used to filter the 100L water samples were back-flushed with 2.5L Tween-80 dH2O, and the back-flushed liquid was filtered through 0.45µm nitrocellulose membranes, similar to the 1L water samples.

A 25g soil sample collected from each fresh-produce sample type was weighed and added to 225mL of BPW. To enumerate coliforms and *E. coli* in soil, a tenfold serial dilution of each BPW sample mixture was plated in duplicate onto *E. coli*/coliform count plates and incubated for 24h at 37ºC according to the manufacturer's instructions (3M Petrifilm, 3M, St. Paul, Minnesota, United States, ISO method 4832). The remaining soil/BPW mixtures were incubated at 37ºC for 24h to detect potential human pathogens.

A 50g composite sample of each respective leafy vegetable bunch was aseptically cut and subsequently placed into a pre-labelled sterile polyethylene-strainer stomacher bag containing 200mL of BPW, in a 1:4 weight to volume ratio (3M, Johannesburg, South Africa) (Richter et al., 2019). For other vegetable sample types (tomatoes, baby carrots, onions, and green peppers), 150g of each was placed into pre-labelled sterile polyethylene-strainer stomacher bags containing 150mL BPW, in a 1:1 weight-to-volume ratio (Richter et al., 2019). To enumerate coliforms and *E. coli* in fresh produce, a tenfold serial dilution of each BPW sample mixture (obtained from the stomacher bag) was plated in duplicate onto coliform/*E. coli* count plates and incubated for 24h at 37ºC according to the manufacturer's instructions (3M Petrifilm, 3M, St. Paul, Minnesota, United States, ISO method 4832). The remaining fresh produce/BPW mixtures were incubated at 37ºC for 24h to detect foodborne pathogens, as described for the soil samples.

### 3.2.2 Detection and isolation of presumptive positive potential human pathogenic bacteria

#### *Escherichia coli* isolation

Following incubation, each of the enriched water, soil, and fresh-produce samples was plated onto RAPID *E. coli* 2 agar plates (Bio-Rad, South Africa) using the streak-plate method, and incubated at 37ºC for 24h to isolate typical *E. coli* colonies. Additionally, 1mL of each sample was incubated in 9mL STEC selective enrichment broth (SEB) (Bio-Rad, South Africa) for the detection of Shiga-toxin *E. coli* O157:H7 and non-O157:H7 *E. coli* and incubated at 37ºC for 24h.

Following enrichment, the samples were streaked onto chromID O157:H7 agar to detect typical O157:H7 colonies using the streak plate method.

#### *Salmonella* spp. Isolation

Of each BPW-enriched sample, 1mL was further incubated in 9mL Rappaport-Vassiliadis (RV) *Salmonella* enrichment broth (Merck, Darmstadt, Germany) at 42ºC for 24h. Subsequently, the presence of *Salmonella* spp. was assessed using the iQ-Check *Salmonella* II kit (BioRad, Johannesburg, South Africa) according to the manufacturer's instructions. The positive results were then plated onto Xylose lysine deoxycholate (XLD) agar plates (Biolabs, Johannesburg, South Africa) and incubated for 24h at 37ºC. The presence of typical black colonies was then visualised, and the identities confirmed using MALDI-TOF MS.

**Isolation of extended-spectrum β-lactamase (ESBL), AmpC β-lactamase-producing Enterobacterales**

Each of the prepared BPW samples (water, soil, and fresh produce) was incubated for 3-4h at 37ºC, after which 1mL was added to 9mL Enterobacteriaceae selective enrichment (EE) broth (Oxoid, Basingstoke, United Kingdom) and incubated for 24h at 30°C (SANS 21528-1:2004). Following selective enrichment, all samples were streaked onto selective chromogenic chromID ESBL agar plates (bioMérieux, Midrand, South Africa) and incubated for 18–24h at 30℃ to isolate presumptive ESBL-producing Enterobacterales.

**Confirmation of ESBL/AmpC production of isolates obtained from the selective media**

All presumptive ESBL-producing isolates were screened for ESBL production by the double-disk synergy test (DDST) using cefotaxime-30µg, ceftazidime-30µg, and cefpodoxime-10µg, alone or in combination with clavulanic acid-10µg (Mast Diagnostics, Bootle, United Kingdom and Oxoid, Basingstoke, United Kingdom) [European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2024]. To determine isolate resistance, zone diameters were measured and compared with EUCAST criteria. Isolates showing resistance to cefoxitin and cefotaxime or ceftazidime were regarded as a phenotypic indicator of AmpC production (EUCAST, 2018). The cefepime ESBL disc set (Cefepime-30µg, cefepime-clavulanic acid-30µg–10µg) and the AmpC detection set (Mast Diagnostics and Oxoid) were used to confirm ESBL and AmpC production, respectively (EUCAST, 2013; CLSI, 2018). According to the manufacturers' instructions, *K. quasipneumoniae* ATCC 700603, *E. coli* NCTC 13351, and *Enterobacter cloacae* NCTC 1406 were used as positive controls, and *E. coli* ATCC 25922 was included as a negative control (Mast Diagnostics).

### 3.2.3 Additional antimicrobial susceptibility testing

Isolates from the water, soil, and fresh-produce samples were phenotypically characterised using the Kirby-Bauer disk diffusion technique (Clinical Laboratory Standard Institute [CLSI], 2020). The isolates were cultured in 9mL Tryptone Soy Broth (TSB) and incubated for 24h at 37ºC. From each TSB sample, 100µl was subsequently cultured in 9mL of Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) and incubated at 37ºC for 24h, and the inoculum was adjusted to the turbidity of the 0.5 McFarland Standard (OD625 0.08–0.12) corresponding to an inoculum containing 1–2 × $10^8$ CFU/mL. A 120µl aliquot was then plated onto Mueller-Hinton (MH) agar plates (Thermo Fisher Scientific, Johannesburg, South Africa) and incubated at 37ºC for 24h, after which the respective zone diameters were measured. The zone diameters were then compared with the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria to determine whether each isolate was resistant, intermediately resistant, or susceptible to each antibiotic (CLSI, 2020; EUCAST, 2024). Additionally, the results were interpreted using BioNumerics 7.6 (Applied Maths, Saint-Martin-Latem, Belgium) and the BioNumerics software program (Applied Mathematics, Johannesburg, South Africa). Isolates resistant to three or more antibiotic classes were defined as MDR. The antibiotics that were tested for resistance or susceptibility of each respective

isolate included gentamicin (10µg), streptomycin (10µg), aztreonam (30µg), chloramphenicol (30µg), ampicillin (10µg), amoxicillin (10µg), imipenem (10µg), meropenem (10µg), norfloxacin (10µg), ciprofloxacin (10µg), tigecycline (15µg), azithromycin (15µg), erythromycin (15µg), nalidixic acid (30µg), trimethoprim-sulfamethoxazole/cotrimoxazole (1,25µg/ 23,75µg), tetracycline (30µg), cefoxitin (30µg), cefpodoxime (30µg), cefotaxime (30µg), ceftazidime (30µg) and cefepime (30µg).

**Molecular screening of *Escherichia coli* O157:h7 and non-O157:h7 isolates**

The presence of enterohaemorrhagic *E. coli* (EHEC) (*eaeA*, *stx1,* and *stx2* genes) virulence genes was analysed by PCR for all isolates, with the *mdh* gene used as an internal control in all reactions (Omar and Barnard, 2010a). Control strains for the PCR reactions included *E. coli* O157:H7 ATCC 43890 (EHEC) as the positive control, and *E. coli* ATCC 25922 as the negative control. Single colonies of each *E. coli* isolate were cultured in 9mL TSB (Merck, Darmstadt, Germany) for 24h at 37ºC. The cells were pelleted by centrifugation (12 500g for 3min), the DNA extracted using the Quick-gDNA mini-prep kit according to the manufacturer's instructions (Zymo Research, Irvine, United States), and the concentration was determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg, South Africa). PCR was performed using a 2 × DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Johannesburg, South Africa) with specific primers and the thermocycling conditions for each gene (Table 2). All reactions were run on a Bio-Rad T100 thermal cycler (Bio-Rad) and the amplicons visualised on a 2% agarose gel stained with Conda®-Safe (Carl Roth GmbH and Co, Karlsruhe, Germany) and analysed using a Bio-Rad GelDoc XR in conjunction with the Image LabTM software (BioRad).

**Genomic fingerprinting of *Escherichia coli* isolates by repetitive PCR**

The same *E. coli* isolates analysed for antimicrobial susceptibility were used to conduct repetitive PCR through the generation of enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprints for each of the isolates from the six individual smallholder farms. The primer sequences and thermocycling conditions used to generate the ERIC-PCR fingerprints are summarised in Table 2. Following the visualisation of the PCR amplicons on a 2% agarose gel, the band patterns were analysed digitally using BioNumerics 7.6 (Applied Maths, Saint-Martin-Latem, Belgium).

**Genomic DNA extraction, whole genome sequencing, and analysis**

The genomic DNA of 19 selected isolates from Farms A–F (10 Enterobacterales and nine *Pseudomonas aeruginosa* isolates) was extracted using the DNeasy PowerSoil kit (Qiagen, South Africa) according to the manufacturer's instructions (Table 3). After gDNA extraction, the concentration and quality of the extracted DNA were determined using the Qubit dsDNA Broad Range Assay (Life Technologies, Johannesburg, South Africa) and NanoDrop 2000 (Thermo Scientific, Johannesburg, South Africa).

**Whole genome sequencing and analysis**

Multiplexed paired-end libraries (2 × 300bp) were generated utilising the Nextera XT DNA sample preparation kit (Illumina, San Diego, California, United States). Sequencing was conducted on an Illumina MiSeq platform with a coverage depth of 100x at the National Institute of Communicable Diseases (NICD) Sequencing Core Facility in South Africa. The initial raw reads underwent quality assessment, trimming, and *de novo* assembly into reads/contigs utilising CLC Genomics Workbench v10 (CLC, Bio-QIAGEN, Aarhus, Denmark). Subsequently, the *de novo* assembled reads/contigs were submitted to GenBank for annotation using the NCBI prokaryotic genome annotation pipeline server (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Genotypic characterisation followed by identifying antibiotic resistance genes (ARGs), multi-locus sequence types (MLST), pathogen likelihood, and mobile genetic elements (MGEs), by searching the ResFinder v4.6.0 (Borolaia et al., 2020; Camacho et al., 2009), MLST v2.0.9, PathogenFinder v1.1 (Camacho et al., 2009), and MGE v1.0.3 (Johansson et al., 2020) databases at the Centre for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/), respectively. Default parameters were considered for all of the software used unless otherwise indicated. Additionally, ABRicate Version 1.0.1 (https://github.com/tseemann/abricate) (Seenan, 2016) was used to assess AMR gene presence. Enterobacterales and *P. aeruginosa* virulence factors and serotypes were identified using VirulenceFinder v2.0 (Joensen et al., 2014; Malberg et al., 2020) and SeroTypeFinder v2.0 (Joensen et al., 2014) on the CGE platforms. Additionally, *Klebsiella pneumoniae* isolates were serotyped based on the capsule polysaccharides (K-type) using Kaptive Web (Wick et al., 2018).

<< Development of fit-for-purpose guidelines for managing microbiological water quality risks>>

**Table 2:** Primer information and thermocycling conditions used for screening of *Escherichia coli* O157:H7, non-O157, and DNA fingerprint generation using ERIC-PCR.

| Organism | Target genes | Primer sequences (5'–3') | Thermocycling conditions | Expected amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| | *eaeA* | F: CTG AAC GGC GAT TAC GCG AA | | 917 | |
| | | R: GAC GAT ACG ATC CAG | | | |
| | *stx1* | F: ACA CTG GAT GAT CTC AGT GG | | 614 | |
| Enterohaemorrhagic *E. coli* (EHEC) O157:H7 | | R: CTG AAT CCC CCT CCA TTA TG | 95°C, 15min; 35 cycles of (94°C, 45s; 56°C, 45s; 68°C; 2min), 72ºC, 5min; hold 4ºC | | Omar and Barnard 2010 |
| | | F: CCA TGA CAA CGG ACA GCA GTT | | | |
| | *stx2* | | | 779 | |
| | | R: CCT GTC AAC TGA GCA CTT TG | | | |

| | | | | | |
|---|---|---|---|---|---|
| | *mdh* | F: GGT ATG GAT CGT TCC GAC CT | | 304 | |
| | | R: GGC AGA ATG GTA ACA CCA GAG T | | | |
| *Escherichia coli* | ERIC | F: ATG TAA GCT CCT GGG GAT TCAC | 95ºC for 4min, 30 cycles (94ºC 30s, 40ºC 1min, 72ºC 8min), hold 72ºC for 15min, 4ºC hold | DNA fingerprint | Soni et al., 2014 |
| | | R: AAG TAA GTG ACT GGG TGA GCG | | | |

**Table 3:** Isolates selected for whole genome sequence analysis from the six smallholder farm environments in South Africa.

| Strain (*UPMP) | Organism identity | Source | Farm | System/scenario |
|---|---|---|---|---|
| *2457 | *Serratia marcescens* | Borehole water | B | Integrated |
| *2464 | *Pseudomonas aeruginosa* | Borehole water | C | Organic |
| *2388 | *Escherichia coli* | River water | D | Conventional or river |
| *2468 | *Escherichia coli* | Baby carrots | D | Conventional or river |
| *2474 | *Enterobacter cloacae* | Leeks | D | Conventional or river |
| *2456 | *Salmonella enterica.* | Baby carrots | D | Conventional or river |
| *2469 | *Pseudomonas aeruginosa* | Baby carrots | D | Conventional or river |
| *2470 | *Pseudomonas aeruginosa* | Spring onions | D | Conventional or river |
| *2472 | *Pseudomonas aeruginosa* | Leeks | D | Conventional or river |
| *2475 | *Pseudomonas aeruginosa* | Rocket | D | Conventional or river |
| *2476 | *Pseudomonas aeruginosa* | Rocket | D | Conventional or river |
| *2478 | *Pseudomonas aeruginosa* | Soil | D | Conventional or river |
| *2488 | *Pseudomonas aeruginosa* | Irrigation water | E | Conventional or dam |
| *2439 | *Escherichia coli* | Soil | F | Conventional or municipal |
| *2492 | *Enterobacter cloacae* | Soil | F | Conventional or municipal |
| *2496 | *Klebsiella pneumoniae* | Spinach | F | Conventional or municipal |
| *2497 | *Klebsiella pneumoniae* | Bell pepper | F | Conventional or municipal |
| *2499 | *Klebsiella pneumoniae* | Onions | F | Conventional or municipal |
| *2502 | *Pseudomonas aeruginosa* | Onions | F | Conventional or municipal |
| *2505 | *Pseudomonas aeruginosa* | Municipal water | F | Conventional or municipal |

### 3.2.4 Data availability

The nucleotide sequences of the selected Enterobacterales and *P. aeruginosa* strains included in this study were deposited in the National Center for Biotechnology Information (NCBI) Genbank Database in Bioproject number: PRJNA642017.

**Confirmation of isolate identities using MALDI-TOF MS**

All presumptive positive *E. coli, Salmonella* spp., and ESBL-producing Enterobacterales colonies from Farms A–F were isolated and purified, after which the isolates were identified

using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Bremen, Germany). MALDI-TOF MS identifies isolates to the species level (Standing et al., 2013) (AOAC-OMA#2017.09) as described by the manufacturer. Briefly, the purified cultures were aseptically transferred to a MALDI-TOF target plate, then overlaid with α-cyano-4-hydroxycinnamic acid matrix (Bruker) and allowed to air-dry. The target plate was then analysed using MicroFlex LT MALDI-TOF (Bruker, Bremen, Germany) in conjunction with the Biotyper automation software and library (Bruker), following calibration with a bacterial standard according to the manufacturer's instructions (Bruker). An organism's identity is considered reliable for identification at the species level, with a score value ranging between 2.30 and 3.00, whilst the best organism match value between 2.00 and 2.29 was considered reliable at the genus level. All isolates with confirmed identities were preserved in 65% glycerol at -20ºC for future use.

**Physicochemical analysis of water samples**

At each water sampling point of Farms A–F, an additional 1L water sample was collected and sent to the CSIR Environmental Laboratory (Pretoria, South Africa) for analysis of biochemical oxygen demand (BOD), chemical oxygen demand (COD), pH, total suspended solids (TSS), and turbidity.

### 3.2.5 Statistical analysis

The total coliform and *E. coli* counts were analysed using SAS v9.3 (SAS/STAT User's Guide, 1999) to determine significant differences between counts across sample types. The Shapiro-Wilk test was performed on the standardised residuals to test for deviations from normality (Shapiro and Wilk, 1965). Student's protected t-LSD (least significant difference) was calculated at a 5% significance level to compare means of significant source effects (Snedecor and Cochran, 1980). The boxplots representing the coliform and *E. coli* counts in Section 3.3.2 below were generated using GraphPad Prism (GraphPad Software, California, United States).

## 3.3 Results

### 3.3.1 Physicochemical analyses of water samples

The overall physicochemical values for the source, storage, and irrigation water samples collected along the irrigation system for Farms A–F are shown in Table 4, and the Department of Water Affairs (DWAF) recommended guidelines for each test. The physicochemical values displayed are measures of the BOD, COD, pH, total dissolved solids TDS, TSS, and turbidity levels in each sample. The river source water sample (Farm D) had a BOD level of 144mg/L, whereas the holding dam and overhead irrigation water samples had levels of 10mg/L (Table 4). The river water exceeded the recommended standard, as suggested by DWAF for irrigation water (Table 4) (DWAF, 1996), and was the highest level amongst all the water samples collected. Similarly to the BOD levels, the COD level in the river water was the highest among the water samples from all the farms in the study. None of the water samples from Farms A–

F exceeded the recommended guidelines (Table 4) (DWAF, 1996). The holding dam and irrigation point water samples collected from Farm E had pH values of 9.7 and 8.3 (Table 4), with the holding dam water having the highest pH level as well as exceeding DWAF regulations (pH=6–9) (Table 4) (DWAF, 1996). The irrigation point sample on Farm D was the only water sample to exceed the recommended standards (Table 4) (DWAF, 1996) and had the highest TSS level amongst all the water samples. The turbidity levels of the entire irrigation system on Farm D exceeded the recommended standards (Table 4) (DWAF, 1996), with the irrigation water point samples recording the highest levels among the other water samples.



**Table 4:** Physicochemical analyses of water samples from various stallholder farms around South Africa, as well as the guidelines for irrigation water recommended by the Department of Water Affairs and Forestry (DWAF).

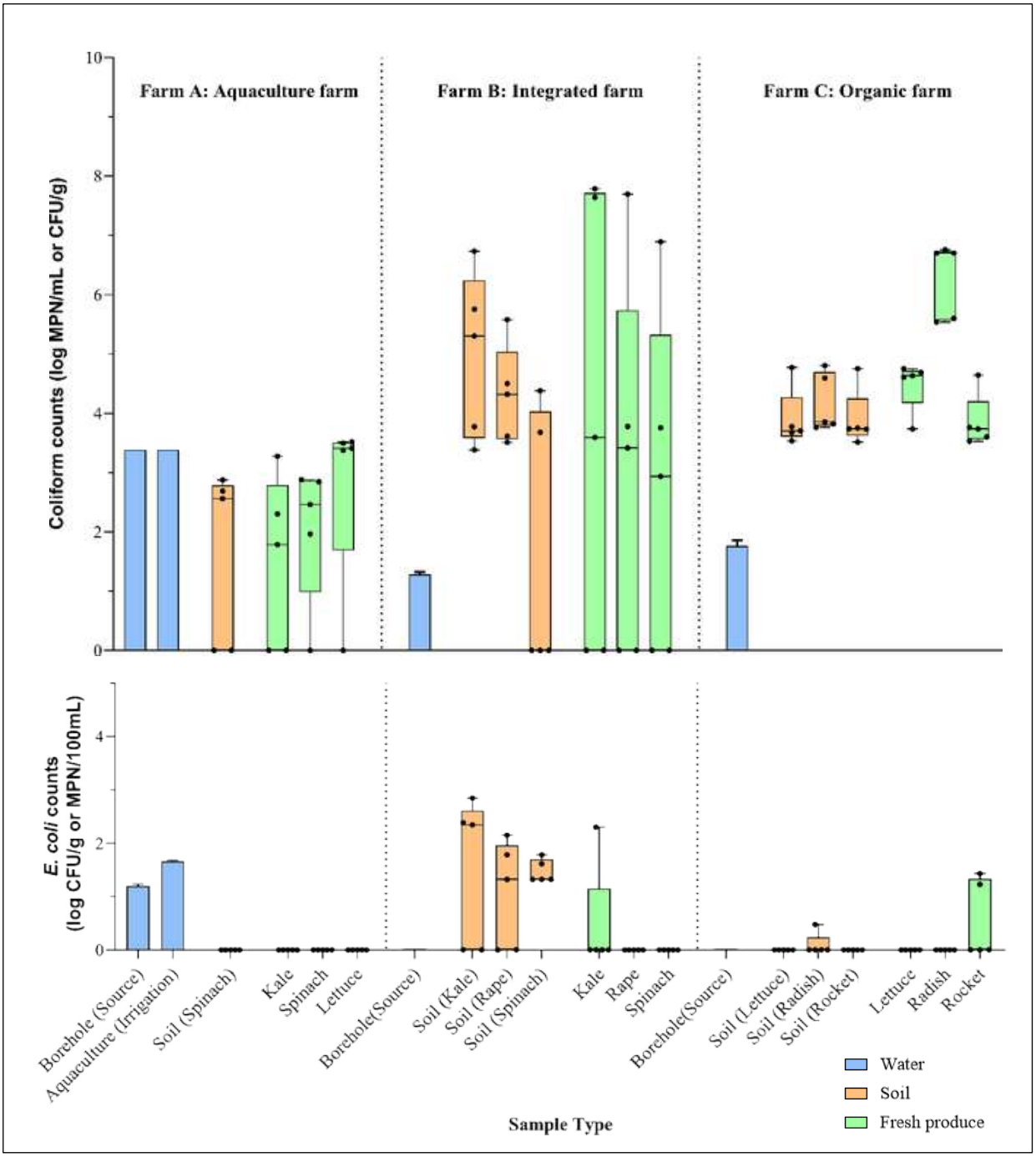
| **Reference – recommended guidelines** | | | **BOD (mg/L)** | **COD (mg/L)** | **pH** | **TDS (mg/L)** | **TSS (mg/L)** | **Turbidity (NTU)** |
|---|---|---|---|---|---|---|---|---|
| **Department of Water Affairs and Forestry (DWAF), 1996** | | | <30 | <250 | 6-9 | <450 | <37.4 | <1 (not >5) |
| **Farm** | | **Sample type** | **BOD (mg/L)** | **COD (mg/L)** | **pH** | **TDS (mg/L)** | **TSS (mg/L)** | **Turbidity (NTU)** |
| **Scenario** | **Number** | | | | | | | |
| Aquaculture | A | Aquaculture | 14 | 125 | 7,21 | 535 | 15 | 6,6 |
| Integrated | B | Borehole source | 10 | 10 | 8,33 | 220 | 0,3 | 0,25 |
| Organic | C | Borehole source | 10 | 16 | 6,76 | 37 | 0,3 | 0,5 |
| | | River source | 144 | 206 | 7,2 | 178 | 24 | 11 |
| Conventional or river | D | Holding dam | 10 | 10 | 8,1 | 176 | 9,3 | 15 |
| | | Overhead irrigation point | 10 | 10 | 7,9 | 186 | 185 | 38 |
| Conventional or dam | E | Holding dam | 10 | 20 | 9,7 | 46 | 3,3 | 2 |
| | | Overhead irrigation point | 10 | 12 | 8,3 | 70 | 5,3 | 17 |
| | | Municipal source | 10 | 12 | 7,8 | 196 | 1 | 0,3 |
| Conventional or municipal | F | Storage tank | 10 | 16 | 7,8 | 168 | 1 | 0,3 |
| | | Drip irrigation point | 10 | 12 | 7,8 | 168 | 1 | 0,3 |
| *BOD – Biological oxygen demand, *COD – Chemical oxygen demand, *TDS – Total dissolved solids, *TSS – Total suspended solids | | | | | | | | |

### 3.3.2 Microbiological quality analyses

The coliform and *E. coli* counts for the water, soil, and fresh-produce samples for Farms A–F are shown in Figures 7 and 8.

No significant difference (p=0.1702) was seen between the coliform counts for the water, soil, and fresh-produce samples on Farm A (Figure 7). Notably, all the coliform counts for the water

samples were the same at 3.38 log MPN/100mL. Coliform counts for soil samples ranged from <limit of detection (LOD) – 2.88 log CFU/g, and for fresh-produce samples from <LOD – 3.52 log CFU/g (Figure 7). *Escherichia coli* counts for the water samples on Farm A ranged between 1.16 and 1.69 log MPN/100mL (Figure 7), with the aquaculture water significantly higher than the borehole source water ($p<0.001$). *Escherichia coli* was not enumerated from any of the soil or fresh-produce samples (Figure 7). The coliform counts for the borehole water from Farm B ranged from 1.24–1.31 log MPN/100mL (Figure 7). The leafy vegetable coliform counts ranged from <LOD – 7.90 log CFU/g, with the soil counts between <LOD and 6.76 log CFU/g, respectively (Figure 7). No significant differences ($p=0.2945$) were observed in any of the coliform counts between all sample types on Farm B, with no *E. coli* enumerated from the borehole water on Farm B (Figure 7). The *E. coli* counts for the soil samples ranged from <LOD – 2.85 log CFU/g, with soil collected during kale harvesting (±2.52 log CFU/g) significantly higher ($p=0.0097$) than the other soil samples that ranged from 0.6–1.57 log CFU/g (Figure 7). This can be attributed to the uneven distribution of untreated animal manure observed in the fields. Additionally, *E. coli* was enumerated in only one fresh produce sample: a kale sample, at 2.3 log CFU/g (Figure 7). The coliform counts for the borehole water from Farm C ranged between 1.67 and 1.86 log MPN/100mL (Figure 7). Coliform counts for the soil samples, collected during lettuce and rocket harvesting, ranged from 3.51–4.8 log CFU/g, with significant differences ($p<0.0001$) observed for the soil collected during radish harvesting, ranging from 3.76–4.8 log CFU/g (Figure 7). Moreover, coliform counts were significantly higher ($p<0.0001$) for the radish samples (5.54–6.76 log CFU/g) when compared to the other leafy fresh produce (3.53–4.75 log CFU/g) (Figure 7). *Escherichia coli* were only enumerated from the rocket and the associated soil samples on Farm C (<LOD – 1.43 log CFU/g) (Figure 7). The higher detection of coliforms in fresh-produce samples compared to soil can be attributed to the higher natural occurrence of Enterobacterales isolates on leafy fresh produce (Blaak et al., 2014; Richter et al., 2021).

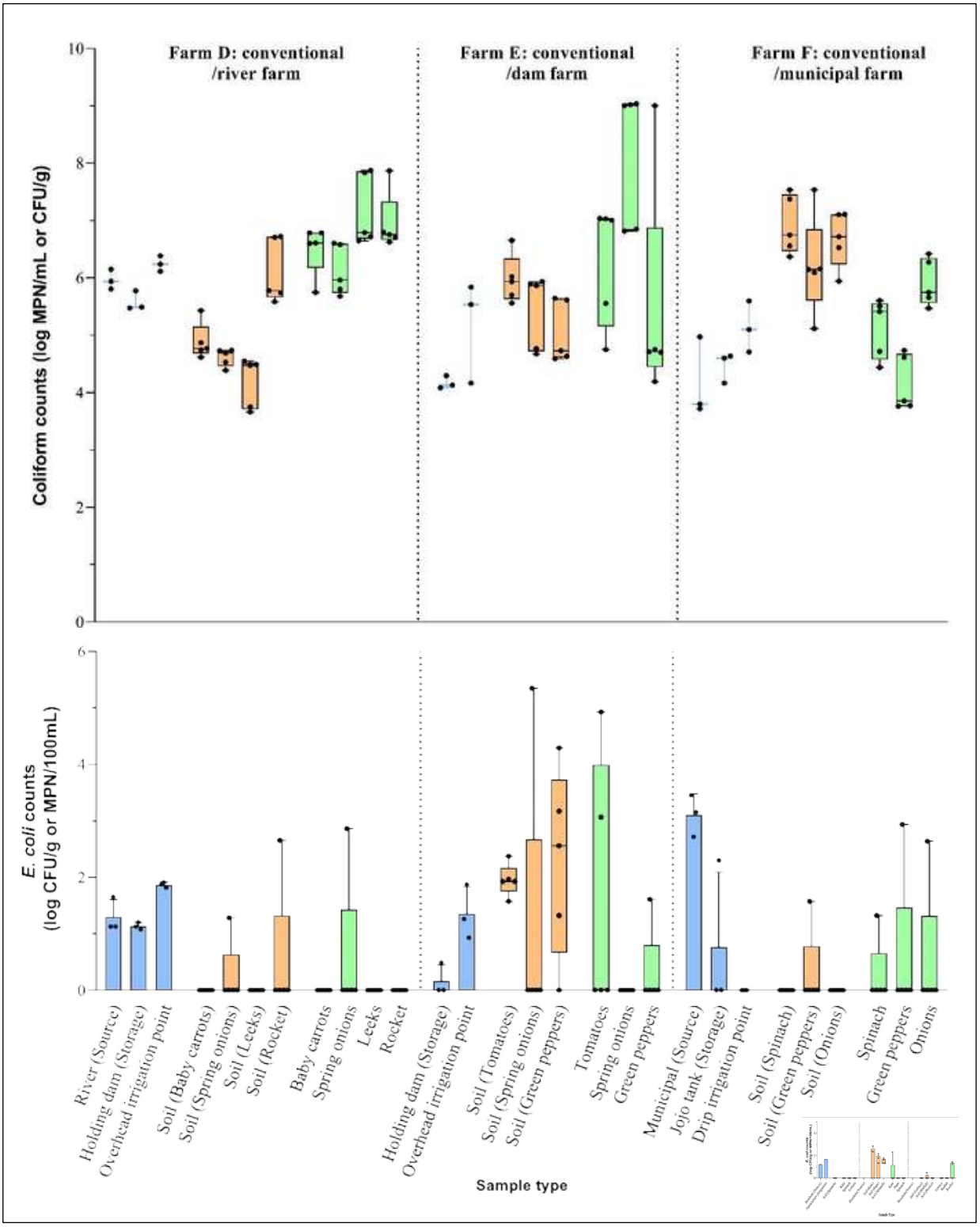



CFU – colony-forming unit; MPN – most probable number. Water samples are represented in blue, soil samples in orange and fresh produce in green.

**Figure 7:** Indicator bacteria levels for water, soil, and fresh-produce samples collected from three smallholder farms (aquaculture, integrated and organic farms), which used borehole water as an irrigation source, as well as manure or compost as a fertiliser for fresh-produce production. Water counts were measured as log MPN/100mL, with soil and fresh-produce counts as log CFU/g.

The coliform counts for the water samples on Farm D ranged from 5.47–6.38 log MPN/100mL, with the irrigation point samples (±6.24 log MPN/100mL) significantly higher ($p<0.0001$) than the rest of the irrigation system samples (±5.8 log MPN/100mL) (Figure 8). The coliform counts

for the soil samples ranged from 4.19–6.11 log CFU/g (Figure 8), with fresh-produce counts ranging between 5.68 and 7.87 log CFU/g (Figure 8). *Escherichia coli* counts in the water samples ranged from 1.08–1.91 log MPN/100mL, with the irrigation point samples (±1.9 log MPN/100mL) significantly higher (p=0.0007) than the other water samples (±1.2 log MPN/100mL). *Escherichia coli* was enumerated from soil samples with levels from <LOD – 2.86 log CFU/g, with counts only observed for spring onion samples between <LOD and 2.86 log CFU/g (Figure 8). Farm E coliform levels from 4.08–5.83 log MPN/100mL were enumerated for the water samples with the irrigation point levels (±5.2 log MPN/100mL), significantly higher (p<0.0001) than the holding dam water levels (±4.2 log MPN/100mL) (Figure 8). The coliform levels for the soil samples ranged from 4.59–6.65 log CFU/g, with the fresh-produce levels from 4.19–9.04 log CFU/g. Coliforms enumerated from spring onions (±8.2 log CFU/g) were significantly higher (p<0.0001) than those from tomatoes and green peppers (±5.9 log CFU/g) (Figure 8). *Escherichia coli* counts for the irrigation water (0.93 – 1.87 log MPN/100mL), on Farm E, were significantly higher (p<0.0001), compared to the holding dam water samples between <LOD and 0.49 log MPN/100mL (Figure 8). The *E. coli* counts for the soil samples ranged from <LOD – 5.35 log CFU/g, with fresh-produce levels from <LOD – 4.93 log CFU/g. *Escherichia coli* levels from tomatoes (±1.02 log CFU/g) were significantly higher (p<0.0001) than those from spring onions and green peppers (±0.3 log CFU/g) (Figure 8). Coliform counts for the irrigation water samples (4.0–5.6 log MPN/100mL) on Farm F were significantly higher (p<0.0001), compared to the rest of the water coliform counts between 3.72 and 4.97 log MPN/100mL (Figure 8). Coliform counts for the soil samples ranged from 5.11–7.54 log CFU/g, with counts for fresh produce from 3.76–6.41 log CFU/g (Figure 8). *Escherichia coli* counts were significantly higher (p=0.0009) in municipal water samples (2.72–3.45 log MPN/100mL) than in storage and irrigation point counts (<LOD and 2.23 log MPN/100mL, respectively; Figure 8). *Escherichia coli* was also enumerated in soil samples, ranging from <LOD – 1.57 log CFU/g, and fresh-produce samples from <LOD – 2.94 log CFU/g (Figure 8). *Escherichia coli* enumerated from green pepper samples (±0.98 log CFU/g) were significantly higher (p=0.0009) compared to the onion and spinach samples (±0.9 log CFU/g) (Figure 8).




CFU – colony-forming unit; MPN – most probable number. Water samples are represented in blue, soil samples in orange and fresh produce in green.

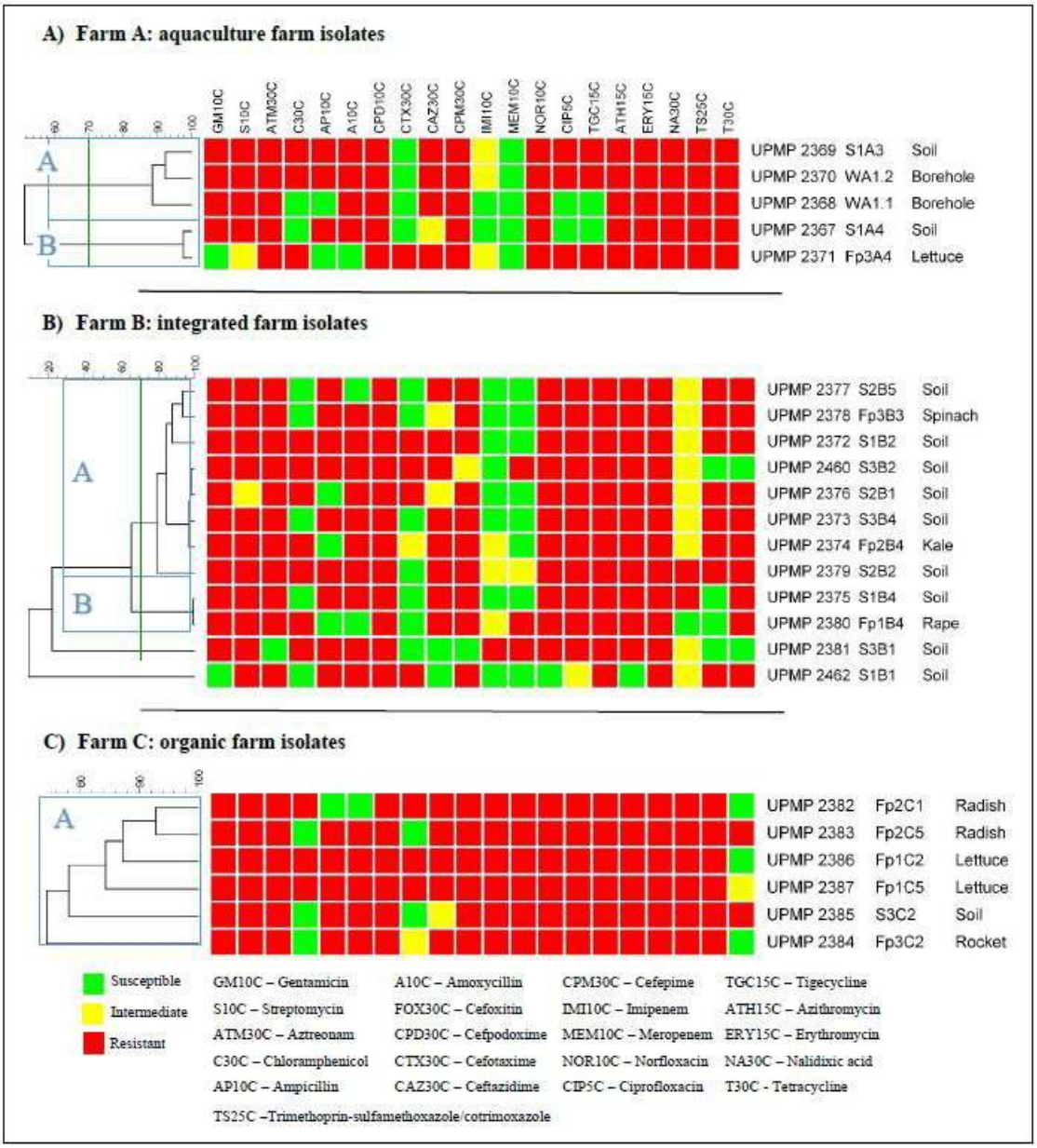
**Figure 8:** Indicator bacteria levels for water, soil, and fresh-produce samples collected from three smallholder farms (all conventional) that used either river (d), dam (e) or municipal (f) water as an irrigation source for fresh-produce production. Water counts were measured as log MPN/100mL, and soil and fresh-produce counts as log CFU/g.

### 3.3.3 Detection of potential foodborne pathogens

In total, 121/295 samples (41,02%) harboured *E. coli* after enrichment, with the most isolates obtained from water (44/85; 53%), followed by soil (43/100; 43%) and then fresh-produce (33/110; 30%) samples. A total of 56 *E. coli* isolates were identified as presumptive Shiga-toxin-producing *E. coli* (STEC) after enrichment. However, none of the isolates from Farms A–F harboured any of the STEC virulence genes, *stx1*, *stx2* and *eaeA*, after an end-point PCR was performed. Of all the samples (n=295), only one *Salmonella* spp. isolate was obtained from a baby carrot sample from Farm D.

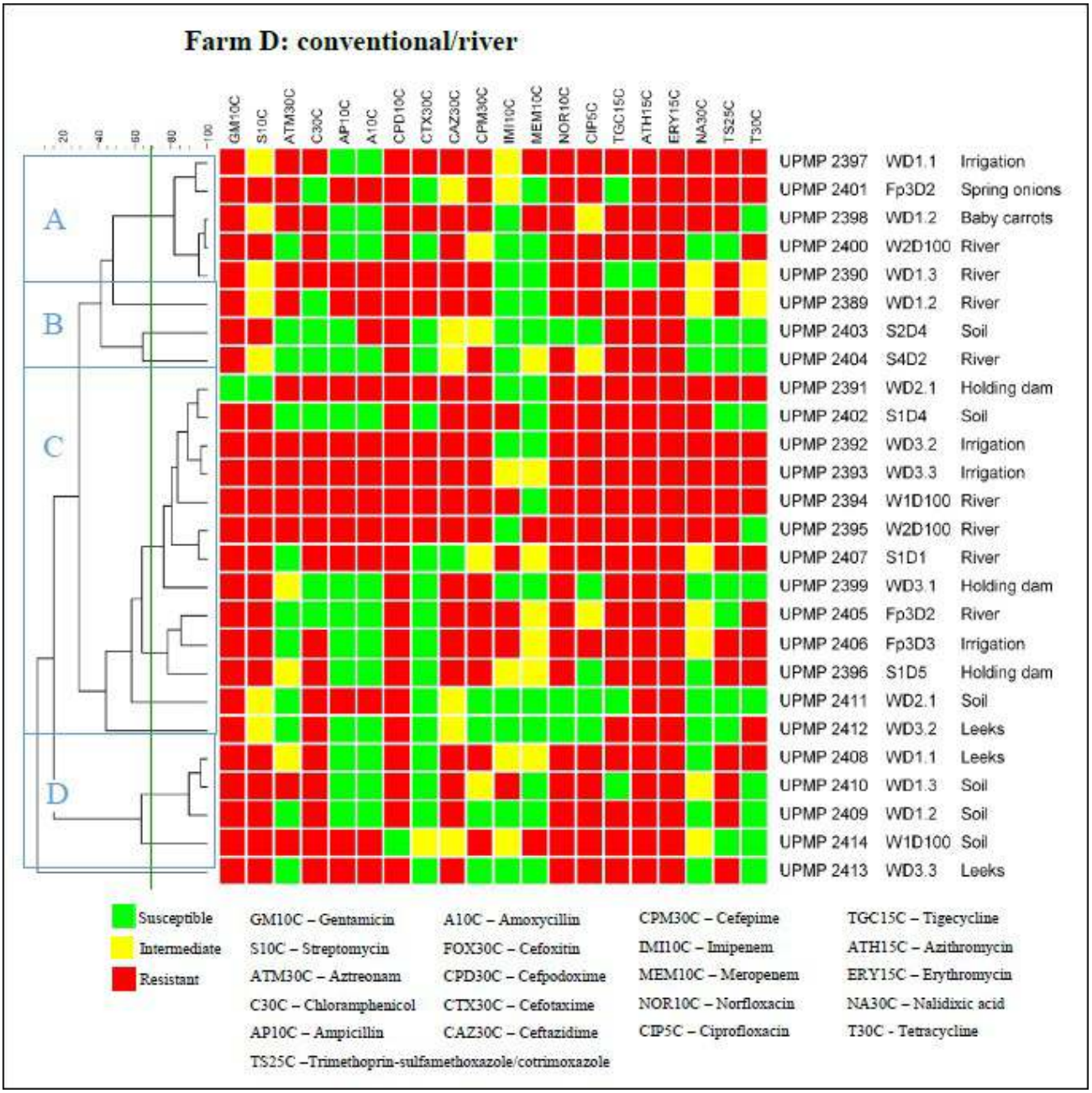
**Results of antimicrobial resistance testing of *Escherichia coli***

The AMR profiles of each *Escherichia coli* isolate, and the source (water, fresh produce, or soil) of the six farms are presented in Figure 9 (Farms A, B, and C), Figure 10 (Farm D) and Figure 11 (Farms E and F). The number of antimicrobials, resistance patterns, and types of antibiotic classes to which generic *Escherichia coli* isolates from various smallholder farms are summarised in Table 5. Of the 90 *E. coli* isolates obtained, all were categorised as MDR with resistance to three or more antibiotic classes. Within the β-lactam class of antibiotics, *E. coli* (n=90) isolates showed the highest resistance to 3rd-generation cephalosporins (98%), followed by 4th-generation cephalosporins (92%), penicillins (83%), and carbapenems (71%). The *E. coli* isolates from the soil samples showed resistance to 17/21 antimicrobials, including antimicrobials from the aminoglycoside, chloramphenicol, β-lactam, fluoroquinolone, glycylcycline, macrolide, sulfonamide, and tetracycline classes. In contrast, isolates recovered from water samples were resistant to fewer antimicrobials (4/21) than those from soil samples. The MDR patterns of all *E. coli* isolates predominantly included resistance to β-lactams combined with aminoglycosides and glycylcyclines (100% of the isolates), followed by β-lactams combined with macrolides and fluoroquinolones (91% of the isolates).



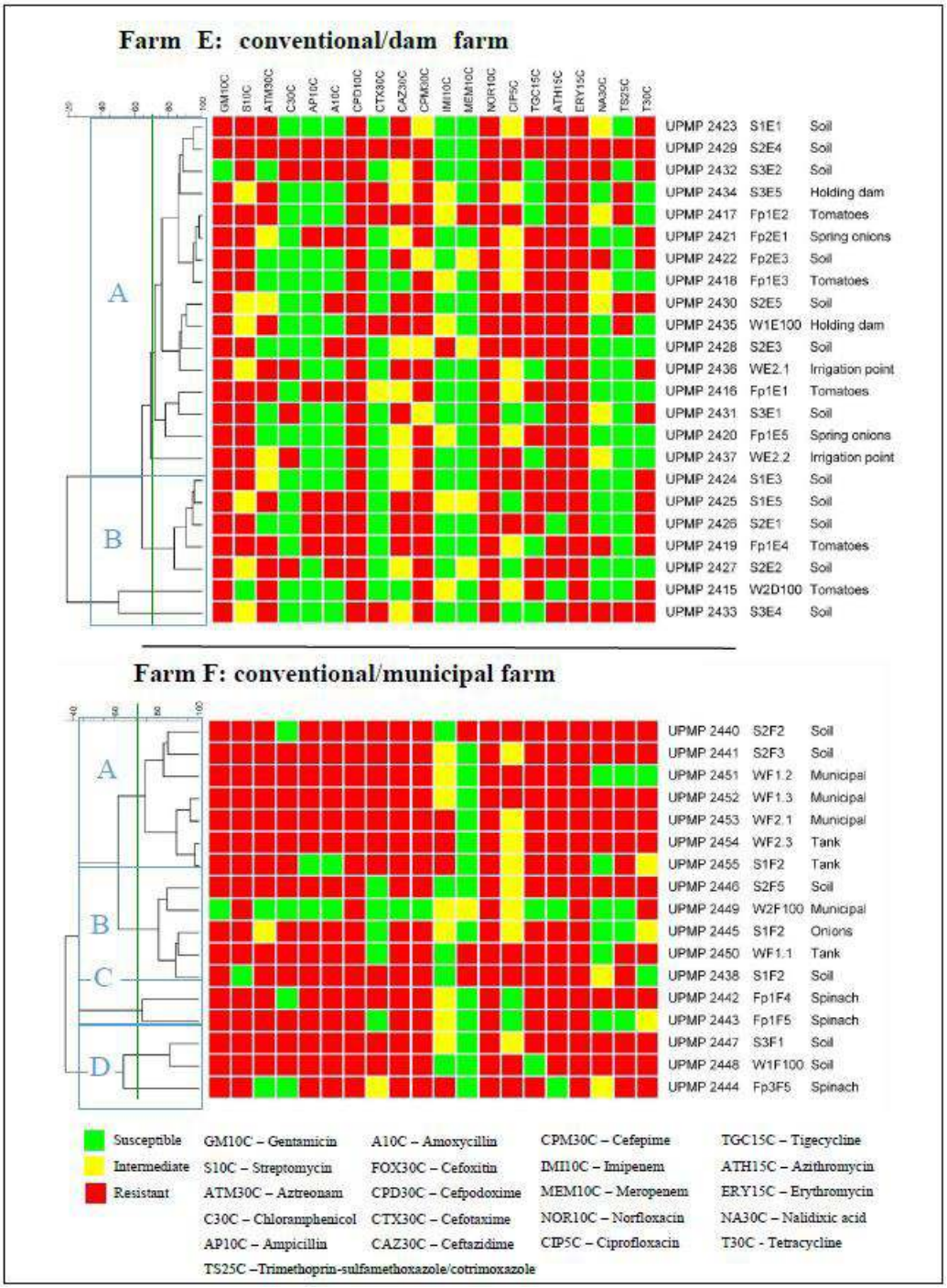

The 70% similarity cut-off is represented by a green vertical line. Green blocks represent susceptible isolates, and yellow and red blocks represent intermediate- and resistant-isolates, respectively.

**Figure 9:** Genetic relatedness and antimicrobial resistance profiles between *Escherichia coli* isolates recovered from the water-soil-plant nexus from (A) the aquaculture farm, (B) the integrated farm and (C) the organic farm. A 70% similarity cut-off was used to determine isolate clusters.




The 70% similarity cut-off is represented by a green vertical line. Green blocks represent susceptible isolates, and yellow and red blocks represent intermediate- and resistant-isolates, respectively.

**Figure 10:** Genetic relatedness and antimicrobial resistance profiles between *Escherichia coli* isolates recovered from the water-soil-plant nexus from (D) the conventional/river farm (Farm D). A 70% similarity cut-off was used to determine clusters of isolates.




The 70% similarity cut-off is represented by a green vertical line. Green blocks represent susceptible isolates, and yellow and red blocks represent intermediate- and resistant-isolates, respectively.

**Figure 11:** Genetic relatedness and antimicrobial resistance profiles between *Escherichia coli* isolates recovered from the water-soil-plant nexus from (E) the conventional/dam farm and (F) the conventional/municipal farm. A 70% similarity cut-off was used to determine isolate clusters.

### 3.3.4 Genomic fingerprinting and phenotypic characterisation of *Escherichia coli* isolates from Farms A–F

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) cluster analysis for the aquaculture farm (Farm A) showed two distinct clusters at a 70% similarity cut-off (Figure 9, A and B). Cluster A contained isolates recovered from borehole water and soil, with >90% similarity among the isolates. Cluster B, with 98% similarity, contained two isolates: one from soil and the other from a lettuce sample (Figure 9, A and B). Similarly, ERIC-PCR cluster analysis for *E. coli* isolates recovered from the mixed farm (Farm B) showed two distinct clusters at a 70% similarity cut-off. Cluster A had *E. coli* isolates at 80% similarity from soil, spinach, and kale samples, while Cluster B had *E. coli* isolates from soil and rape samples at a near 100% similarity (Figure 9, A and B). Genomic fingerprinting of all six *E. coli* isolates from the organic farm (Farm C) revealed a cluster at 75% similarity, including isolates recovered from soil and fresh-produce samples (Figure 9A).

At a 70% similarity cut-off, DNA fingerprinting generated four distinct clusters for the isolates recovered from Farm D (Figure 10, A to D). Cluster A included isolates (n=5) from the river, irrigation point and leek samples at >95% similarity. Cluster B contained three isolates from the holding dam and soil, with>65% similarity (Figure 10, A to B). The largest cluster (Cluster C) comprised 13 isolates recovered from the entire water-soil-plant nexus, with>80% similarity among all isolates. Lastly, Cluster D (n=5) had isolates recovered from the water system (Figure 10, D).

The genetic fingerprinting of the *E. coli* isolates from Farm E had two distinct clusters at a 70% similarity cut-off (Figure 11, A and B). Cluster A, the largest (n=16), contained isolates recovered from the water-soil-plant nexus, with close clustering (>90% similarity) among water, soil, and fresh-produce samples. On the other hand, Cluster B (n=5) had isolates from soil and tomato samples with high similarity (>80%) (Figure 11, A and B). The ERIC-PCR analysis of the *E. coli* isolates recovered from Farm F showed four distinct clusters at a 70% similarity cut-off (Figure 11, A-D). The largest cluster (Cluster A) comprised seven isolates recovered from tank, soil, onion, and spinach samples, with>80% similarity. Similarly, Cluster B (n=5) had isolates from the water system and one spinach sample, whereas Clusters C and D had isolates from municipal, soil, and spinach samples at >70% similarity (Figure 11, A to D).

**Table 5:** Summary of the number of antimicrobials, resistance patterns, and type of antibiotic classes to which generic *Escherichia coli* isolates from smallholder farms (Farms A–F) were resistant.

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| 3 | 1 | 1 | ERY15C-T30C-CPD10C | 3 | Aminoglycosides, Tetracyclines, Cephalosporins II |
| 4 | 5 | 3 | S10C-TGC15C-ATH15C-ERY15C | 3 | Aminoglycosides, Glycylcyclines, Macrolides |
| | | 1 | TGC15C-ATH15C-ERY15C-CPD10C | 3 | Glycylcyclines, Macrolides, Cephalosporins II |
| | | 1 | ATM30C-TGC15C-ATH15C-ERY15C | 3 | Aminoglycosides, Glycylcyclines, Macrolides |
| 5 | 7 | 1 | S10C-TGC15C-ERY15C-NA30C-T30C | 5 | Aminoglycosides, Glycylcyclines, Macrolides, Quinolones, Tetracyclines |
| | | 1 | S10C-C30C-CIP5C-TGC15C-ERY15C | 5 | Aminoglycosides, Amphenicols, Fluoroquinolones, Glycylcyclines, Macrolides |
| | | 1 | S10C-A10C-TGC15C-ATH15C-ERY15C | 4 | Aminoglycosides, Beta-lactams, Glycylcyclines, Macrolides |
| | | 1 | C30C-TGC15C-ATH15C-ERY15C-T30C | 4 | Amphenicols, Glycylcyclines, Macrolides, Tetracyclines |
| | | 1 | S10C-TGC15C-ATH15C-ERY15C-T30C | 4 | Aminoglycosides, Glycylcyclines, Macrolides, Tetracyclines |
| | | 1 | AP10CA10CATH15C-ERY15C-CPD10C | 3 | Beta-lactams, Macrolides, Cephalosporins II |
| | | 1 | ATM30C-TGC15C-ERY15C-T30C-CTX30C | 5 | Aminoglycosides, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins III |
| 6 | 7 | 1 | S10C-C30C-ATH15C-ERY15C-T30C-FOX30C | 5 | Aminoglycosides, Amphenicols, Macrolides, Tetracyclines, Cephalosporins II |
| | | 1 | S10C-CIP5C-TGC15C-ERY15C-T30C-CTX30C | 6 | Aminoglycosides, Fluoroquinolones, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins III |
| | | 1 | S10C-TGC15C-ATH15C-ERY15C-T30C-CPD10C | 5 | Aminoglycosides, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins II |
| | | 1 | S10C-ATM30C-TGC15C-ATH15C-ERY15C-T30C | 4 | Aminoglycosides, Glycylcyclines, Macrolides, Tetracyclines |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | GM10C-ATM30C-ATH15C-ERY15C-T30C-CPD10C | 4 | Aminoglycosides, Macrolides, Tetracyclines, Cephalosporins II |
| | | 1 | S10C-ATM30C-IMI10C-CIP5C-ATH15C-ERY15C | 4 | Aminoglycosides, Carbapenems, Fluoroquinolones, Macrolides |
| | | 1 | S10C-IMI10C-MEM10C-TGC15C-ATH15C-ERY15C | 4 | Aminoglycosides, Carbapenems, Glycylcyclines, Macrolides |
| 7 | 8 | 1 | S10C-AP10C-A10C-TGC15CERY15C-T30C-ATH15C | 5 | Aminoglycosides, Beta-lactams, Glycylcyclines, Macrolides, Tetracyclines |
| | | 1 | S10C-TGC15C-ATH15C-ERY15C-TS25C-T30C-CAZ30C | 6 | Aminoglycosides, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins III |
| | | 1 | S10C-IMI10C-TGC15C-ATH15C-ERY15C-T30C-CTX30C | 6 | Aminoglycosides, Carbapenems, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins III |
| | | 1 | S10C-ATM30C-ATH15C-ERY15C-NA30C-T30C-CAZ30C | 5 | Aminoglycosides, Macrolides, Quinolones, Tetracyclines, Cephalosporins III |
| | | 1 | S10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C--CPD10C | 6 | Aminoglycosides, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins III |
| | | 1 | ATH15C-ERY15C-NA30C -T30C-FOX30C-CPD10C-CPM30C | 5 | Macrolides, Quinolones, Tetracyclines, Cephalosporins II, Cephalosporins IV |

1 ATM30C – TGC15C – ATH15C – ERY15C – T30C – CPD10C – CTX30C

1 ATM30C – ATH15C – ERY15C – TS25C – CPD10C – CTX30C – CPM30C

6 Aminoglycosides, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins II, Cephalosporins IV

6 Aminoglycosides, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| 8 | 5 | 1 | S10C-ATM30C-A10C-ATH15C-ERY15C-NA30C-TS25C-T30C | 6 | Aminoglycosides, Tetracyclines Beta-lactams, Macrolides, Quinolones, Sulfonamides, |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1<br>1 | S10C-ATM30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-CTX30C-ATM30C-ATH15C-ERY15C-NA30C | 5<br>7 | Aminoglycosides, Beta-lactams, Glycylcyclines, Macrolides, Cephalosporins III<br>Aminoglycosides, Macrolides, Quinolones, Sulfonamides, Tetracyclines, |
| | | 1 | TS25C-T30C-CPD10C-CTX30C ATM30C-TGC15C-ATH15C-ERY15C | 7 | Cephalosporins II, Cephalosporins III Aminoglycosides, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, |
| | | 1 | TS25C-T30C-CTX30C-CPM30C-TGC15C-ATH15C-ERY15C-TS25C-T30C-FOX30C-CPD10C-CTX30C | 6 | Cephalosporins III, Cephalosporins IV Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |
| 9 | 5 | 1<br>1 | S10C-C30C-IMI10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-T30C-S10C-NOR10C-CIP5C-TGC15C | 8<br>7 | Aminoglycosides, Amphenicol, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines<br>Aminoglycosids, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, |
| | | 1 | ATH15C-ERY15C-TS25C-T30C-CPD10C-S10C-IMI10C-CIP5C-TGC15C-ATH15C | 8 | Tetracyclines, Cephalosporins II Aminoglycosides, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, |
| | | 1 | ERY15C-NA30C-CPD10C-CTX30C S10C-ATM30C-MEM10C-TGC15C- | 6 | Quinolones, Cephalosporins II, Cephalosporins III Aminoglycosides, Carbapenems, Glycylcyclines, Macrolides, Sulfonamides, |
| | | 1 | ATH15C-ERY15C-TS25C-CTX30C-CAZ30C-ATM30C-TGC15C-ATH15C-ERY15C-TS25C-CPD10C-CTX30C-CAZ30C-CPM30C | 7 | Cephalosporins III Aminoglycosides, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 10 | 5 | 1<br>1 | S10C-AP10C-A10C-IMI10C-CIP5C-TGC15C-ERY15C-TS25C-T30C-CTX30C-S10C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-CPD10C- CAZ30C | 9<br>8 | Sulfonamides, Cephalosporins II, Cephalosporins III |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-CTX30C-CPM30C | 7 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Cephalosporins III, Cephalosporins IV |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | S10C-AP10C-A10C-TGC15C-ATH15C-ERY15C-FOX30C-CPD10C-CAZ30C-CPM30C | 7 | Aminoglycosides, Beta-lactams, Glycylcyclines, Macrolides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | ATM30C-C30C-MEM10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-CTX30C-CAZ30C | 8 | Aminoglycosides, Amphenicols, Carbapenems, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins III |
| 11 | 8 | 1 | ATM30C-C30C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CTX30C-CAZ30C | 9 | Aminoglycosides, Amphenicols, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins III |
| | | 1 | ATM30C-C30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-FOX30C-CTX30C | 9 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Cephalosporins II, Cephalosporins III |
| | | 3 | ATM30C-AP10C-A10C-CIP5C-TGC15C-ERY15C-TS25C-CPD10C-CTX30C-CAZ30C-CPM30C | 9 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | ATM30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-CPD10C-CTX30C-CAZ30C | 8 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Cephalosporins II, Cephalosporins III |
| | | 1 | ATM30C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-T30C-FOX30C-CPD10C-CTX30C-CPM30C | 9 | Aminoglycosides, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-TGC15C-ATH15C-ERY15C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 7 | Aminoglycosides, Amphenicols, Glycylcyclines, Macrolides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 12 | 6 | 1 | S10C-AP10C-A10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-T30C-CPD10C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CAZ30C | 9 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |

S10C – ATM30C – A10C – ATH15C – ERY15C – NA30C – TS25C – T30C – CPD10C – CTX30C – CAZ30C – CPM30C

1 S10C – ATM30C – A10C – CIP5C – TGC15C – ATH15C – ERY15C – TS25C – T30C – FOX30C – CPD10C – CPM30C

1 S10C – ATM30C – C30C – TGC15C – ATH15C

9 Aminoglycosides, Beta-lactams, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV

9 Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins IV

9 Aminoglycosides, Amphenicols, Glycylcyclines, Macrolides, Quinolones

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| 12 | 6 | 1 | S10C-ATM30C-C30C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-T30C-CPD10C-CAZ30C-CPM30C | 10 | Aminoglycosides, Amphenicols, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-AP10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-CTX30C | 9 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins III |
| 11 | 1 | 1 | S10C-ATM30C-IMI10C-MEM10C-CIP5C-TGC15CATH15C-ERY15C-NA30C-TS25C-CTX30C-CPM30C | 9 | Aminoglycosides, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins III, Cephalosporins IV |
| 13 | 7 | 1 | GM10C-S10C-ATM30C-C30C-A10C-TGC15C-ATH15C-ERY15C-TS25C-T30C-FOX30C-CTX30C-CPM30C | 10 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-T30C-CAZ30C | 10 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins III |
| | | 1 | S10C-ATM30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-T30C-FOX30C-CPD10C-CPM30C | 9 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins IV |
| | | 1 | S10C-ATM30C-AP10C-A10C-NOR10C-CIP5C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C | 9 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | S10C-ATM30C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-T30C-CPD10C-CTX30C-CAZ30C-CPM30C | 10 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | ATM30C-C30C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CTX30C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins IV |
| | | 1 | S10C-ATM30C-AP10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-CPD10C-CTX30C | 10 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins II, Cephalosporins III |
| 14 | 10 | 1 | S10C-ATM30C-C30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CTX30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CAZ30C | 10 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |
| | | 1 | S10C-ATM30C-AP10C-A10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CTX30C | 10 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins III |
| | | 1 | ATM30C-C30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CPM30C | 12 | Aminoglycosides, Amphenicols, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | S10C-ATM30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C–T30C-FOX30C-CPD10C-CTX30C-CPM30C | 10 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CPD10C-CTX30C-CAZ30C | 9 | Aminoglycosides, Beta-lactams, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |
| 14 | 10 | 1 | S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-TS25C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 9 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 9 | Aminoglycosides, Amphenicols, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | ATM30C-C30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-TS25C-CPD10C-CTX30C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 15 | 4 | 1 | GM10C-S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-AP10C-A10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C | 11 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |

| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | S10C-ATM30C-AP10C-A10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CPM30C | 11 | Aminoglycosides, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 16 | 3 | 1 | S10C-ATM30C-C30C-AP10C-A10C-IMI10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CPD10C-CTX30C-CAZ30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III |
| | | 1 | S10C-ATM30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CAZ30C-CPM30C | 12 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins II |
| | | 1 | S10C-ATM30C-AP10C-A10C-IMI10C-MEM10C-CIP5C-TGC15C-ATH15C–ERY15C-NA30C-TS25C-FOX30C-CTX30C-CAZ30C-CPM30C | 11 | Aminoglycosides, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 17 | 6 | 1 | S10C-ATM30C-C30C-AP10C-A10C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CAZ30C-CPM30C | 13 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-CPD10C-CTX30C-CAZ30C-CPM30C | 13 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 2 | S10C-ATM30C-C30C-AP10C-A10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 12 | Aminoglycosides, Amphenicols, Beta-lactams, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-NOR10C-CIP5C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 11 | Aminoglycosides, Amphenicols, Beta-lactams, Fluoroquinolones, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |

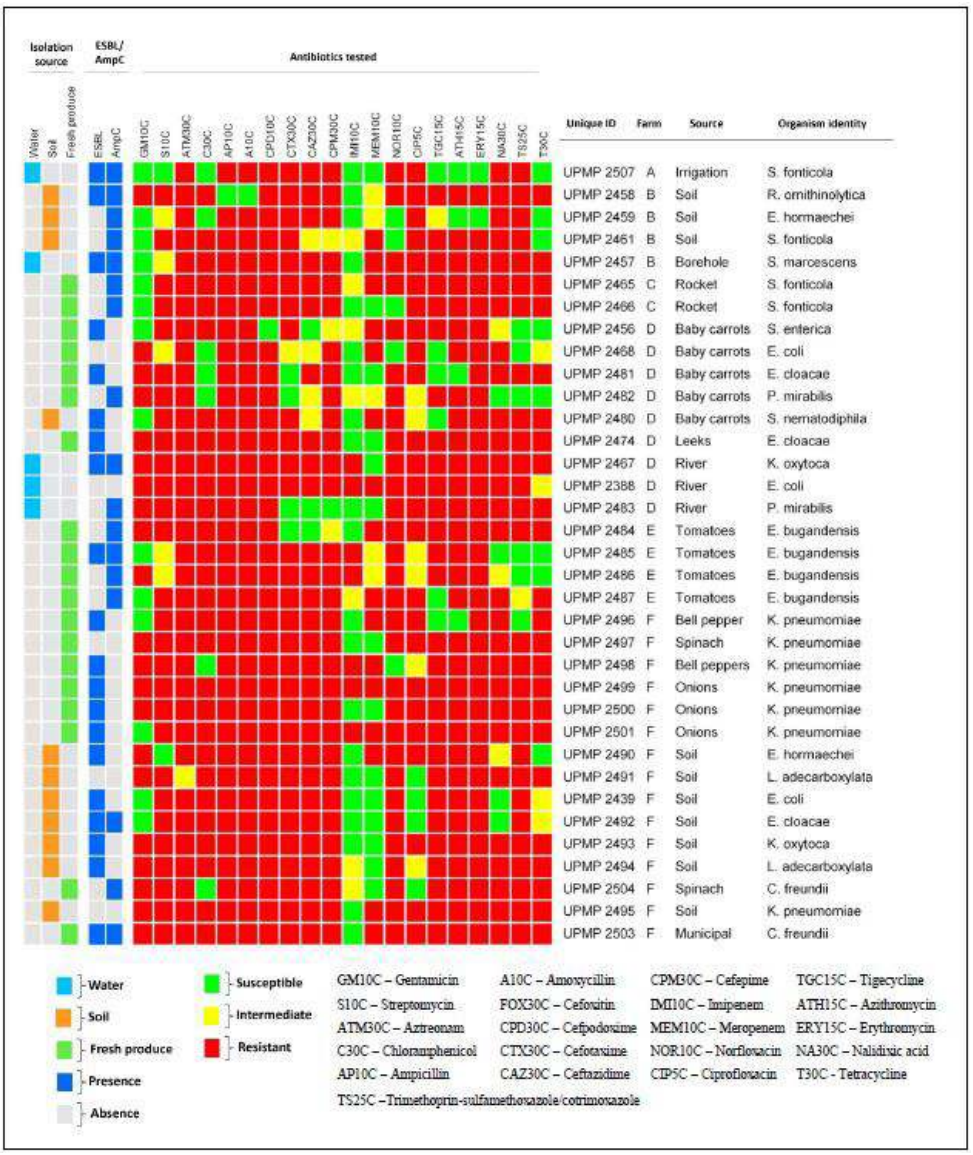
| No of antimicrobials to which isolates were resistant | No of isolates (n =90) | No of isolates with a specific resistant pattern | Resistance pattern | No of antibiotic classes to which isolates were resistant | Antibiotic class(es) |
|---|---|---|---|---|---|
| | | 1 | S10C-ATM30C-C30C-AP10C-A10C-MEM10C-CIP5C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-FOX30C-CPD10C-CTX30C-CAZ30C-CPM30C | 12 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Fluoroquinolones, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| | | 1 | GM10C-S10C-ATM30C-C30C-AP10C-A10C-IMI10C-TGC15C-ATH15C-ERY15C-NA30C-TS25C-T30C-FOX30C | 12 | Aminoglycosides, Amphenicols, Beta-lactams, Carbapenems, Glycylcyclines, Macrolides, Quinolones, Sulfonamides, Tetracyclines, Cephalosporins II, Cephalosporins III, Cephalosporins IV |
| 18 | 1 | | CPD10C-CTX30C-CAZ30C-CPM30C | | |

GM10C – Gentamicin, S10C – Streptomycin, ATM30C – Aztreonam, C30C – Chloramphenicol, AP10C – Ampicillin, A10C – Amoxycillin, FOX30C – Cefoxitin, CPD10C – Cefpodoxime, CAZ30C – Ceftazidime, CTX30C – Cefotaxime, CPM30C – Cefepime, IMI10C – Imipenem, MEM10C – Meropenem, NOR10C – Norfloxacin, CIP5C – Ciprofloxacin, TGC15C – Tigecycline, ATH15C – Azithromycin, ERY15C – Erythromycin, NA30C – Nalidixic acid, TS25C – Trimethoprin-sulfamethoxazole/ cotrimoxazole, T30C – Tetracycline

### 3.3.5 Detection of ESBL-producing Enterobacterales

From Farms A–F, 21.43% of the samples (n=48/224) harboured presumptive ESBL/AmpC-producing organisms. These isolates were predominantly obtained from fresh produce (n=23), followed by soil (n=13), and water (n=12) samples. 126 (33.7%) of the 374 presumptive extended-spectrum/AmpC β-lactamase-producing isolates were confirmed as Enterobacterales belonging to 10 genera. The four non-Enterobacterales isolates were predominantly identified as *Pseudomonas* spp. (92,62%) and *Aeromonas* spp. (7.38%). The 36/126 Enterobacterales isolates, selected for phenotypic characterisation in this study, included *Enterobacter* spp. (n=9, 25%), *Klebsiella* spp. (n=9, 25%, with seven isolates identified as *K. pneumoniae*), *Serratia* spp. (n=6, 17%), *Escherichia coli* (n=3, 8,33%); *Proteus* spp.; *Citrobacter* spp.; *Leclercia adecarboxylata* (6% or two isolates each) and one *Salmonella enterica*, and *Raoultella ornithinolytica* isolate each (Figure 12). Most Enterobacterales isolates were obtained from fresh-produce samples (22/36, 61%), followed by soil (13/36, 36%), and water (6/36, 17%) (Figure 12). In contrast, the 15 *P. aeruginosa* isolates selected for further characterisation in this study were predominantly obtained from fresh produce (8/15, 53%), followed by water (5/15, 33%), and soil samples (2/15, 14%) (Figure 12).

Across the various smallholder farms and sample types, Farm D (the conventional farm that used river water for irrigation) harboured the most genera (8/11 total), including clinically significant presumptive ESBL-producing isolates such as *Serratia marcescens*, *P. aeruginosa*, *Salmonella enterica, Enterobacter cloacae* and *E. coli* (Figure 12). Farm F (the conventional farm that used municipal water for irrigation and chicken manure as soil amendment) had the second most, with six of the 11 genera, including *P. aeruginosa*, *E. cloacae*, *K. pneumoniae*, *Leclercia adecarboxylata,* and *E. coli* (Figure 12). The occurrence of the selected ESKAPE strains across the various water source types was highest in borehole and river water (four isolates each), followed by municipal (three isolates) and dam (one isolate) water. Notably, the highest occurrence of isolates from soil was observed for Farms F (seven isolates) and E (four isolates), and both farms utilised animal manure as a soil amendment, compared to Farms C and E (no isolates), where plant-based compost was used as a soil amendment (Figure 12).




Green blocks represent susceptible isolates, and yellow and red blocks represent intermediate- and resistant-isolates, respectively.

**Figure 12:** Extended-spectrum and AmpC β-lactamase-producing Enterobacterales (including *Serratia fonticola* and *Serratia marcescens*, *Enterobacter hormaechei, Enterobacter cloacae* and *Enterobacter bugandensis, Escherichia coli, Klebsiella pneumoniae* and *Klebsiella oxytoca, Leclercia adecarboxylata* and *Citrobacter freundii),* isolated from the environment of diverse smallholder farms.

### 3.3.6 Whole genome sequence results (Farms A–F)

The 19 isolates selected for whole-genome sequencing belonged to six genera. The isolates were from Farms B, C, D, E, and F and included five isolates from water, three from soil, and 12 isolates from fresh produce, respectively (Figure 13). Except for a single isolate (namely, the *Salmonella* enterica isolate) from the water-soil-plant nexus, all carried at least one genetic determinant of carbapenem and/or cephalosporin resistance. The most prevalent β-lactamase genes, $bla_{\text{OXA-486}}$ and $bla_{\text{PAO_3}}$, were observed in 57.89% (11/19) and 52.63% (10/19) of the

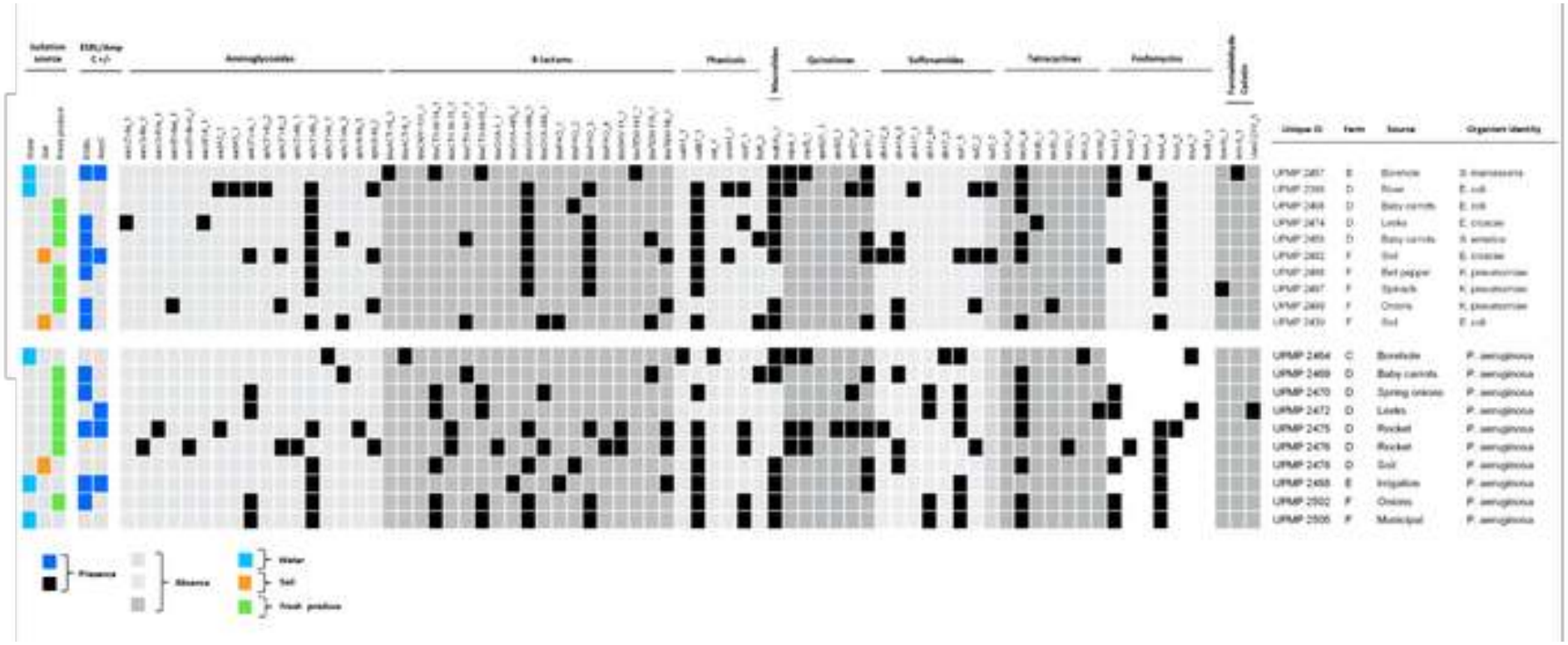
isolates, respectively. Furthermore, the chloramphenicol acetyltransferase gene, *catB,* and its variants were detected in 78.95% (15/19) of the isolates, while the *sul* (sulfonamide resistance) and *tet(A)* (tetracycline resistance) genes were present in 57.9% (11/19) and 73.68% (14/19) of the isolates, respectively. Across all isolates, only one macrolide-resistance gene (*mdf*(A)_1) was detected, present in 73.68% (14/19) of the isolates. Notably, a high diversity of aminoglycosides and β-lactam resistance genes was observed, with 17 and 19 variants, respectively.

*In silico* analysis identified 35 virulence genes across the isolates using VirulenceFinder. At least 11 virulence genes were common across 12 or more isolates, with *hlyE* present in all isolates, followed by *nlpI* in 90%, *terC* in 85%, and *flmH* in 80%. Other shared genes included the *yehBCD* complex in 65%, *ompT* and *hlyF* in 75%, and *csgA* and *traT* in 60%. A *Serratia marcescens* isolate from borehole water on Farm B had the most virulence genes (27), followed by a *Pseudomonas aeruginosa* isolate from dam water on Farm E and an *Escherichia coli* isolate from river water on Farm D, each with 17 genes. The 10 Pseudomonas isolates carried 10–17 virulence genes, with genes *traT, terC, ompT, flmH, nlpI,* and *hlyE* commonly shared across strains from soil, vegetables, and water sources.

Multilocus sequence typing (MLST) of three *E. coli* isolates revealed distinct sequence types: ST48 (borehole water, Farm C), ST254 (baby carrots, Farm D), and ST1141 (soil, Farm F). Three *Klebsiella pneumoniae* isolates belonged to two sequence types: ST147 (two isolates from onions and bell peppers) and ST36 (spinach), all from Farm F. Two Enterobacter cloacae strains shared ST514, while 10 *P. aeruginosa* isolates belonged to four distinct sequence types: ST313, ST514, ST564, and ST3017. *In silico* serotyping identified three *E. coli* serotypes (O45:H9, O113:H4, O156:H4), two *K. pneumoniae* K-loci serotypes (KL113, KL178), and three *P. aeruginosa* serotype groups (O1, O3, O9).

Eight distinct plasmid replicons were identified across Enterobacterales and *Pseudomonas aeruginosa* isolates. These included pO111, the Col plasmid family (Col3M), and several Inc plasmid groups, such as IncFII, IncHI2, IncFIB, IncI, and IncX1. The IncF plasmid group, known for its role in bacterial conjugation, was detected in four isolates and was associated with multiple virulence genes. More specifically, IncFIC(FII) and IncFII were present in two of the four IncF-positive isolates and were associated with three distinct virulence genes (*anr*, *traT*, and *traJ*). In contrast, the IncHI2A plasmid, more commonly associated with MDR, was detected in only two *P. aeruginosa* isolates, one from municipal water and the other from irrigated produce on the same farm. This plasmid harbours several resistance genes, including *aadA1*, *dfrA1*, *qacE*, *tet(A)*, and *sul1*, which confer resistance to multiple antibiotic classes (such as aminoglycosides, sulfonamides, quinolones, and tetracyclines). The Col plasmid family, which consists of small, often non-conjugative plasmids primarily found in Enterobacterales, typically carries genes that encode bacteriocins known as colicins (Partridge et al., 2018; Rozwandowicz et al., 2018). In this study, only one Col plasmid was identified: Col3M, which was associated with resistance genes qnrD1 and qnrS1 (encoding sulfonamide resistance) in one *E. coli* and two *P. aeruginosa* isolates from borehole water and fresh

produce (rocket and spring onions), respectively. Lastly, the phage-like plasmid pO111 was detected in an *E. coli* isolate from chicken manure-amended soil and was associated with several resistance genes, including *aph(3')-Ia*, $bla_{TEM-176}$, and $bla_{CTX-M-27}$.



Green blocks represent susceptible isolates, and yellow and red blocks represent intermediate- and resistant-isolates, respectively.

**Figure 13:** Antimicrobial resistance genes detected, using whole-genome sequencing and ResFinder, in 10 Enterobacterales and *Pseudomonas aeruginosa* strains each, isolated from smallholder farming environments in South Africa.

## 3.4 Discussion

This study highlights the link between *E. coli* isolated throughout the water-soil-plant nexus and contaminated irrigation water implicated in fresh-produce contamination. This finding emphasises the importance of monitoring irrigation water and enhancing water quality control methods for smallholder farmers, who depend on this limited resource. Irrigation water is a known significant risk factor for bacterial contamination of fresh produce (Gurtler and Gibson 2022). The typical irrigation water sources used by smallholder farmers in the current study were municipal, borehole, and river water, similar to those reported by Beharielal et al. (2018) for smallholder farmers in KwaZulu-Natal. Recently, the risk-based site-specific water irrigation guidelines (IrrigWQ) were developed, which provide greater site specificity during the evaluation process (du Plessis, Annandale and Benade, 2021). However, this tool presents certain limitations as it only indicates the risk associated with an intended use linked to a specific chemical analysis that should be included. To develop fit-for-purpose guidelines for managing microbiological water quality risks, the current study measured chemical parameters, including pH, COD, and BOD; however, the bacterial load and its impact across various production systems were the primary focus. This follows many studies indicating that the presence of *E. coli* alone as an indicator might not provide sufficient information about overall bacterial pathogen presence within a specific crop production system (Richiardi et al., 2023).

From the current study, only one water source had unsatisfactory *E. coli* levels according to the DWAF and WHO guidelines: the municipal water source at 3.12 log MPN/100mL (DWAF, 1996; WHO, 2006). This contaminated source is concerning as municipal water is supplied as potable water for communities, and thus the municipal water sampled at the specific time point in the current study potentially poses a contamination risk in the supply chain. The water quality results for the municipal water samples in the current study, however, differed from previous reports that reported satisfactory levels for municipal water samples in the Marianhill Agri-hub in KwaZulu-Natal and the Vhembe District in Limpopo, respectively (Beharielal et al., 2018; Edokpayi et al., 2018). Low levels (1.16–1.24 log MPN.100mL-1) of *E. coli* were enumerated from the borehole water used on the aquaculture farm (Farm A), which is similar to other South African studies where low levels of *E. coli* were enumerated from borehole water used for irrigation purposes (Iwu et al., 2020; Richter et al., 2021). Currently, the DWAF guidelines stipulate that water used for vegetable and crop irrigation should have coliform levels <1 000CFU.100mL-1 and that there is a likelihood of contamination of vegetables and other crops eaten raw if the *E. coli* levels range between 1–1 000CFU.100mL-1 (DWAF, 1996). Although enumeration of *E. coli* is routinely used globally as an indicator of faecal contamination in irrigation water used in fresh-produce production, no standardised global guidelines exist. In South Africa, the fit-for-purpose guidelines for managing microbiological water quality risks are currently being developed, specifically for smallholder farmers, in line with the FAO risk-based water quality guidelines (Drechsel et al., 2023).

The general pH of surface water in South Africa has been reported to fluctuate around neutral (Mekonnen et al., 2018). Similarly, 5 of the 6 farms in the current study had neutral pH levels in their irrigation water samples, except for a surface water sample from Farm E (conventional/dam), which was more alkaline (pH 9.7). Moreover, reports have noted reduced activity of nitrifying bacteria and reduced bioavailability of essential nutrients for crops grown at alkaline pH levels (FAO, 2014; Cerozi and Fitzsimmons, 2016). The BOD level for the river water source sample (Farm D) exceeded DWAF regulations (<30mg/L), at 144mg/L, indicating potential pollution of the river. Similarly, the COD levels for the same sample were at least 15 times higher than those of the other water samples tested in the study. Since the BOD and COD levels of the river water exceeded the water quality regulations, the source may have a reduced ability to sustain aquatic life (FAO, 2014; Prambudy et al., 2019). The turbidity levels throughout the entire irrigation system on Farm D (conventional/river) exceeded regulations of less than 5 NTU, which is similar to reports for other rivers around South Africa and can be attributed to the current pollution in water systems in the country (Bonthuys, 2018; Rangeti and Dzwairo, 2021; Gqomfa et al., 2022). These high turbidity levels can reduce light penetration through water, leading to reduced photosynthesis and lower survival of aquatic microorganisms (Kuutondokwa, 2021; Rangeti and Dzwairo, 2021).

In this study, 40.2% of generic *E. coli* were isolated (after enrichment) from the water (32%), soil (39%) and fresh-produce (28%) samples, which is similar to previous reports in smallholder fresh-produce farms in South Africa (Beharielal et al., 2018). The ERIC-PCR profiles showed a clear link between the *E. coli* isolated from the water-soil-plant nexus. The >80% similarity observed is consistent with other studies in the commercial food supply chain (Richter et al., 2021; Ratshilingano et al., 2022). To further elucidate the genetic relatedness between *E. coli* isolates from various environmental samples, more detailed genetic typing methods, such as whole-genome sequencing, should be included in future studies.

Notably, the *E. coli* isolates from each farm in the current study (regardless of farm scenario or water source type) clustered together with isolates from irrigation water, soil, and fresh produce. The results further reiterate the importance of irrigation water as a contamination source during fresh-produce production, in line with previous studies that showed links between *E. coli* in irrigation water and contaminated fresh produce (Decol et al., 2017; Douti et al., 2021; du Plessis et al., 2021). Notably, no STEC virulence genes were detected in any *E. coli* isolates from Farms A–F, despite the 100% MDR resistance observed. This is similar to previous studies in South Africa, which reported a low incidence of virulence genes in *E. coli* (Baloyi et al., 2022; Ratshilingano et al., 2022). Understanding the patterns of AMR, particularly the presence of foodborne pathogens across fresh-produce farms, is crucial for reducing treatment failures in the event of an outbreak (Kim et al., 2019).

The MDR *E. coli* isolates from the current study displayed >70% prevalence of resistance against 2nd-generation, 3rd-generation and 4th-generation cephalosporins, at 99%, 96% and 96%, respectively. This raises concern, as cephalosporins (2nd, 3rd, and 4th-generation) are considered the last-resort medications for treating MDR *E. coli* infections (WHO 2018). In

Ghana, similar results were observed, with 100% resistance to β-lactams and tetracyclines among *E. coli* isolates from fresh produce (Adzitey 2018). In the current study, a higher prevalence of MDR *E. coli* was observed in soil samples (36/90), followed by water (29/90 and fresh-produce samples (25/90). Moreover, previous studies in LMICs reported the prevalence of antimicrobial resistance in *E. coli* isolates recovered from the environment (Canizalez-Roman et al., 2019; Richter et al., 2021). Surveillance of foodborne pathogens and AMR is a crucial aspect of disease outbreak assessment and food safety. Unfortunately, in South Africa, foodborne illnesses are often not reported, under-reported, or inadequately investigated (Bisholo et al., 2018; Richter et al., 2023).

Building on a South African study by Richter et al. (2019) on ESBL/AmpC-producing Enterobacteriaceae in fresh produce from informal markets, the current study adds that the production environment and farm practices serve as key reservoirs of antibiotic-resistant bacteria, contributing to the overall spread of AMR in informal fresh-produce supply chains. In particular, from four of the six farms in the current study, water and/or soil samples, as well as corresponding fresh-produce samples, harboured MDR isolates with similar phenotypic resistance profiles.

The high percentage of MDR organisms in the current study (100%) is consistent with reports from South Africa (Richter et al., 2019), which documented a MDR rate of 98% in fresh produce at the point of sale and in Tunisia, where the rate was 93% in a similar study (Ben Said et al., 2015). In contrast, Klaui et al. (2024) reported only 20.5% Enterobacterales, yet 100% *Pseudomonas* spp. isolates from retailed vegetables originating from conventional agriculture (non-organic) farms in Switzerland were MDR. Among the non-β-lactam resistance profiles, the most prevalent in the current study was aminoglycoside resistance (100%), followed by chloramphenicol (69%) and tetracycline (72%). Notably, isolates from the current study, including those from boreholes, rivers, and municipal water sources used for irrigation, had similar resistance profiles. Despite being limited to targeted water samples, this study confirms the serious extent of MDR bacteria in irrigation water used in small-scale fresh-produce production, aligning with previous research in Lebanon (Osman et al., 2024) and Ecuador (Montero et al., 2021).

In total, 17.65% (9/51) of the isolates from Farms A–F were confirmed as ESBL and AmpC producers. Phenotypic confirmation of ESBL and/or AmpC production among the Enterobacterales isolates was observed in 56% and 50% of isolates in the current study, respectively. In contrast, Richter et al. (2020) reported that 100% and 18.6% of the isolates from South African commercial spinach supply chains were phenotypically confirmed as ESBL- and AmpC-producing Enterobacterales, as reported through the previously funded Water Research Commission project 2706/1/21. Notably, the prevalence of presumptive ESBL-producing bacteria across all samples in the current study was higher compared to the 14.58% reported in samples from commercial spinach production systems in South Africa (Richter et al., 2020).

## 3.5 Conclusion

This case study demonstrated that irrigation water serves as a primary vector for microbial contamination and the dissemination of AMR bacteria across the water-soil-plant nexus in fresh produce production. Despite varying water sources (borehole, river, dam, municipal) and farming systems (aquaculture, integrated livestock, organic, conventional), high levels of indicator bacteria (total coliforms and *E. coli*), widespread multidrug-resistant *E. coli* (100% of the isolates), and ESBL/AmpC-producing Enterobacterales were consistently detected in water, soil, and fresh produce samples. Whole-genome sequencing further revealed shared resistance genes, virulence factors and genetic relatedness between environmental and crop isolates, underscoring the transfer of pathogens and AMR determinants from irrigation water to edible crops, particularly under overhead irrigation and when using untreated manure. Physicochemical parameters occasionally exceeded established guidelines, indicating potential risks to crop health and microbial dynamics. However, the current study did not include longitudinal data or follow-up analyses.

The case study revealed the limitations of relying solely on generic *E. coli* counts as an indicator organism, since high levels of MDR and ESBL-producing bacteria were frequently detected even when indicator levels remained relatively low. It emphasised the need for risk-based, site-specific, and production-system-tailored approaches to water quality management, building on the SA IrrigWQ Decision Support System, which explicitly accounts for local water sources, irrigation methods (such as overhead vs drip), fertilizer practices, crop types, and seasonal variations. Furthermore, the research provided valuable empirical evidence drawn from real smallholder farming contexts in SA, thereby supporting the development of more nuanced and practical microbiological water quality guidelines that align with FAO/WHO risk-based frameworks. Finally, the study highlighted the value of advanced molecular tools, such as WGS, for establishing clear linkages between contamination sources across the water-soil-plant nexus and for strengthening surveillance systems in settings where foodborne illnesses are often under-reported.

## CHAPTER 4: CASE STUDY 2: Small-scale Vegetable-Pig Production Systems

*M. Dlangalala, S. Schmidt, L. Richter-Mouton, and L. Korsten*

### 4.1 Introduction

Integrated agricultural systems, which combine crop and livestock production on a single farm, promote sustainability by optimising resource use and enhancing food security (Sekaran et al., 2021). In vegetable-pig ecosystems, pig waste serves as fertiliser for crops, while crop residues provide feed for pigs, fostering a symbiotic relationship (Hilimire, 2011). However, this interconnectedness heightens the risk of microbial contamination, as pig faeces can harbour pathogens such as *Staphylococcus* spp., *Escherichia coli*, *Clostridium* spp., *Campylobacter* spp., and *Salmonella* spp. (Abdalla et al., 2021). Proximity between animals and crops creates transmission pathways, potentially compromising produce safety and posing public health hazards.

Foodborne pathogens, including Shiga toxin-producing *E. coli* (STEC, e.g., O157:H7), *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Yersinia* spp., and *Campylobacter* spp., are significant global causes of illness (Abebe et al., 2020; Imre et al., 2020; WHO, 2022). Fresh produce, such as leafy greens, cucumbers, and tomatoes, can act as reservoirs for these, often contaminated pre-harvest via irrigation water, soils, or manure, or post-harvest through handling (Callejón et al., 2015; Olaimat and Holley, 2012). In integrated systems, risks potentially escalate due to overlapping waste management and crop practices, with inadequate composting or sanitation allowing pathogen persistence in the environment. Small-scale systems, common in peri-urban South Africa and supplying informal markets, face heightened vulnerabilities due to limited resources, infrastructure, and biosecurity (Queenan et al., 2020; Carelsen et al., 2021). Reliance on traditional methods, polluted water sources, and poor hygiene, exacerbated by water scarcity and wastewater issues (Gemmell and Schmidt, 2012), increases the likelihood of contamination. Commercial systems, serving formal markets, benefit from advanced protocols and typically require the implementation of more stringent controls (e.g., Pork 360, Global GAP); however, these systems are still susceptible to contamination risks, especially considering the high animal densities and complex supply chain structures involved (Queenan et al., 2020).

Factors such as manure handling, irrigation with contaminated water, environmental conditions (e.g., temperature and humidity), and farmer awareness influence pathogen prevalence (Uyttendaele et al., 2015; Wadamori et al., 2017). In LMIC contexts, this threatens consumer health, economic viability, and market access. The aim of this case study was to assess microbial quality across the water-soil-crop continuum in small-scale and commercial vegetable-pig production systems, with a focus on investigating the prevalence of key foodborne pathogens, including Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp., and *Listeria monocytogenes*. By comparing contamination hazards across system

scales, identifying key control points, and proposing targeted mitigation strategies, the study aims to address gaps in current practices and inform evidence-based interventions.

### 4.2 Experimental Procedures

The three vegetable-pig small-scale farms (Farms A_S, B_S, and C_S) all used borehole water for irrigation of the fresh produce as well as drinking water for the pigs (Figure 14). The small-scale farms for this study were defined as a farm that grows fresh produce (spinach) and rears pigs (≤25 pigs – average 132-day-old female and male pigs) for both household consumption and income generation. All three farms housed approximately 20–25 pigs in individual pens, with one farm in Limpopo that also had free-roaming pigs in the enclosure. Spinach was grown on all three farms using overhead irrigation. The farm workers cleaned the pig pens every day, and one farmer noted that pig faeces were occasionally applied to the farm fields to fertilise the soil.



**Figure 14**: Examples of the vegetable-pig small-scale farms where irrigation water, soil, fresh produce, and drinking water for the pigs were collected for microbiological quality analysis.

### 4.2.1 Sample collection, processing, and enumeration of coliforms/*Escherichia coli*

In total, 30 samples were collected from each of the three small-scale farms during a single sampling event conducted between September and November 2022. The collected samples included spinach (n=5), soil (n=5), irrigation water (n=5), animal feed (n=5), drinking water (n=5), and faeces (n=5). Although results from the animal feed and pig faecal samples are selectively presented in this report, these samples fall outside the main scope of the current study and are therefore not discussed in detail; they are included only to provide broader contextual insight. The samples (water, soil, and fresh produce) were collected and processed as described for those in Case Study 1.

### 4.2.2 Detection and isolation of presumptive positive potential human pathogenic bacteria

The same methods described in Case Study 1 were used to isolate *E. coli*, *Salmonella*, and ESBL-producing Enterobacterales. In addition, *Listeria monocytogenes* isolation was included in this study.

#### *Listeria monocytogenes* isolation

Following incubation of 3–4h at 37ºC of the prepared Buffered Listeria Enrichment Broth (BLEB)-sample mixtures, 1mL *Listeria* Selective Enrichment Supplement (BioRad) was added to the BLEB-sample mixtures, followed by 48h incubation at 30ºC. The presence of *Listeria monocytogenes* was assessed using the iQ-Check *Listeria monocytogenes* II Kit AOAC 010802 (BioRad Laboratories, Hercules, California, United States) according to the manufacturer's instructions. Enriched mixtures that yielded positive results were inoculated onto Agar Listeria Ottavani and Agosti (ALOA) (Biomérieux SA, Marcy-l'Étoile, France) using the streak plate method and subsequently incubated for 24h at 37ºC for *Listeria monocytogenes* isolation; blue-green colonies with a halo were considered presumptive *Listeria monocytogenes*.

As described in Chapter 3, all presumptive positive isolates were identified using MALDI-TOF analysis. Furthermore, antimicrobial susceptibility testing, molecular screening of Escherichia coli O157:H7 and non-O157:H7 isolates, and ERIC-PCR analysis were performed using the same antibiotics, methods, and primers as described for Case Study 1.

#### Genomic DNA extraction

Single colonies of each isolate (generic *E. coli*, STEC, and ESBL- and AmpC-producing *E. coli*) were transferred into 9mL tryptone soy broth (TSB) (Merck SA, Johannesburg, South Africa) and incubated for 24h at 37ºC. The cells were pelleted by centrifugation (12 500g for 3min), followed by DNA extraction using the Quick-gDNA Mini-Prep kit (Zymo Research, Irvine, United States). The DNA quality was determined using the NanoDrop 2000 (Thermo Scientific, Johannesburg, South Africa).

**Whole genome sequencing and analysis**

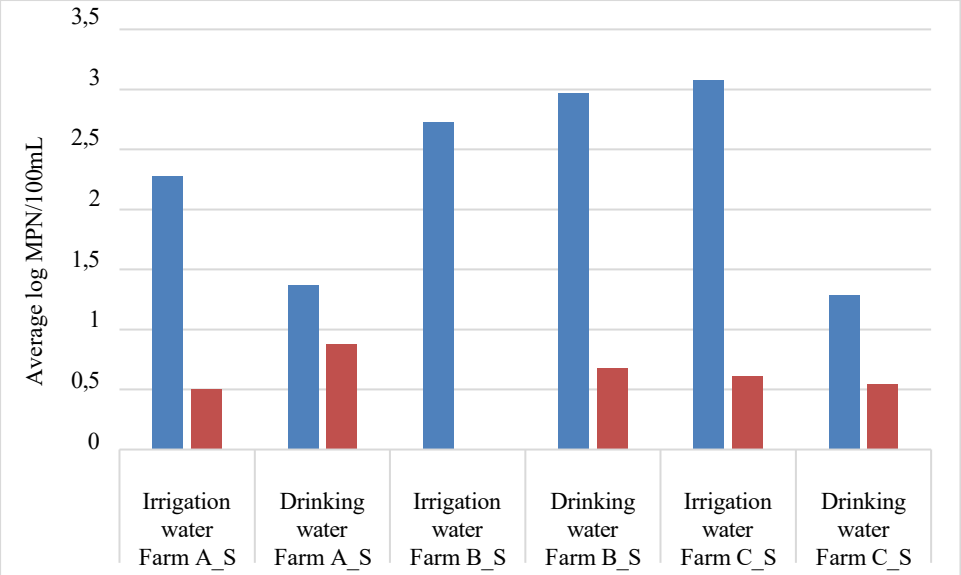
Similar to the WGS for Case Study 1, multiplexed paired-end libraries (2 × 300bp) were generated using the Nextera XT DNA sample preparation kit (Illumina, San Diego, California, United States). Sequencing was conducted on an Illumina MiSeq platform with a coverage depth of 100 × at the National Institute of Communicable Diseases (NICD) Sequencing Core Facility in South Africa. Similar assembly methods were used as described for Case Study 1. The KBase platform was used to obtain genome assemblies for the nine selected *E. coli* isolates. Sequence quality was evaluated using FastQC v0.12.1, followed by trimming with Trimmomatic v0.39. The trimmed sequences were then assembled using Unicycler v0.4.8, and genome quality was assessed with CheckM v1.0.18. The final assemblies were imported into Solu.genomics v1.0.399.

## 4.3 Results

### 4.3.1 Microbiological quality results from Farms A_S–C_S

The most widely referenced global drinking-water quality guidelines are issued by the WHO and state a zero tolerance for microbiological contaminants, requiring 0 CFU/100mL of *E. coli* and faecal coliforms to ensure water is free from faecal pollution (WHO, 2024). South Africa adheres to the South African National Standard (SANS) 241, which mirrors WHO recommendations by mandating 0 CFU/100mL for both total coliforms and *E. coli*, thereby aligning with international benchmarks to ensure microbiologically safe drinking water free of contamination. In this study, the drinking water of all three farms exceeded acceptable limits, as coliforms and *E. coli* were enumerated at 1.3–3 log MPN/100mL and 0–0.89 log MPN/100mL, respectively (Figure 15).

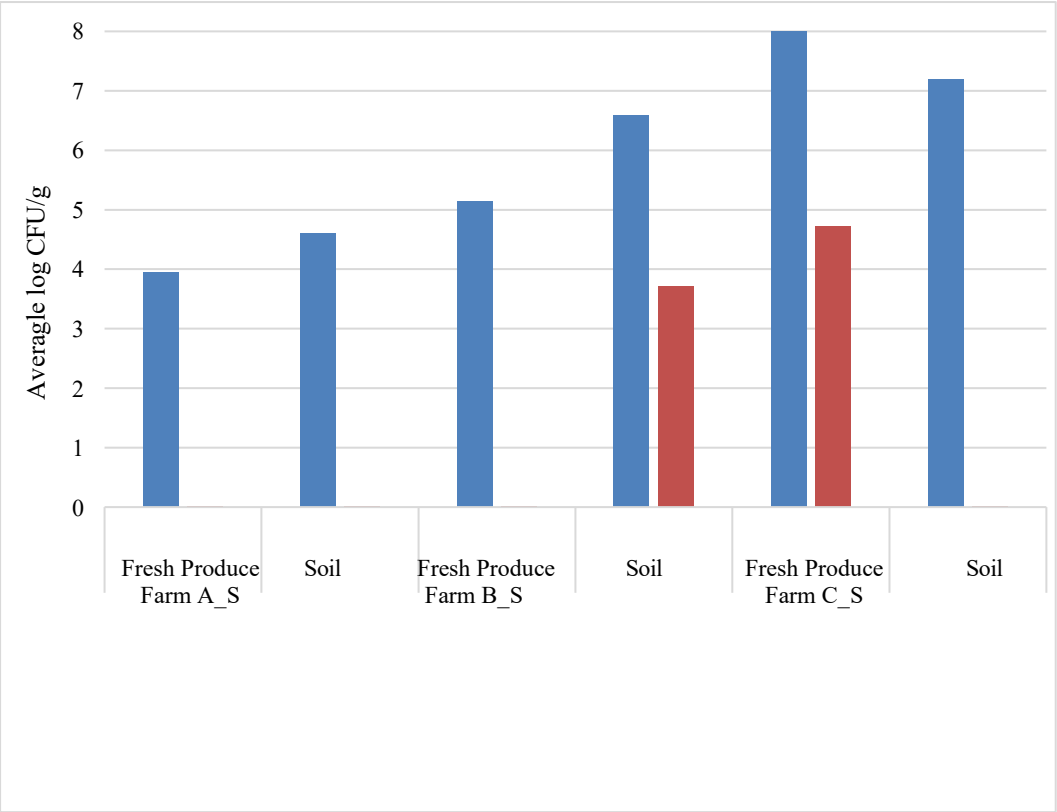
In these small-scale vegetable-pig production systems, irrigation water was applied overhead to crops typically consumed raw or minimally processed, such as leafy greens. According to WHO guidelines (FAO and WHO, 2021), acceptable limits for faecal coliforms are ≤1 000MPN/100mL, with the recommendations being risk-based and requiring local adaptation depending on factors such as irrigation method (e.g., spray or sprinkler systems) and crop type (with raw-consumed crops necessitating higher water quality). In this study, the average log MPN/100mL *E. coli* counts fell within these acceptable limits. Overall, the findings suggest that animal interaction may be a potential source of contamination in these integrated vegetable-pig production systems, as irrigation and drinking water (borehole water) originate from the same source.




Average total coliforms (blue) and *E. coli* (red)

**Figure 15:** Average total coliforms and *E. coli* levels in drinking and irrigation water from Farms A_S, B_S, and C_S.

From the fresh produce and soil samples, coliforms were, as expected, enumerated from all samples (Figure 16). Additionally, on Farm B_S, *E. coli* were enumerated from soil samples at an average of log 3,7CFU/g and from fresh-produce samples on Farm C_S at an average of log 4,7CFU/g (Figure 16). Although no soil amendments were observed on these farms, the animals were allowed to roam freely on Farm C_S, which may have contributed to the higher counts observed in the fresh-produce samples.




Average total coliforms (blue) and *E. coli* (red)

**Figure 16:** Average total coliforms and *E. coli* levels in fresh produce and soil from farms A_S, B_S, and C_S. In Farm C_S, the coliform counts were so high that they could not be enumerated at >8 log CFU/g.

### Detection of potential pathogenic bacteria

The presence of diarrhoeagenic *E. coli* (enterohaemorrhagic *E. coli,* EHEC*)* was confirmed by analysing candidate isolates for the virulence genes *eaeA, stx1,* and *stx2* using PCR. Only one of 30 isolates (D30s-UPMP 2623, presumptive STEC from faecal sample) harboured *mdh* and the diarrhoeagenic Shiga toxin encoding gene *stx1* (Figure 17). Additional isolates shown in the figure are beyond the scope of the current study, as selected commercial farms were included in a broader leverage-funded study.

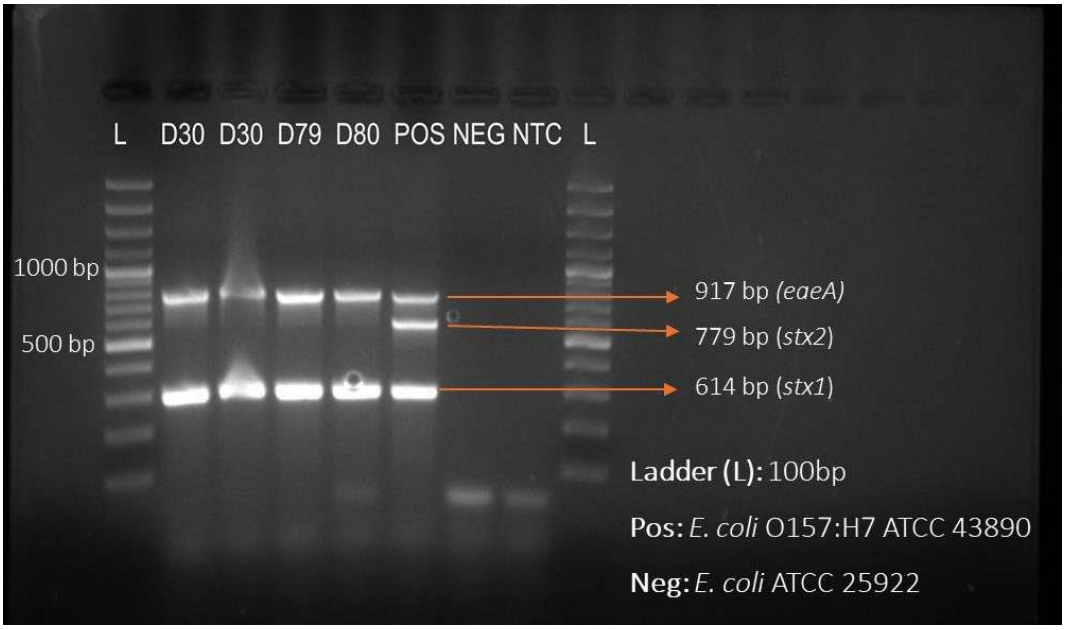



**Figure 17:** Gel electrophoresis results showing the detection of diarrhoeagenic *Escherichia coli* (EHEC) through PCR amplification of virulence genes *eaeA*, *stx1*, and *stx2*. Distinct bands confirm the presence of these genes, indicating potential pathogenicity.

From Farms A-S, B_S, and C_S, fourteen ESBL/AmpC-producing Enterobacterales were isolated from soil and water samples. This included *Serratia fonticola* (n=6) from soil and pig drinking-water samples, *Citrobacter freundii* (n=2) from irrigation water, and *Escherichia coli* isolates (n=6) from borehole water at the irrigation and drinking-water sampling points.

The AMR profiles of the isolates from the respective sources (water, fresh produce, or soil) of the three vegetable-pig farms are presented in Figure 18 (Farm A_S), Figure 19 (Farm B_S) and Figure 20 (Farm C_S). Of the 38 *E. coli* isolates obtained, all were categorised as MDR with resistance to antibiotics in three or more classes observed (Table 6). Within the β-lactam class of antibiotics, resistance against penicillins, 2nd-generation cephalosporins, and carbapenems was observed in 78.95% (30/38), 60.53% (23/38), and 73.68% (28/38) of the isolates, respectively. In total, 97.37% (37/38) of *E. coli* isolates were resistant to antibiotics in the class aminoglycosides.

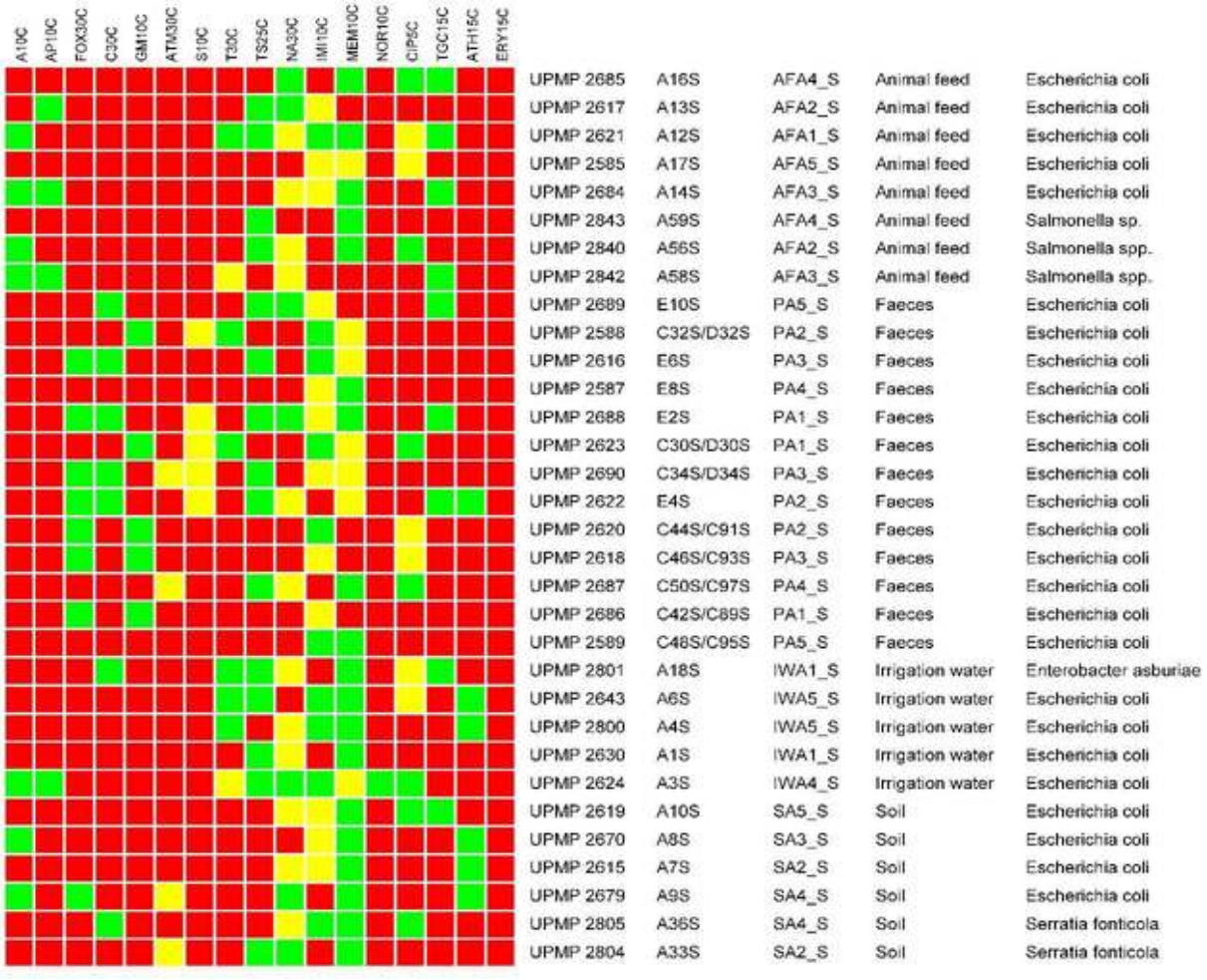



**Figure 18:** *Escherichia coli* and ESBL-producing Enterobacterales isolated from vegetable-pig production farms (Farm A_S). In total, eight *E. coli* isolates were obtained from water and soil after enrichment, while no *E. coli* was isolated from any of the fresh-produce samples.

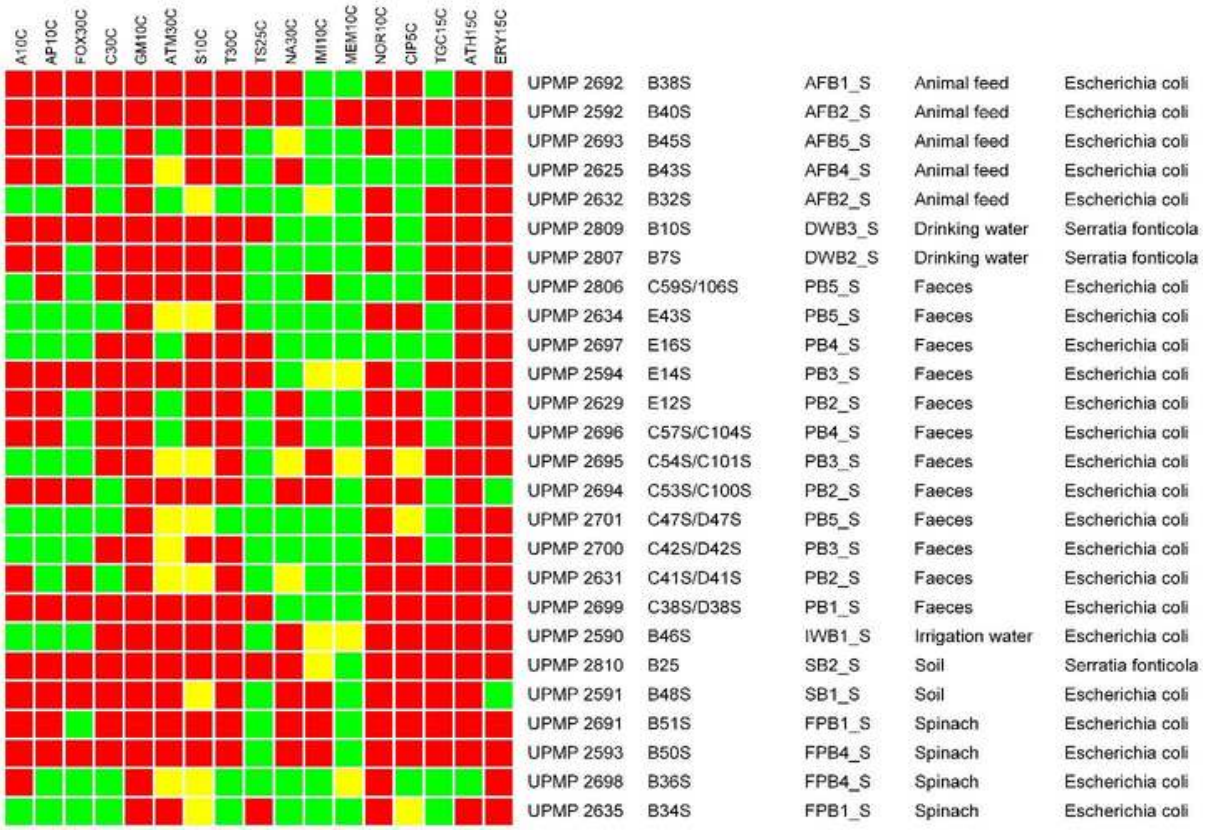
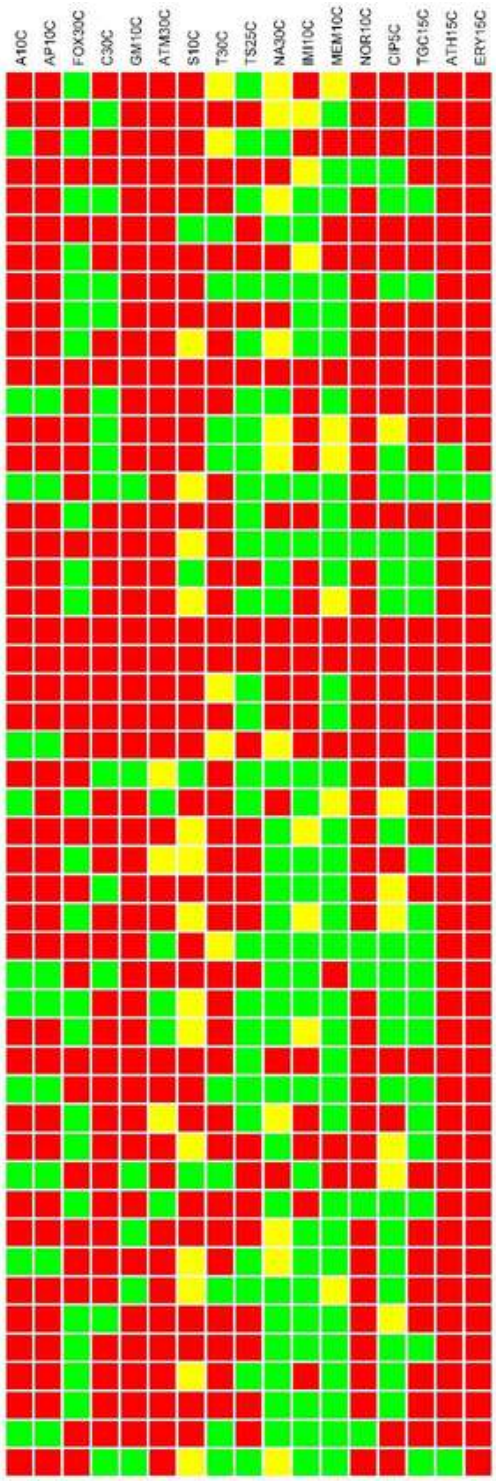



**Figure 19:** *Escherichia coli* and ESBL-producing Enterobacterales isolated from vegetable-pig production farms (Farm B_S). In total, six *E. coli* isolates were obtained from water, soil, and fresh-produce samples after enrichment.




| | | | | |
|---|---|---|---|---|
| UPMP 2818 | C25S/D25S | AFC2_S | Animal feed | Escherichia coli |
| UPMP 2598 | C36S | AFC2_S | Animal feed | Citrobacter sedlakii |
| UPMP 2728 | C28S/D28S | AFC5_S | Animal feed | Escherichia coli |
| UPMP 2716 | C42S | AFC5_S | Animal feed | Escherichia coli |
| UPMP 2638 | C40S/C87S | AFC5_S | Animal feed | Escherichia coli |
| UPMP 2708 | C36S/C83S | AFC3_S | Animal feed | Escherichia coli |
| UPMP 2596 | C34S/C81S | AFC2_S | Animal feed | Escherichia coli |
| UPMP 2707 | C32S/C79S | AFC1_S | Animal feed | Escherichia coli |
| UPMP 2715 | C41S | AFC5_S | Animal feed | Escherichia coli typed as KPC positive |
| UPMP 2813 | C7S | DWC1_S | Drinking water | Escherichia coli |
| UPMP 2734 | C82S/D82S | DWC2_S | Drinking water | Escherichia coli |
| UPMP 2722 | C12S/D12S | DWC5_S | Drinking water | Escherichia coli |
| UPMP 2721 | C10S/D10S | DWC4_S | Drinking water | Escherichia coli |
| UPMP 2639 | C8S/D8S | DWC3_S | Drinking water | Escherichia coli |
| UPMP 2720 | C6S/D6S | DWC2_S | Drinking water | Escherichia coli |
| UPMP 2599 | C4S/D4S | DWC1_S | Drinking water | Escherichia coli |
| UPMP 2714 | C15S | DWC5_S | Drinking water | Escherichia coli |
| UPMP 2713 | C11S | DWC3_S | Drinking water | Escherichia coli |
| UPMP 2712 | C9S | DWC2_S | Drinking water | Escherichia coli |
| UPMP 2733 | C57S/D57S | PC5_S | Faeces | Escherichia coli |
| UPMP 2732 | C54S/D54S | PC4_S | Faeces | Escherichia coli |
| UPMP 2731 | C53S/D53S | PC3_S | Faeces | Escherichia coli |
| UPMP 2730 | C50S/D50S | PC2_S | Faeces | Escherichia coli |
| UPMP 2729 | C48S/D48S | PC1_S | Faeces | Escherichia coli |
| UPMP 2719 | C47S | PC3_S | Faeces | Escherichia coli |
| UPMP 2718 | C45S | PC2_S | Faeces | Escherichia coli |
| UPMP 2717 | C44S | PC1_S | Faeces | Escherichia coli |
| UPMP 2711 | C70S/C117S | PC5_S | Faeces | Escherichia coli |
| UPMP 2637 | C69S/C116S | PC4_S | Faeces | Escherichia coli |
| UPMP 2710 | C66S/C113S | PC3_S | Faeces | Escherichia coli |
| UPMP 2636 | C65S/C112S | PC2_S | Faeces | Escherichia coli |
| UPMP 2709 | C62S/C109S | PC1_S | Faeces | Escherichia coli |
| UPMP 2812 | C4S | IWC3_S | Irrigation water | Citrobacter freundii |
| UPMP 2811 | C2S | IWC2_S | Irrigation water | Citrobacter freundii |
| UPMP 2595 | C2S/D2S | IWC1_S | Irrigation water | Escherichia coli |
| UPMP 2815 | C25S | SC5_S | Soil | Serratia fonticola |
| UPMP 2814 | C18S | SC1_S | Soil | Serratia fonticola |
| UPMP 2640 | C16S/D16S | SC3_S | Soil | Escherichia coli |
| UPMP 2723 | C14S/D14S | SC1_S | Soil | Escherichia coli |
| UPMP 2726 | C24S/D24S | FPC5_S | Spinach | Escherichia coli |
| UPMP 2597 | C22S/D22S | FPC4_S | Spinach | Escherichia coli |
| UPMP 2725 | C20S/D20S | FPC2_S | Spinach | Escherichia coli |
| UPMP 2724 | C18S/D18S | FPC1_S | Spinach | Escherichia coli |
| UPMP 2706 | C31S/C76S | FCP4_S | Spinach | Escherichia coli |
| UPMP 2705 | C30S/C73S | FCP4_S | Spinach | Escherichia coli |
| UPMP 2704 | C29S/C72S | FCP5_S | Spinach | Escherichia coli |
| UPMP 2703 | C27S/C77S | FCP3_S | Spinach | Escherichia coli |
| UPMP 2642 | C25S/C74S | FCP5_S | Spinach | Escherichia coli |
| UPMP 2702 | C23S/C70S | FPC2_S | Spinach | Escherichia coli |

**Figure 20:** *Escherichia coli* and ESBL-producing Enterobacterales isolated from vegetable-pig production farms (Farm C_S). In total, 23 *E. coli* isolates were obtained from water and soil and fresh-produce samples after enrichment.

**Table 6:** Summary of the number of antimicrobials, resistant patterns, and type of antibiotic classes to which generic *Escherichia coli* isolates from smallholder farms (Farms A_S, B_S and C_S) were resistant.

| Farm | Isolation source | No of antimicrobials to which isolates were resistant | No of antibiotic classes to which isolates were resistant | Resistance pattern | Antibiotic class(es) |
|---|---|---|---|---|---|
| C_S | Drinking water | 6 | 6 | FOX30C-ATM30C-S10C-T30C-NOR10C-TGC15C | Cephalosporins II, Monobactam, Aminoglygoside, Tetracycline, Quinolone, Glycylcycline |
| B_S | Spinach | 8 | 7 | A10C-GM10C-ATM30C-S10C-MEM10C-NOR10C-TGC15C-ERY15C | Penicillin, Aminoglycoside, Monobactam, Carbapenem, Quinolone, Glycylcycline, Macrolide |
| B_S | Spinach | 9 | 6 | GM10C-ATM30C-S10C-TS25C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Aminoglycoside, Monobactam, Sulfonamide, Quinolone, Glycylcycline, Macrolide |
| A_S | Irrigation water | 10 | 9 | FOX30C-C30C-GM10C-ATM30C-S10C-T30C-MEM10C-TGC15C-ATH15C-ERY15C | Cephalosporins II, Amphenicol, Aminoglycoside, Monobactam, Sulfonamide, Tetracycline, Carbapenem, Glycylcycline, Macrolide |
| C_S | Spinach | 10 | 9 | FOX30C-C30C-GM10C-ATM30C-S10C-TS25C-CIP5C-TGC15C-ATH15C-ERY15C | Cephalosporins II, Amphenicol, Aminoglycoside, Monobactam, Sulfonamide, Tetracycline, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 11 | 8 | A10C-AP10C-C30C-GM10C-S10C-T30C-TS25C-IMI10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 11 | 9 | A10C-AP10C-FOX30C-C30C-ATM30C-S10C-MEM10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Monobactam, Aminoglycoside, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 11 | 9 | A10C-AP10C-FOX30C-C30C-ATM30C-S10C-MEM10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Monobactam, Aminoglycoside, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Irrigation water | 11 | 9 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-NA30C-NOR10C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Monobactam, Aminoglycoside, Quinolone, Carbapenem, Fluoroquinolone, Macrolide |

| Farm | Isolation source | No of antimicrobials to which isolates were resistant | No of antibiotic classes to which isolates were resistant | Resistance pattern | Antibiotic class(es) |
|---|---|---|---|---|---|
| C_S | Drinking water | 11 | 9 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 11 | 9 | A10C-AP10C-FOX30C-GM10C-ATM30C-S10C-NA30C-IMI10C-NOR10C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Aminoglycoside, Monobactam, Quinolone, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Soil | 11 | 9 | FOX30C-C30C-ATM30C-TS25C-NA30C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Cephalosporin II, Amphenicol, Monobactam, Sulfonamide, Aminoglycoside, Carbapenem, Fluoquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 11 | 8 | FOX30C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-NOR10C-TGC15C-ATH15C-ERY15C | Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 11 | 9 | FOX30C-GM10C-ATM30C-S10C-T30C-IMI10C-NOR10C-CIP15C-TGC15C-ATH15C-ERY15C | Cephalosporin II, Aminoglycoside, Monobactam II, Sulfonamide, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 12 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-IMI10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam,Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 12 | 10 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-IMI10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 12 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 12 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-T30C-NA30C-IMI10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |

| Farm | Isolation source | No of antimicrobials to which isolates were resistant | No of antibiotic classes to which isolates were resistant | Resistance pattern | Antibiotic class(es) |
|---|---|---|---|---|---|
| C_S | Spinach | 12 | 8 | A10C-AP10C-GM10C-ATM30C-S10C-T30C-TS25C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 13 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-IMI10C-MEM10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 13 | 8 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 13 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-MEM10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Spinach | 13 | 8 | A10C-AP10C-FOX30C-C30C-ATM30C-S10C-T30C-TS25C-NA30C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Sulfonamide, Tetracycline, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Irrigation water | 13 | 9 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-TS25C-NA30-NOR10C-CIP5C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Sulfonamide, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Soil | 13 | 10 | AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-IMI10C-NOR10C-CIP5C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Macrolide |
| B_S | Irrigation water | 13 | 7 | C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-MEM10C-NOR10C-CIP5C-TCG15C-ATH15C-ERY15C | Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline |
| B_S | Soil | 14 | 9 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ATH15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline |
| C_S | Drinking water | 14 | 8 | A10C-AP10C-FOX30C-GM10C-ATM30C-S10C-NA30C- | Penicillin, Cephalosporin II, Aminoglycoside, Monobactam, |
| | | | | IMI10C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |

| Farm | Isolation source | No of antimicrobials to which isolates were resistant | No of antibiotic classes to which isolates were resistant | Resistance pattern | Antibiotic class(es) |
|---|---|---|---|---|---|
| A_S | Soil | 14 | 10 | AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Macrolide |
| B_S | Spinach | 15 | 9 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Soil | 15 | 10 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-TS25C- IMI10C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Drinking water | 15 | 10 | A10C-AP10C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Irrigation water | 15 | 10 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| B_S | Spinach | 15 | 10 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Soil | 15 | 11 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NA30-IMI10C-NOR10C-CIP5C-TGC15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| C_S | Irrigation water | 16 | 10 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-NA30C-IMI10C-MEM10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |

| Farm | Isolation source | No of antimicrobials to which isolates were resistant | No of antibiotic classes to which isolates were resistant | Resistance pattern | Antibiotic class(es) |
|---|---|---|---|---|---|
| C_S | Drinking water | 16 | 11 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NA30C-IMI10C-NOR10C-CIP5C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |
| A_S | Soil | 16 | 11 | A10C-AP10C-FOX30C-C30C-GM10C-ATM30C-S10C-T30C-TS25C-NA30-IMI10C-MEM10C-NOR10C-TGC15C-ATH15C-ERY15C | Penicillin, Cephalosporin II, Amphenicol, Aminoglycoside, Monobactam, Tetracycline, Sulfonamide, Carbapenem, Fluoroquinolone, Glycylcycline, Macrolide |

GM10C – Gentamicin, S10C – Streptomycin, ATM30C – Aztreonam, C30C – Chloramphenicol, AP10C – Ampicillin, A10C – Amoxycillin, FOX30C – Cefoxitin, IMI10C – Imipenem, MEM10C – Meropenem, NOR10C – Norfloxacin, CIP5C – Ciprofloxacin, TGC15C – Tigecycline, ATH15C – Azithromycin, ERY15C – Erythromycin, NA30C – Nalidixic acid, TS25C – Trimethoprin-sulfamethoxazole/ cotrimoxazole, T30C – Tetracycline

**Genomic fingerprinting and phenotypic characterisation of *Escherichia coli* isolates from Farms A_S, B_S, and C_S**

The figures of the ERIC-PCR profiles and antibiograms from Farms A-S, B_S, and C_S include results from animal feed and pig faecal samples, which were included for a broader context. However, these results are outside the scope of the current project and will therefore not be discussed in detail.

The ERIC-PCR cluster analysis for Farm A_S showed five distinct clusters at a >80% similarity cut-off (Figure 21). Cluster C had isolates recovered from soil, and animal faeces, while Clusters A and D had isolates with a >90% similarity for isolates from irrigation water and faeces, respectively (Figure 21).

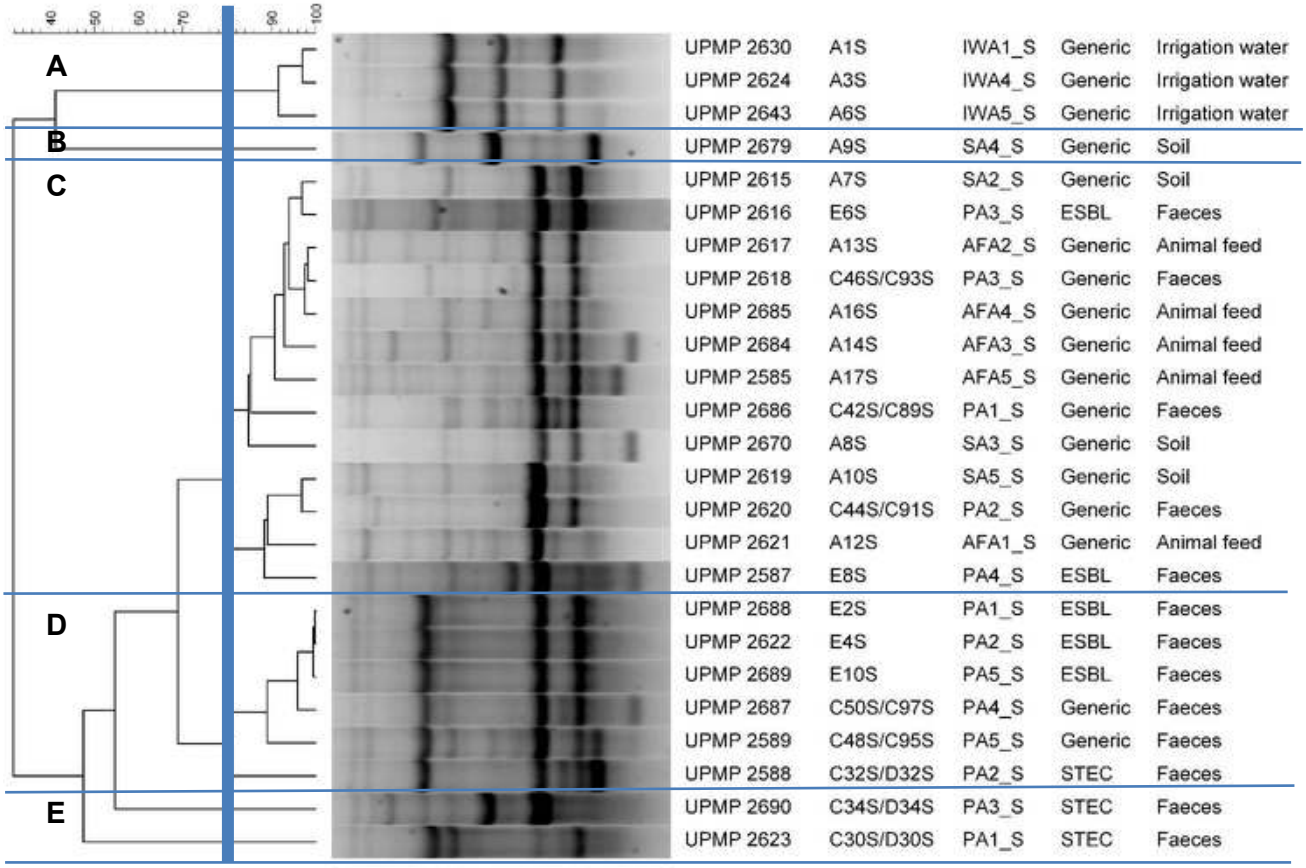



**Figure 21:** Genetic relatedness of *Escherichia coli* isolated from water, soil, and fresh produce on Farm A_S in vegetable-pig small-scale production farms.

On Farm B_S, four distinct clusters were identified at an 80% similarity cut-off value (Figure 22). In Cluster A, *E. coli* from spinach, soil, irrigation water, faeces, and animal feed clustered together, with *E. coli* from spinach and irrigation water that had a >95% similarity. In Cluster B, isolates from spinach clustered with isolates from faeces and animal feed.

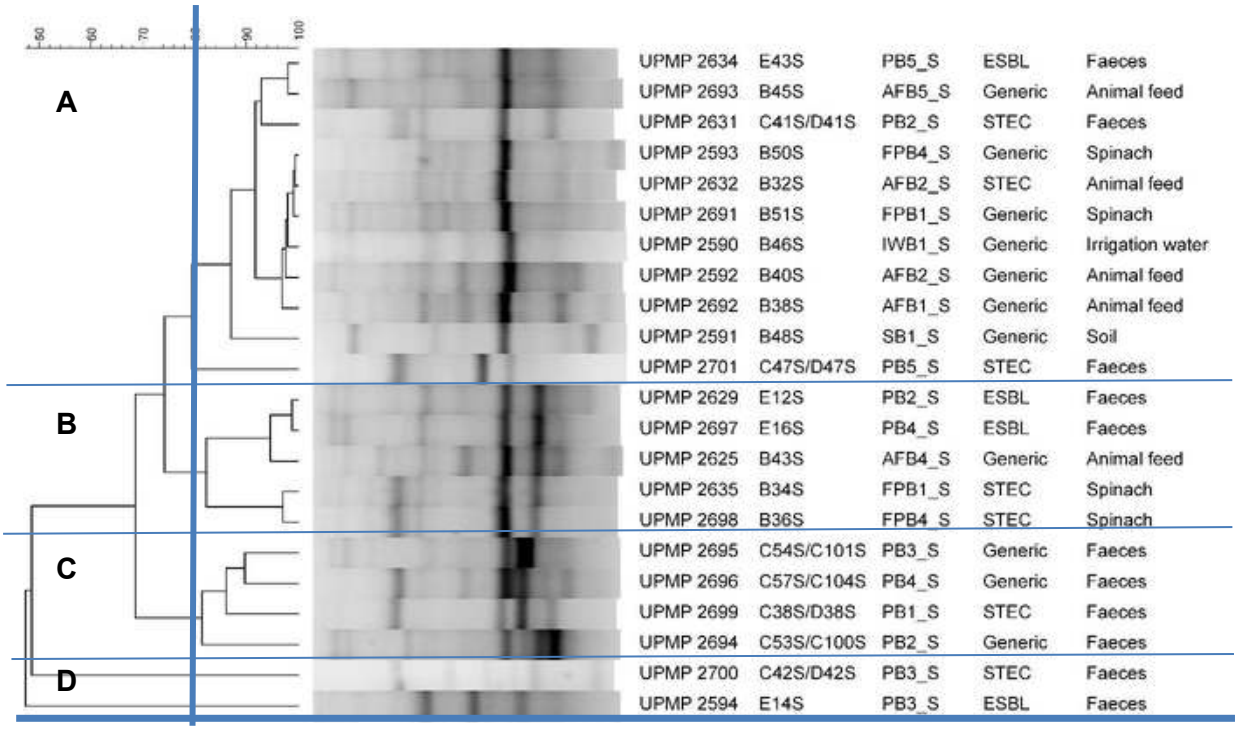



**Figure 22:** Genetic relatedness of *Escherichia coli* isolated from water, soil, and fresh produce on Farm B_S in vegetable-pig small-scale production farms.

On Farm C_S, 10 *E. coli* clusters were identified at an 80% similarity cut-off value (Figure 23). Overall, *E. coli* from the pig drinking water clustered mostly with *E. coli* from faecal samples. In Cluster A, the similarity of *E. coli* isolated from the pig drinking-water samples, spinach, and isolates from the faeces samples was >90%. Moreover, *E. coli* isolates from spinach, soil, and irrigation water clustered at >90% similarity (Figure 23).

Notably, none of the presumptive ESBL/AmpC-producing isolates from Farms A_S, B_S, or C_S tested positive for ESBL/AmpC-production using the double disk synergy test. However, resistance to imipenem, a carbapenem antibiotic, was observed in selected isolates, with the highest number of isolates obtained from pig faeces (73.3%), followed by soil (33.3%), pig feed (33.3%), and drinking water (33.3%).

### Whole genome sequencing (Farms A_S–C_S)

From the small-scale pig production farms, selected *E. coli* isolates were subjected to WGS. In contrast to the isolates from the water-plant-food scenarios (Farms A–F), the *E. coli* isolates from the vegetable-pig production systems harboured fewer resistance genes (Table 7). However, the fosfomycin resistance gene *glpT_E448K* and the colistin resistance gene pmrB_Y358N were present in most *E. coli* isolates across all three farms, isolated from various sources, including spinach, soil, and irrigation water.

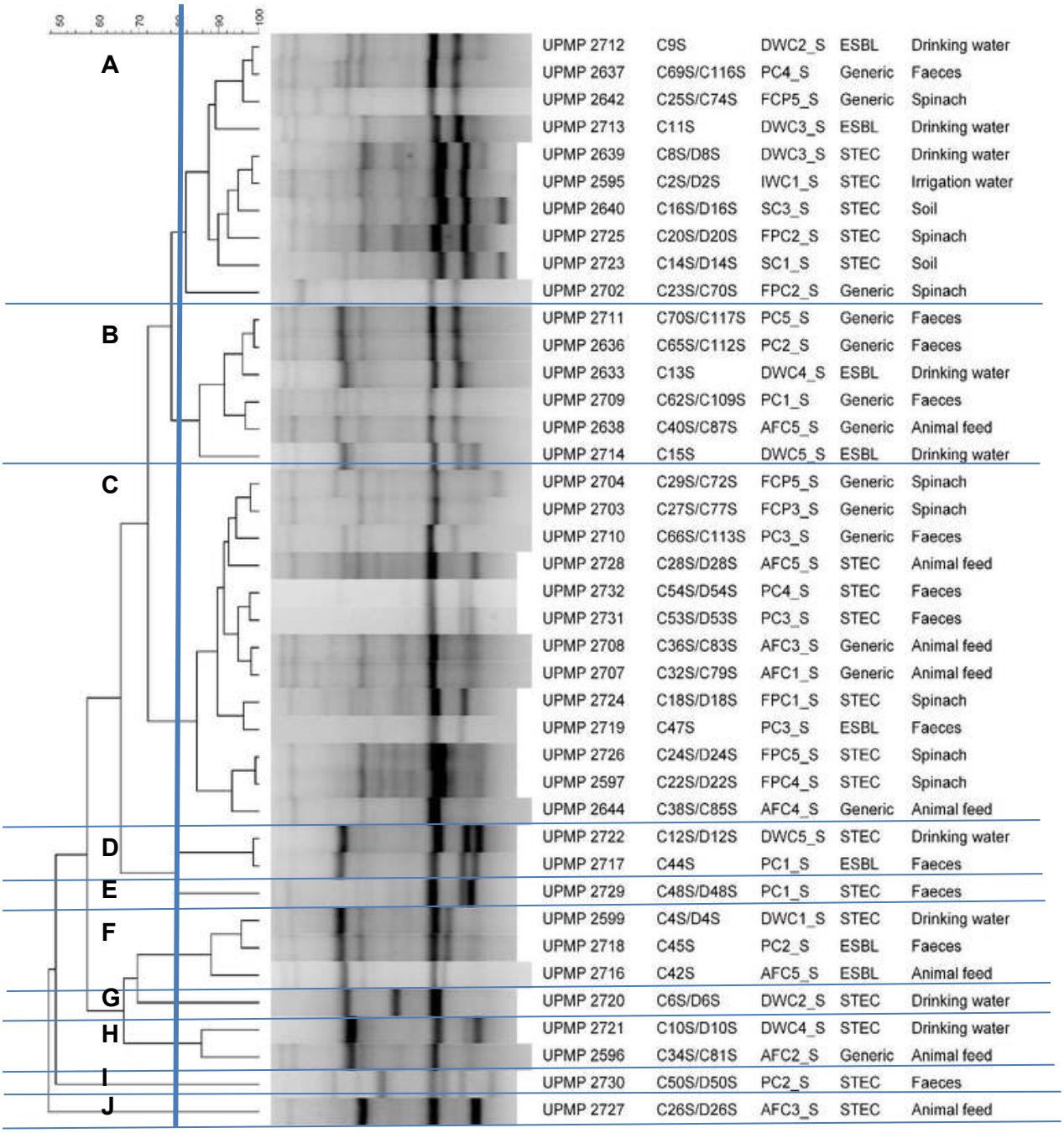



**Figure 23:** Genetic relatedness of *Escherichia coli* isolated from water, soil, and fresh produce on Farm C_S in vegetable-pig small-scale production farms.

**Table 7:** Whole genome sequence characterisation of selected *Escherichia coli* isolates from smallholder vegetable-pig production systems, indicating the sequence types (MLST), serotypes and antimicrobial resistance genes detected, grouped according to various classes of antibiotics.

| Unique ID | Farm | Isolation source | MLST | Serotype | Beta-lactams | Quinolones | Streptomycin | | Sulfonamides | Tetracyclines | Fosfomycin | | Colistin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UPMP 2587 | A_S | Faeces | 212 | H49:O18ac/O18 | CTX-M-14 | gyrA_S8 3L | | | | tet(A) | glpT_E4 48K | fosA3 | pmrB_Y3 58N |
| UPMP 2658 | A_S | Animal feed | 1147 | H2/H47:O128ac/O128ab | | | | | | | glpT_E44 8K | | pmrB_Y3 |
| UPMP 2590 | B_S | Irrigation water | 7631 | H16:O76 | | | | | | | glpT_E4 48K | | pmrB_Y3 58N |
| UPMP 2592 | B_S | Animal feed | 6422 | H8:O82 | | | | | | | glpT_E44 8K | | pmrB_Y3 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| UPMP 2593 | B_S | Spinach | 388 | H2:O112ab | | blaTEM-1 | aph(3")-Ib | aph(6)-Id | *sul2* | tet(A) | glpT_E4 48K | pmrB_Y3 58N |
| UPMP 2591 | B_S | Soil | | H19:O139 | | | | | | | glpT_E4 48K | pmrB_Y3 58N |
| UPMP 2699 | B_S | Faeces | 165 | H51:O180 | | | | | | | | glpT_E4 48K |
| UPMP 2597 | C_S | Spinach | Unknown sequence type | H16:O8 | | | | | | tet(B) | glpT_E4 48K | pmrB_Y3 58N |
| UPMP 2640 | C_S | Soil | 58 | H30:O154 | | | | | | tet(A) | glpT_E4 48K | pmrB_Y3 58N |

## 4.4 Discussion

The overall results of this case study demonstrated that small-scale, integrated vegetable-pig production systems in South Africa can serve as a potential pathway for contamination across water, soil, animal, and crop environments. Similarly, Yang et al. (2024) reported on the interconnected nature of these environments and the potential for cross-contamination among them. In the current study, *E. coli* was detected in drinking/irrigation water (0–0.89 log MPN/100mL), soil (up to log 3.7CFU/g), and produce (up to log 4.7CFU/g), that exceeded the WHO zero-tolerance for drinking water but were within irrigation limits for faecal coliforms (≤1 000MPN/100mL; FAO and WHO, 2021; WHO, 2024).

Compared to a similar South African study (du Plessis et al., 2015), which associated irrigation water quality with produce safety across broader agricultural contexts (e.g., phenotypic or genotypic correlations for *E. coli* in onions and lettuce showing 67–92% similarity), this study expands upon those findings by focusing on integrated pig farming systems. It demonstrated a greater incidence of animal-related contamination, evidenced by the high similarity of *E. coli* strains between samples from free-roaming pigs on Farm C_S and elevated *E. coli* levels observed on the produce. Although classical foodborne pathogens were infrequently detected, the microbial profiles identified a more concerning risk: all recovered *E. coli* isolates demonstrated MDR to three or more antibiotic classes. This MDR contrasts with the study by du Plessis et al. (2015), which placed less emphasis on antimicrobial resistance, yet aligns with other research indicating borehole vulnerabilities in peri-urban South African production systems (Richter et al., 2021; Viviers et al., 2024; Gemmell and Schmidt, 2012). Moreover, broader studies across Africa and LMICs corroborate the high prevalence of MDR organisms in pig production systems. For example, in Uganda, semi-intensive pig farms exhibited higher carriage of AMR genes than free-range systems, likely due to higher transmission risks (Muwonge et al., 2025). The close genetic relationship observed among isolates from water, soil, crops, and animals provides clear evidence of ongoing microbial exchange within these interconnected systems. This highlights irrigation water and animal contact as significant transmission routes.

## 4.5 Conclusion

This case study revealed how integrated crop-livestock farming intensifies microbial contamination pathways across the water-soil-animal-crop continuum, building directly on findings from the diversified fresh produce farms in Case Study 1. Borehole water used for both irrigation and pig drinking exceeded WHO zero-tolerance limits for *E. coli*, while coliform and *E. coli* levels on spinach and soil reached up to 4.7 log CFU/g, with free-roaming pigs directly linked to elevated produce contamination. All recovered *E. coli* isolates were MDR), showing resistance to three or more antibiotic classes. Similar to results from Case Study 1, ERIC-PCR and whole-genome sequencing confirmed strong genetic linkages and shared resistance genes between water, soil, spinach, and pig faeces, underscoring animal waste and shared water sources as primary transmission routes. These results advance the

development of fit-for-purpose microbiological water quality guidelines by exposing the heightened risks unique to integrated systems, where dual-use water and animal contact rapidly transfer MDR bacteria to raw-consumed crops. By demonstrating the power of molecular tools (ERIC-PCR and WGS) for source tracking and AMR surveillance, the research strengthens calls for targeted interventions—such as water treatment, restricted animal access, proper manure composting, and separate water supplies—to safeguard produce safety, limit resistance spread and enhance sustainable smallholder production in South Africa.

## CHAPTER 5: CASE STUDY 3: River Irrigation Water as a Source of Contamination in Fresh Produce Sold at Market

*N. Ndlazi, T. Khwela, and S. Schmidt*

### 5.1 Introduction

The microbiological quality of surface water used for irrigation is increasingly recognised as a crucial determinant of fresh-produce safety, particularly in LMICs where contaminated rivers are widely used for smallholder and informal agriculture. Irrigation water contaminated with faecal matter is a well-established pathway for the transmission of enteric pathogens and antimicrobial-resistant bacteria to crops consumed raw, thereby posing direct risks to public health (WHO, 2006; EC, 2020).

Beyond classical pathogen risks, the emergence and dissemination of AMR in aquatic environments has become a global One Health priority. Rivers impacted by wastewater inputs serve as reservoirs and transmission corridors for resistant bacteria and resistance genes, which can be transferred to crops via irrigation and subsequently enter the food chain (WHO, 2017). The use of indicator organisms such as *Escherichia coli* and intestinal enterococci, alongside pathogen detection (e.g. *Salmonella* spp.), therefore provides an integrated measure of both faecal pollution pressure and potential food safety risks (WHO, 2006).

In peri-urban areas of KwaZulu-Natal, such as the Sobantu Township in Pietermaritzburg, small-scale farmers rely on the uMsunduzi River as a primary irrigation source for crops such as cabbage and spinach, amid broader water scarcity and pollution challenges (Gemmell and Schmidt, 2012; du Plessis et al., 2015). This river, often contaminated by deteriorating wastewater infrastructure, poses a risk of microbial transfer to fresh produce via overhead irrigation, potentially exacerbating the dissemination of foodborne pathogens in informal supply chains.

Against this backdrop, the present study aimed to characterise the seasonal physicochemical quality, microbial contamination patterns, pathogen occurrence, and antimicrobial resistance profiles of bacteria recovered from the uMsunduzi River water over 13 months, and to relate these findings to historical data from the same site. This comparative approach enables an assessment of long-term trends in environmental degradation and provides evidence to inform risk-based irrigation water management to produce minimally processed leafy vegetables within smallholder and informal food systems.

### 5.2 Experimental Procedures

In KwaZulu-Natal, the Sobantu Township is in Pietermaritzburg. The uMsunduzi River (Figure 24) runs through the township and serves as an irrigation source for local small-scale farmers, who grow fresh produce such as cabbage and spinach. River water samples were collected

over four seasons at a fast-flowing point at the uMsunduzi River, as well as from leafy vegetables of various origins sold by street vendors in downtown Pietermaritzburg.

River water samples from the uMsunduzi River were generally collected between 08h00 and 12h00 at a fast-flowing point in Sobantu using sterile 1L Schott bottles. Sampling was conducted about 30cm below the water surface. The samples were transported to the laboratory on ice, and analysis was performed within 2h. Water samples were used directly or decimally diluted using sterile saline as diluent (8.5g of NaCl per 1L of distilled water, pH 7.0), usually up to $10^{-8}$. The analyses of leafy vegetables of various origins were undertaken, focusing on the general burden (APC procedure as reported previously [Gemmell and Schmidt, 2012]), the detection of *E. coli* by employing the same decimal dilutions as used for the water samples and directly plating out in duplicate on TBX agar (ISO 16649-2, 2001), and testing for the presence of antibiotic-resistant plant associated heterotrophic bacteria by employing PCA with an added representative cephalosporin (cephalexin) antibiotic.

The pH and the temperature of the water samples from the uMsunduzi River were measured on site with a pH/°C meter model HI98108 (Hanna Instruments, Villafranca Padovan, Italy), while the COD was determined using COD test kits (10-150mg/L) and the NOVA 60 system following the manufacturer's (Merck, Darmstadt, Germany) instructions.



**Figure 24:** uMsunduzi River water sampling site and students sampling water that is used in Sobantu in Pietermaritzburg, and a typical field (photo on the top right) used for vegetable production in the river proximity.



**Figure 25:** Representative samples of fresh produce from various origins that were analysed and street vendor locations in downtown Pietermaritzburg.

### 5.2.1 Sample processing and enumeration for aerobic plate counts, faecal coliforms, *Escherichia coli* and intestinal enterococci

River water samples were generally collected between 8h00 and 12h00 at a fast-flowing point on the uMsunduzi River in Sobantu using sterile 1L Schott bottles. Sampling was conducted about 30cm below the water surface. The samples were transported to the laboratory on ice, and the sample analysis was carried out within 2h. Water samples were used directly or diluted decimally with sterile saline as the diluent (8.5g of NaCl per 1L of distilled water, pH 7.0), usually up to $10^{-8}$.

Aerobic plate counts were established using Plate Count agar (Merck, Darmstadt, Germany) and duplicate spread plating of 100µL samples according to SANS 4823 (SANS, 2007), followed by incubation at 30°C. In addition, Tryptone Bile X-glucuronide Agar (Oxoid, Basingstoke, United Kingdom) was used either to determine *E. coli* counts by duplicate plating of 100µL river water samples and decimal dilutions, as outlined in ISO 16692-2 (2001), or to confirm presumptive *E. coli* isolates phenotypically, in addition to EMB agar. TBX agar was incubated at 37°C for 2h, then at 44°C for 24h. Colonies exhibiting blue colour due to β-glucuronidase-activity were enumerated.

Enumeration of total and faecal coliforms and *E. coli* from river water used for irrigation employed the most probable number method (MFHPB-19) as previously described (Gemmell and Schmidt, 2013; Beharielal et al., 2018). MPN values were expressed as MPN/100mL according to de Man (1983). Confirmation of presumptive *E. coli* isolates was carried out using Eosin Methylene Blue (Merck, Darmstadt, Germany) and Tryptone Bile X-glucuronide Agar (Oxoid, Basingstoke, United Kingdom), prescribed biochemical testing (GIMViC), and PCR (*gadA*) as reported previously (Gemmel and Schmidt, 2013).

Intestinal enterococci were quantified in irrigation water following SANS ISO 7899-2 (2004) with 100mL of river water or decimal dilutions thereof filtered through a sterile 0.45µm membrane filter (Whatman, Maidstone, United Kingdom), followed by transfer of membrane filters onto Slanetz Bartley agar (Merck, Darmstadt, Germany), incubated at 35°C for 44h and subsequent transfer onto preheated (44°C) Bile Esculin Azide agar (Merck) incubated for 2h at 44°C to confirm candidate colonies detected on Slanetz Bartley agar as intestinal enterococci. Additional confirmation of isolated intestinal enterococci was done by PCR (Desay et al., 2000).

Since September 2024, the presence of presumptive *Klebsiella* spp. has been verified in irrigation water samples by spreading 100µL of river water or decimal dilutions thereof onto *Klebsiella* Chromo Select Agar (Sigma, Kanagawa, Japan) and incubating at 37°C for 24h. Purple-magenta coloured colonies were regarded as presumptive *Klebsiella* spp.

### 5.2.2 Detection and isolation of presumptive positive potential human pathogenic bacteria from river water in KwaZulu-Natal

The detection of *Salmonella* spp. in irrigation water followed the ISO 6579 procedure (2017). However, 100mL, 10mL, and 1mL river water samples were used for pre-enrichment, employing 100mL and 10mL of prewarmed double-strength and 9mL of single-strength sterile buffered peptone water (Oxoid, Basingstoke, United Kingdom), with incubation at 37°C for 24h. Subsequent selective enrichment in Mueller-Kaufmann Tetrathionate-Novobiocin broth (Merck, Darmstadt, Germany) and Rappaport Vassiliadis Soy broth (Oxoid) with 24h incubation (37/41,5°C for 24h) was followed by plating out samples on Xylose Lysine Desoxycholate (Merck) and a chromogenic agar (*Salmonella* Chromoselect improved) (Merck), incubated for 24h at 37°C. Typical colonies (black centre on XLD, pale-pink to mauve

on SCI agar) were considered presumptive *Salmonella* spp. Confirmation of presumptive *Salmonella* spp. isolates were tested using the prescribed biochemical tests and PCR (*invA*).

For selected and confirmed *E. coli* isolates from the uMsunduzi River in Sobantu, the antibiotic susceptibility profiles were established employing the EUCAST AST procedure and breakpoints as reported previously. The following antibiotic test disks (Oxoid, Basingstoke, United Kingdom) were employed for *E. coli* and *Salmonella* spp. at the prescribed concentrations: ampicillin, amoxicillin-clavulanate, aztreonam, cephalexin, cefotaxime, ceftazidime, tobramycin, norfloxacin, tigecycline, and meropenem. Similarly, for selected, confirmed *Enterococcus* spp. isolates, the following antibiotic test disks were used at the prescribed concentrations: imipenem, ampicillin, linezolid, teicoplanin, vancomycin, norfloxacin, tigecycline, gentamicin, and trimethoprim. A MDR *E. coli* isolate from the uMsunduzi River was further analysed by whole-genome sequencing using isolated genomic DNA and established bioinformatic tools (Ndlazi et al., 2025). For selected *E. coli* isolates, biofilm-forming capacity was assessed as previously reported (Beharielal et al., 2018).

### 5.3 Results and Discussion

According to DWAF (1996), the acceptable range of pH values of raw water used for irrigation is between 6.5 and 8.4, which was met on 36 of the 37 sampling occasions, as the pH for uMsunduzi River water samples ranged from 6.49 to 7.49 at the time of sampling (Figures 26 and 27). In an earlier study, the pH values for the same river ranged from 7.18 to 8.47 (Gemmell and Schmidt, 2013).

The COD values determined for the river water at the time of sampling ranged from 10–46mg $O_2$ perL, meeting irrigation water requirements. An earlier report analysing uMsunduzi River water upstream and downstream of the Darvill WWTP found maximum COD values of 43.2mg/L $O_2$ (Dickens and Graham, 1998).

The aerobic plate counts for the uMsunduzi River ranged from $\log_{10}$ 3.83 (October) to 6.18 (February) CFU/mL, clearly exceeding the DWAF (1996c) recommendation for domestic use of 100CFU/mL. An earlier study sampled the same river in Sobantu in 2010 and reported values ranging from $\log_{10}$ 3.45 to 4.26CFU/mL, indicating that the current microbial burden of the river is substantially higher, based on the APC values obtained.

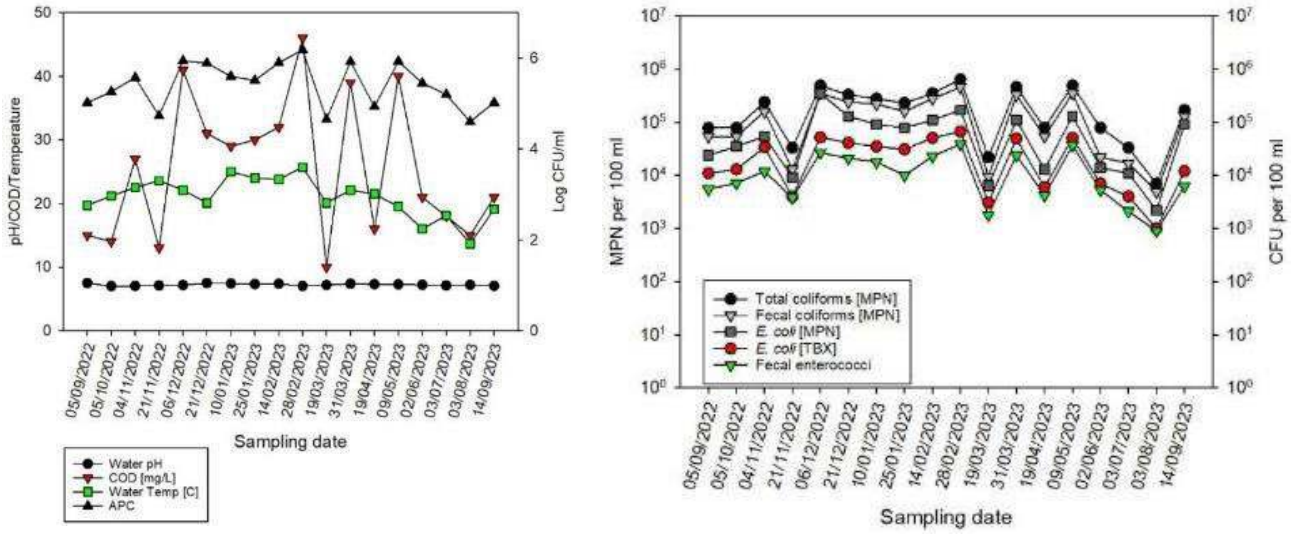



**Figure 26:** Physicochemical parameters and the microbial burden of the uMsunduzi River water samples collected in Sobantu from May 2022 to September 2023 over the four seasons.

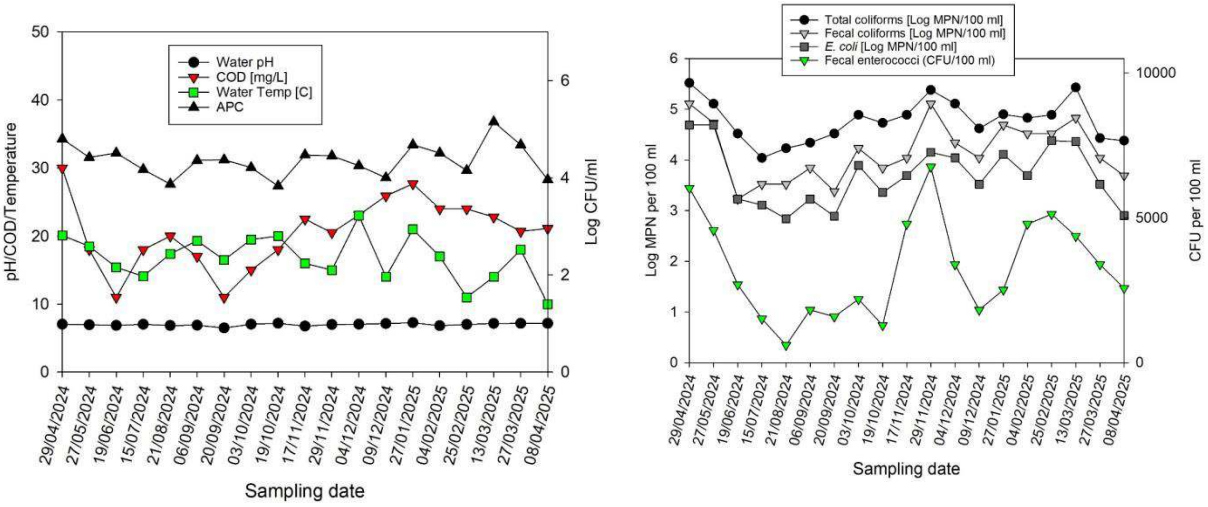



**Figure 27:** Physicochemical parameters and the microbial burden of the uMsunduzi River water samples collected in Sobantu from April 2024 to April 2025 over the four seasons.

The MPN values for total coliforms in the uMsunduzi river water on the 37 sampling occasions ranged from 7 000 to 790 000 MPN/100mL, and those for faecal coliforms from 4 900 (winter) to 460 000 (summer) (Figures 25 and 26). The values for faecal coliforms exceeded the acceptable surface water quality threshold of 1 000/100mL suggested by UNEP (2016) on all 37 sampling occasions. The WHO (2006) threshold for the safe use of irrigation water (1 000 *E. coli*/100mL) was exceeded on most (34/37) sampling occasions, with the *E. coli* burden ranging from 700 (winter) to 170 000 (summer) MPN/100mL. These values are substantially higher than the most recent guideline values suggested by the European Union for safe irrigation at 10 *E. coli*/100mL when direct contact between water and plants can occur via irrigation (EC, 2020).

Notably, *E. coli* counts obtained using TBX agar ranged from 1 000 (winter) to 64 000 (summer) CFU/100mL and were thus lower than the corresponding MPN values for *E. coli*, even though the trend over the four seasons followed that obtained using the MPN procedure (Figure 25). The lower counts can be explained by the higher selectivity of TBX agar, which is less efficient in recovering stressed or sublethally damaged *E. coli* cells than a MPN procedure (Vergine et al., 2017).

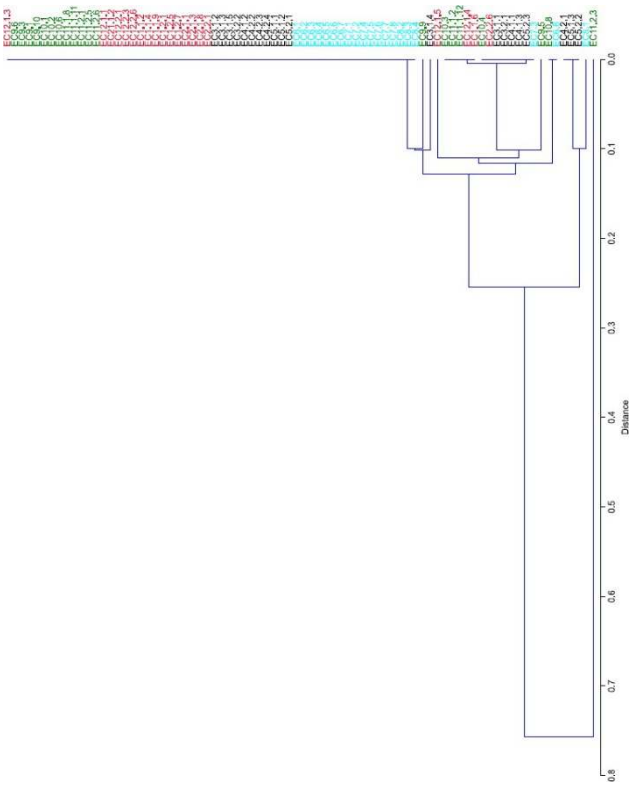
Thus, Gemmell and Schmidt (2013) reported substantially lower total coliform counts (ranging from 490 to 84 000 MPN/100mL) for the same river more than a decade ago. For faecal coliforms and *E. coli,* the highest reported MPN/100mL values were established as 63 000 and 7 900 MPN/100mL, respectively. The comparison of current results for total and faecal coliforms and *E. coli*, using the same methodology as that reported in 2010, highlights the continuously deteriorating microbiological quality of the uMsunduzi River, sadly confirming its reputation as a serious health risk to domestic and recreational users of river water (Ngubane et al., 2022). The burden of intestinal enterococci, bacteria considered an additional useful indicator for faecal pollution, ranged in the uMsunduzi River water samples from 600 (winter) to 39 000 (summer) CFU/100mL (Figures 25 and 26) and exceeded on all 37 sampling occasions the suggested threshold value of 100 intestinal enterococci per 100mL for full contact irrigation of ready-to-eat vegetables (DIN 19650, 1999).

Since September 2024, river water samples have been additionally analysed for the presence of presumptive *Klebsiella* spp., revealing its presence on all sampling occasions. These results, in combination, confirmed the high microbial burden of the river water and its poor microbiological quality. Confirmed *Salmonella* spp. (*invA* PCR positive) were detected in the uMsunduzi River water at seven of the 37 sampling occasions, possibly due to faecal matter entering the river via sewage. An earlier study reported similar results regarding the presence of *Salmonella* spp. in uMsunduzi River water samples (Gemmell and Schmidt, 2013). However, none of these confirmed *Salmonella* spp. isolates exhibited phenotypic antibiotic resistance when undergoing AST. Most of the 80 selected *E. coli* isolates originating from the uMsunduzi River between September 2022 and September 2023 were susceptible to all 10 tested antibiotics (e.g., AMP, AMC, CAZ, CL, ATM, CTX, MEM, NOR, TGC, TOB). However, resistances were detected in more than 20% (high level) of isolates against ampicillin and at lower frequencies (low level) against amoxicillin-clavulanate, aztreonam, cephalexin, cefotaxime, ceftazidime, tobramycin, and norfloxacin. The cluster analysis based on these results is shown in Figure 27.

While no riverine *E. coli* isolate showed resistance to tigecycline or the tested carbapenem, meropenem, one MDR *E. coli* isolate, strain 1123, was further analysed using a procedure closely matching a previously reported WGS analysis of a fresh-produce-associated AmpC-producing *Serratia marcescens* (Claussen and Schmidt, 2023).

The *E. coli* strain 1123 (genome size = 4,949,272bp, G+C content = 50.70%) was assigned to sequence type 131 (ST 506$^{P}$), a global clonal lineage involved in bloodstream and urinary

tract infections. The isolate belonged to phylogroup B2, serotype O16:H5, and *fimH*41. It exhibited the properties of an extraintestinal pathogenic *E. coli* (ExPEC), with typical virulence genes (e.g., *fyuA*) detected. The isolated strain produced cefotaximase (*bla*$_{CTX-M15}$), a class A ESBL-type enzyme. The ESBL activity was confirmed phenotypically using an established screening procedure (MAST D67C ESBL Davies Diagnostics) and by demonstrating the ability of resting cells to hydrolyse the chromogenic cephalosporin, nitrocefin. The strain exhibited phenotypic resistance against antibiotics representing more than three classes (e.g., ampicillin, aztreonam, cefalexin, cefotaxime, ceftaroline, ceftazidime, ciprofloxacin, gentamicin, levofloxacin, moxifloxacin, norfloxacin, tobramycin, trimethoprim, and trimethoprim-sulfamethoxazole), thus qualifying as MDR. It carried genes encoding acquired antibiotic resistance (*aac(3)-IId, aph(3")-Ib, aph(6)-Id*, *bla*$_{CTX-M15}$, *bla*$_{TEM-1B}$, *dfrA17, mph*(A), *qacE, sul1, sul2, and tet*(A)) and chromosomal mutations enabling fluoroquinolone resistance (*gyrA* (p.S83L), *gyrA* (p.D87N), *parC* (p.S80I), and *parC* (p.E84V)). Strains of serotype O16:H5, thus matching our uMsunduzi *E. coli* isolate, have recently found in a river in Japan (Sato et al., 2024).




Green = spring isolate. Red = summer isolate. Black = autumn isolate. Aquamarine = winter isolate.

**Figure 28:** Cluster analysis of the 80 *E. coli* isolates from the uMsunduzi River collected between May 2022 and September 2023 based on the resistance profiles obtained using the 10 antibiotics specified.

**Table 8:** Summary of the resistance profiles of 60 *E. coli* river isolates collected between April 2024 and April 2025.

| Isolates tested | Antibiotics tested | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | AMP | AMC | CL | CTX | ATM | NOR | TGC | MEM | TOB | CAZ |
| A1.1 | R | S | S | S | S | S | S | S | S | S |
| A1.2 | R | R | S | S | S | S | S | S | S | S |
| A4.1 | R | R | S | S | S | S | S | S | S | S |
| A6.1 | R | S | S | S | S | S | S | S | S | S |
| A8.1 | R | S | R | R | S | S | S | S | S | S |
| M1 | S | S | R | S | S | S | S | S | S | S |
| M3 | S | S | S | S | S | S | S | S | S | S |
| M5 | R | S | R | R | S | R | S | S | S | S |
| M7 | S | S | S | S | S | S | S | S | S | S |
| M10 | R | S | S | S | S | S | S | S | S | S |
| June1 | R | R | R | R | R | S | S | S | S | R |
| June3 | S | S | S | S | S | S | S | S | S | S |
| June6 | S | S | S | S | S | S | S | S | S | S |
| June7 | S | S | S | S | S | S | S | S | S | S |
| June5 | S | S | S | S | S | S | S | S | S | S |
| July1 | S | S | S | S | S | S | S | S | S | S |
| July4 | S | S | S | S | S | S | S | S | S | S |
| July5 | S | S | S | S | S | S | S | S | S | S |
| July8 | S | S | S | S | S | S | S | S | S | S |
| July10 | S | S | S | S | S | S | S | S | S | S |
| Aug3 | S | S | S | S | S | S | S | S | S | S |
| Aug1 | S | S | S | S | S | S | S | S | S | S |
| Aug5 | R | R | S | S | S | S | S | S | S | S |
| Aug7 | R | R | S | S | S | S | S | S | S | S |
| Aug8 | S | S | S | S | S | S | S | S | S | S |
| Sep1.1 | S | S | S | S | S | S | S | S | S | S |
| Sep1.12 | S | S | S | S | S | S | S | S | S | S |
| Sep1.5 | S | S | S | S | S | S | S | S | S | S |
| Sep1.9 | S | S | S | S | S | S | S | S | S | S |
| Sep2.5 | S | S | S | S | S | S | S | S | S | S |
| Oct1.1 | S | S | S | S | S | S | S | S | S | S |
| Oct1.3 | S | S | S | S | S | R | S | S | S | S |
| Oct1.6 | S | S | S | S | S | S | S | S | S | S |
| Oct2.4 | R | S | R | R | R | S | S | S | R | R |
| Oct2.6 | S | S | S | S | S | S | S | S | S | S |
| Nov1.1 | R | S | S | S | S | S | S | S | S | S |
| Nov1.3 | S | S | S | S | S | S | S | S | S | S |
| Nov1.6 | S | S | S | S | S | S | S | S | S | S |
| Nov2.4 | S | S | S | S | S | S | S | S | S | S |
| No2.6 | R | S | S | S | S | R | S | S | S | S |
| Dec1.2 | R | R | S | S | S | S | S | S | S | S |
| Dec1.6 | S | S | S | S | S | S | S | S | S | S |
| Dec1.10 | S | S | S | S | S | S | S | S | S | S |
| Dec2.3 | S | S | S | S | S | S | S | S | S | S |
| Dec2.7 | S | S | S | S | S | S | S | S | S | S |
| Jan1 | S | R | S | S | S | S | S | S | S | S |
| Jan3 | S | S | S | S | S | S | S | S | S | S |
| Jan5 | S | S | S | S | S | S | S | S | S | S |
| Jan6 | R | S | S | S | S | S | S | S | S | S |
| Jan9 | R | R | S | S | S | S | S | S | S | S |
| Feb1.2 | S | S | S | S | S | S | S | S | S | S |
| Feb1.4 | R | S | S | S | S | S | S | S | S | S |
| Feb1.9 | R | R | R | R | R | R | R | S | R | R |
| Feb2.7 | S | R | S | S | S | S | S | S | S | S |
| Feb2.8 | S | S | S | S | S | S | S | S | S | S |
| Mar1.2 | S | S | S | S | S | S | S | S | S | S |
| Mar1.6 | S | S | S | S | S | S | S | S | S | S |
| Mar2.5 | S | S | S | S | S | S | S | S | S | S |
| Mar2.6 | S | S | S | S | S | S | S | S | S | S |
| Mar2.1 | S | S | S | S | S | S | S | S | S | S |

As for the *E. coli* isolates collected between May 2022 and September 2023, most of the 60 *E. coli* isolates specified in Table 8 were susceptible to the 10 tested antibiotics. While again,

no resistance against the tested carbapenem (meropenem) was detected; one MDR isolate exhibited resistance against tigecycline.

For riverine *Enterococcus* spp. isolates obtained between September 2022 and September 2023, the AST profiling showed that, similar to the *E. coli* isolates from the same period, most of the 40 tested *Enterococcus* spp. isolates was susceptible to the nine tested antibiotics (Figure 28). However, more than 10% of riverine *Enterococcus* spp. isolates displayed antibiotic resistance against at least one clinically relevant antibiotic.

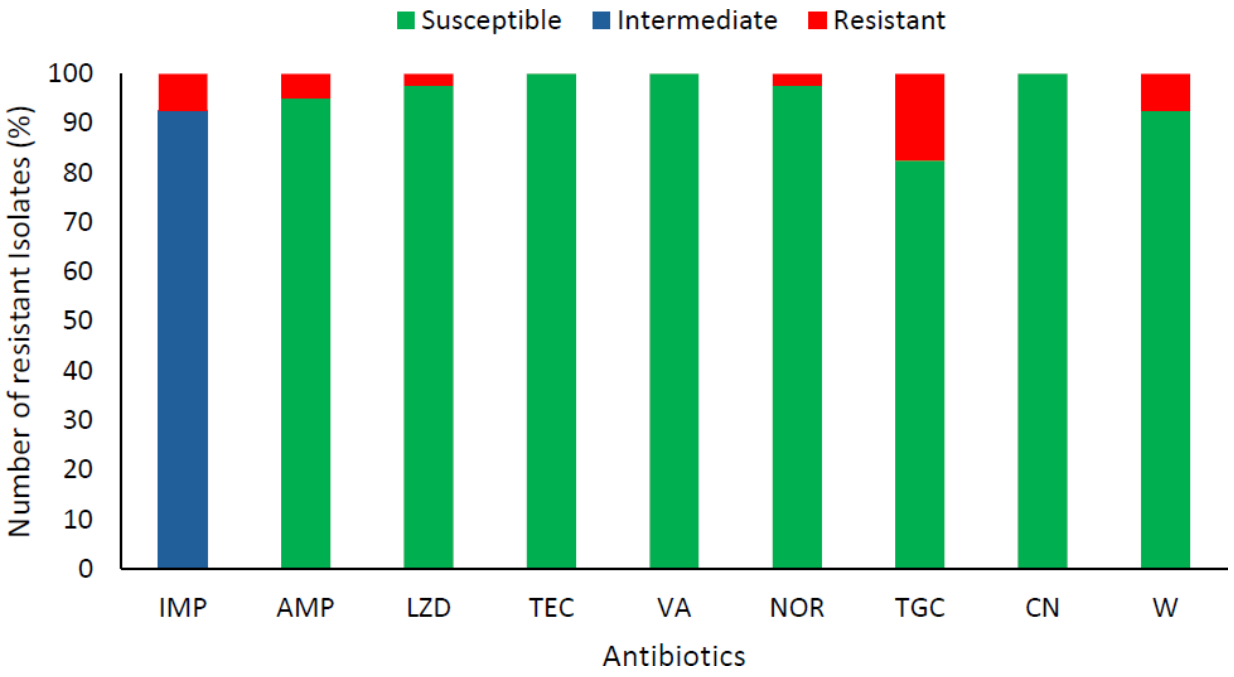



**Figure 29:** Antibiotic resistance to nine tested antibiotics and susceptibility of 40 *Enterococcus* spp. isolates from the uMsunduzi River.

Riverine *E. coli* and *Enterococcus* spp. isolates formed biofilms, as demonstrated in Figures 30 and 31. This ability to form biofilms on natural and artificial surfaces has implications for production hygiene and surface contamination in downstream processing.

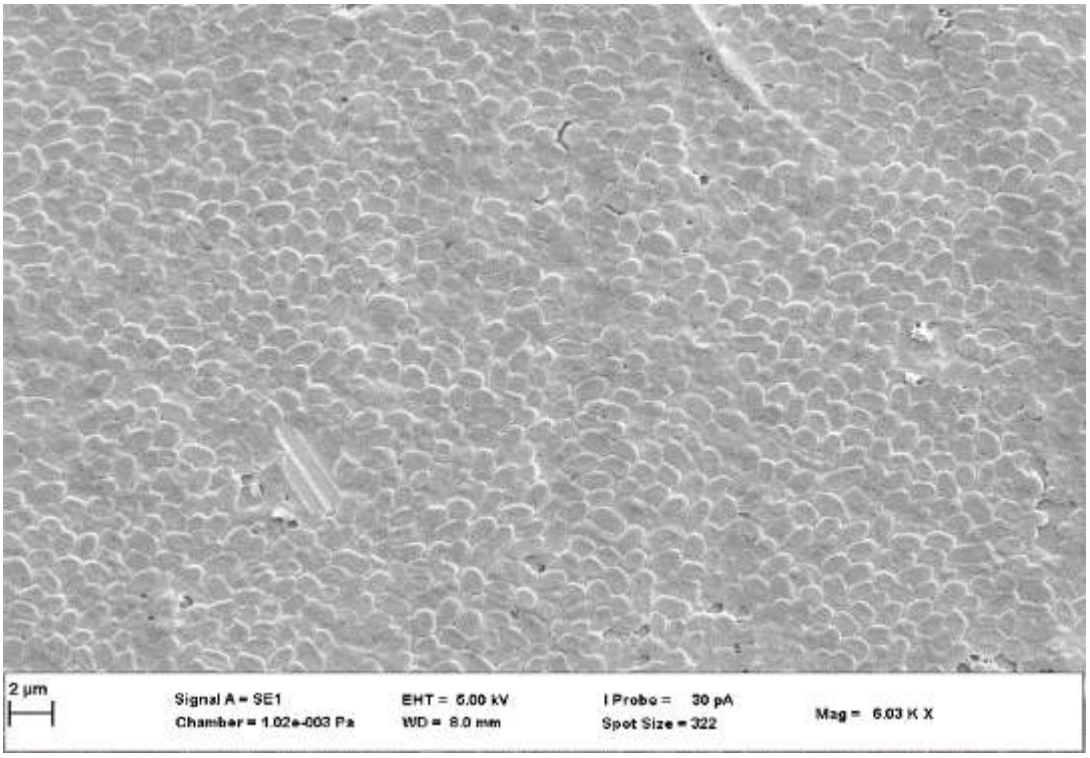



**Figure 30:** Biofilm formation by *E. coli* strain 5.1.3. on stainless steel after 48h, as detected by scanning electron microscopy.

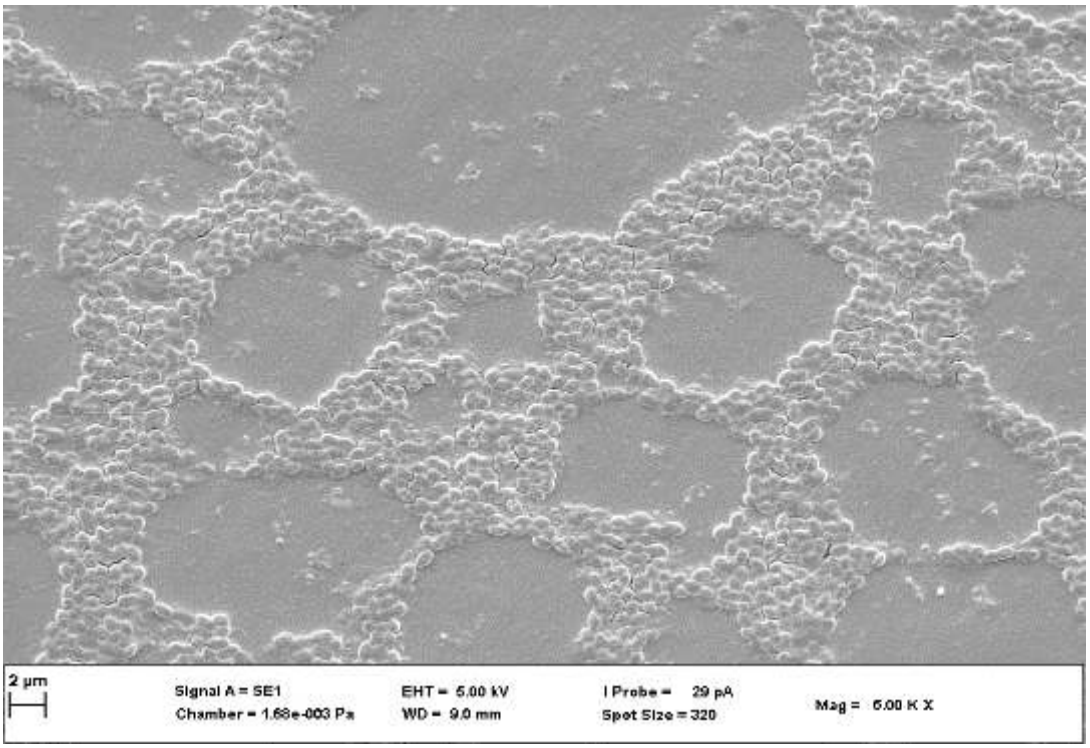



**Figure 31:** Biofilm formation by *Enterococcus* sp. strain 9.1. on stainless steel after 96h, as detected by scanning electron microscopy.

Finally, the results obtained so far for various leafy fresh produce originating from farms, street vendors, and retailers, such as kale and spinach, showed that the APC/g burden ranged for kale from $\log_{10}$ 5.15 to $\log_{10}$ 6.64CFU/g, for Swiss chard and true spinach from $\log_{10}$ 4.39 to $\log_{10}$ 7.44CFU/g, for cabbage from $\log_{10}$ 4.55 to $\log_{10}$ 7.11CFU/g, for spring onion from $\log_{10}$ 5.37 to $\log_{10}$ 7.11CFU/g, and for lettuce (green, red, Romaine) from $\log_{10}$ 5.22 to $\log_{10}$ 6.48CFU/g. These values are in a range previously reported for similar fresh produce from a local location (Gemmell and Schmidt, 2012) and an international location with fresh produce of comparable origins analysed (Schuh et al., 2020). However, for the fresh-produce samples tested so far, the *E. coli* burden was below the limit of detection when analysed directly using TBX agar.

Isolates, provisionally assigned to the phylum *Pseudomonadota* based on basic morphological and physiological characteristics, were obtained from most of the produce samples analysed (e.g., spinach and kale) using cefalexin-enhanced PCA.

The MALDI-ToF-MS results obtained at the University of Pretoria indicate that the selected plant-associated isolates identified to the genus level (score ≥1.7) and sometimes even to the species level (score ≥2) are bacteria well known to be associated with plants and soil or water bodies.

Thus, most isolates yielding at least genus-level identification using the Bruker Sirius system at the University of Pretoria belonged to the genus *Pseudomonas* (family *Pseudomonadaceae)*, with a smaller number of isolates assigned to the genera *Chryseomonas* (family *Pseudomonadaceae)*, *Acinetobacter* (family *Moraxellaceae),* *Xanthomonas* (family *Xanthomonadaceae)*, *Stenotrophomonas* (family *Lysobacteraceae)*, *Sphingomonas* (family *Sphingomonadaceae)*, *Pantoea* (family *Erwiniaceae)*, *Brevundimonas* (family *Caulobacteraceae)*, and *Enterobacter* (family *Enterobacteriaceae)*.

Among these isolates, species-level assignments (score >2) were established for *Pseudomonas rhodesiae*, *P. koreensis*, *P. oryzihabitans*, *P. fulva, Chryseomonas aquaticum, Enterobacter bugandensis,* and *Stenotrophomonas maltophilia*. The latter two are of interest, as they are regarded as opportunistic pathogens (Pati et al., 2018; Sapula et al., 2024). Initial AST profiling indicates that the *E. bugandensis* isolate exhibits resistance against selected β-lactam antibiotics (e.g., $Cl^R$) and a tested fluoroquinolone ($Nor^R$).

However, species-level assignment was not achieved for isolates identified as *Pantoea*, even though scores above two were obtained in some cases.

The uMsunduzi River water is evidently unsuitable for the safe irrigation of minimally processed produce, as on all 37 sampling occasions, the guideline values recommended for direct contact irrigation and the two faecal indicators, *E. coli* and intestinal enterococci, were exceeded. Additionally, *Salmonella* spp. was detected in several river water samples. Finally, WGS analysis confirmed one *E. coli* isolate as an ExPEC strain, representing a global lineage involved in urinary tract and bloodstream infections. Furthermore, the presence of antibiotic-

resistant bacteria, mostly gamma-Proteobacteria, on various fresh produce from multiple origins, including opportunistic pathogens such as *Stenotrophomonas maltophilia*, was observed, as identified by MALDI-ToF-MS.

While the levels of antibiotic resistance established for the 140 selected *E. coli* river water isolates ranged from high (e.g., ampicillin) to low (e.g., tobramycin), with no carbapenem resistance detected, the presence of antibiotic-resistant indicator bacteria, such as *E. coli* (including MDR *E. coli* strains and intestinal enterococci) at elevated levels in river water (even including a pathogenic MDR *E. coli* strain) used for irrigation of ready-to-eat produce, is a reason for concern. Similarly, the presence of cephalosporin-resistant heterotrophic bacteria, possibly due to the notoriously poor outer membrane permeability for β-lactam antibiotics of members of the *Pseudomonadaceae*, including opportunistic pathogens on fresh produce of various origins with and without a known irrigation history, is a serious concern in terms of the efficacy of possible antibiotic therapy for customers consuming such minimally processed contaminated fresh produce.

## 5.4 Conclusion

This case study on the uMsunduzi River in KwaZulu-Natal confirms and intensifies the concerns raised in the previous two case studies regarding irrigation water as a major vector for microbial contamination and AMR in smallholder fresh-produce systems. Long-term seasonal monitoring over 13 months revealed consistently poor water quality, with *E. coli* and faecal coliform levels far exceeding WHO, DWAF, and EU irrigation guidelines on nearly all occasions, alongside frequent detection of *Salmonella* spp. and the presence of a clinically significant MDR extraintestinal pathogenic *E. coli* (ExPEC) strain carrying blaCTX-M-15, as confirmed by whole-genome sequencing. Compared to the diversified farms in Case Study 1 and the integrated vegetable-pig systems in Case Study 2, this study highlighted the particularly severe and deteriorating risk posed by polluted surface water sources, showing a marked decline in microbiological quality over the past decade and the downstream presence of resistant opportunistic pathogens on market-sold leafy vegetables.

These findings strongly contribute to the development of fit-for-purpose microbiological water quality guidelines by providing robust, longitudinal evidence from a real-world, high-risk river system commonly used by smallholders in LMICs. They underscore the critical limitations of relying solely on indicator organisms and generic thresholds, especially in polluted environments where AMR and pathogenic strains persist even when basic physicochemical parameters appear acceptable. When viewed alongside Case Studies 1 and 2, this research supports the shift toward more advanced, risk-based, site-specific guidelines that integrate seasonal variation, molecular surveillance of AMR and pathogens, and practical interventions tailored to informal supply chains.

# CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

## 6.1 General Conclusions

This comprehensive investigation into microbial contamination and AMR in South African smallholder fresh-produce systems, including diversified farms, integrated vegetable-pig operations, and informal river irrigation, reveals a multifaceted contamination cycle driven by persistent faecal pollution, inadequate wastewater infrastructure, rising AMR, and gaps in risk management. Collectively, the three case studies offer baseline data on the occurrence and spread of ESBL/AmpC-producing and other MDR bacteria within smallholder farming systems. The three case studies advocate for context-appropriate guidelines that promote water treatment, alternative irrigation methods, and targeted monitoring to reduce contamination from farm to market, thereby protecting public health and enabling safer smallholder production in water-stressed regions of South Africa. The detection of antibiotic-resistant Enterobacterales and *Pseudomonas aeruginosa* across various environmental matrices emphasises the interconnectedness of water, soil, and produce, and highlights the potential for horizontal transfer of antimicrobial resistance determinants among agricultural, environmental, and clinical reservoirs.

While some water samples met the acceptable microbial and physicochemical standards, others showed signs of contamination, highlighting the importance of inclusive water quality assessments and regulatory guidelines. The presence of generic *E. coli* across water, soil, and fresh-produce samples indicates potential contamination throughout the water-soil-plant nexus. The water-soil-produce-integrated animal farming interface serves as an effective pathway for the transmission of potentially pathogenic microorganisms and resistance genes, with irrigation water as the primary point of entry. The results from this study build on the previous WRC project 2021/2021-C2707, where it was also shown that, for produce at the point of sale, traditional hygiene indicator-based guidelines focusing exclusively on *E. coli* and faecal coliform detection may substantially underestimate actual microbiological hazards by failing to account for antimicrobial-resistant pathogens that represent distinct clinical threats (du Plessis et al., 2021). The presence of microbial pathogens exhibiting antibiotic resistance mechanisms in irrigation water, combined with their detection in harvested and marketed produce, represents an emerging food safety and public health concern inadequately addressed by current guideline frameworks.

Microbiological analyses revealed variable but elevated levels of faecal coliforms and *Escherichia coli*, with produce frequently exceeding matching soil microbiological counts. These findings suggest that irrigation water is a primary source of contamination. While pathogen detection was limited, *Salmonella* spp. was identified in irrigation water from Gauteng and KwaZulu-Natal without in-depth genomic antimicrobial resistance profiling; diarrhoeagenic *E. coli* were found in animal samples; and no Shiga toxin-producing *E. coli* was detected in produce; however, the results indicate potential cross-contamination within mixed systems. Antimicrobial resistance was a prominent threat, with all *E. coli* isolates from

Gauteng, isolated under diversified farm scenarios, exhibiting a MDR phenotype with resistance to cephalosporins and other classes. WGS provided deeper insights, revealing prevalent resistance genes such as *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$, as well as virulence factors. Single-visit assessments of integrated pig-vegetable operations demonstrated pervasive microbial contamination in irrigation water and produce, linked to proximity between animal waste, runoff, and crops. The results of the analyses emphasise the imperative for interventions on diversified smallholder farms that lack proper separation or waste handling.

Residents of Sobantu use the uMsunduzi River for domestic, recreational, and agricultural purposes. The analysis of the microbial burden of river water across the four seasons showed that, on all 37 sampling occasions, the river water exceeded the recommended hygiene indicator levels set by national and international safe irrigation guidelines for fresh produce that is potentially consumed raw. Therefore, the river water is unsuitable for overhead irrigation of fresh produce such as leafy, minimally processed vegetables. When comparing current results to results obtained for the same river in Sobantu in 2010, the deterioration of local river water quality is evident. There is cause for concern, as the recent cholera outbreak between 2022 and 2023 in South Africa highlighted the potential risks posed by deteriorating wastewater treatment systems and highly contaminated river water. Previous studies have shown that farmers are unaware that the microbial burden of irrigation water can negatively affect the quality of fresh produce when using irrigation strategies (e.g., overhead), leading to full contact between the plant and the water. Hence, the farmers need water pre-treatment options to enable safe overhead irrigation when using the uMsunduzi River water for minimally processed fresh vegetables, unless the government or another funding body provides financial assistance to establish drip irrigation. To ensure food safety and sustain farmer livelihoods, the implementation of good agricultural practices (GAPs) is essential. Additionally, routine microbial surveillance of irrigation water and agricultural environments, the application of water treatment or filtration technologies, and the adoption of safer irrigation methods (e.g., drip irrigation where possible) could contribute to improved overall food safety. For resource-limited smallholders, financial and technical support from governmental or external stakeholders is essential to facilitate these interventions.

Budgetary constraints from the solicited project prevented the use of in-depth questionnaires and analyses of farmers' food safety knowledge, attitudes, and practices in the case studies. However, behavioural and capacity assessments from 106 smallholder farmers in Gauteng from a European Union co-funded project titled *Food Safety for Africa (FS4A)* were selectively included in this report, as this complementary project further contextualised these contamination patterns. Farmers demonstrated strong knowledge (mean 85.22%) and positive attitudes (87.44%) toward food safety; however, practical implementation lagged (mean 76.84%). The mean knowledge-practice gap (7.73), more pronounced among female farmers (11.66 vs 1.07 in male farmers), was driven by resource constraints, low risk perception (e.g., only 56.6% were concerned about manure contamination despite 86% of use), and limited access to infrastructure. These socio-economic and behavioural barriers directly intersect with the microbiological findings, illustrating why contamination persists even where awareness is

relatively high. Although smallholder farmers play a crucial role in supplying staples, evidently vulnerabilities in on-farm contamination management persist. This additional evidence also aligns with the findings of Drechsel, Marjani Sadeh, and Pedro (2023), who reported that smallholder farmers often face structural barriers beyond technical knowledge. These include formal guidelines that restrict the irrigation of certain crops or mandate costly drip irrigation systems, which may not be economically feasible for informal farming operations. Additionally, post-harvest recontamination via informal marketing channels can undermine any water-quality improvements implemented at the farm level.

An integrated analysis of physicochemical, microbiological, genomic, and socio-economic data reveals a complex contamination cycle driven by compromised water sources, heterogeneous production practices, and insufficient risk management capabilities. Confirmed transmission of MDR *E. coli* from water to crops, alongside microbiological pollution indicator exceedances and informal chain susceptibilities, necessitates tailored, risk-based irrigation water guidelines for smallholders. These should encompass source categorisation, microbial thresholds, multi-barrier approaches, and farmer-focused training to sever contamination pathways along the water-soil-produce continuum. These outcomes reaffirm the role of irrigation water as a key conduit of contamination, with pathogenic and resistant microbes potentially infiltrating the food chain from farm to retail, endangering consumer health and system sustainability. Mitigation demands One Health frameworks, including dedicated treated-water supplies, enhanced oversight of livestock, and ongoing AMR tracking in agricultural settings. Interventions must account for the varied socio-economic landscapes of South African smallholders. Prioritising customised capacity enhancement, hands-on hygiene education, and adaptive support mechanisms is crucial to bolster pre- and post-harvest safety, minimise microbial threats, and expand market opportunities for fresh and minimally processed produce. Through targeted farmer empowerment, environmental stewardship, and AMR countermeasures, this research paves the way for resilient, safer small-scale agriculture and enhanced public health.

### 6.2 Limitations

Although this comprehensive project provided important evidence to inform irrigation water microbiological quality guidelines for smallholder fresh-produce farmers in South Africa, several factors may affect the interpretation and wider applicability of the results.

1. The number of farms included for each production scenario was limited, with single sampling events at the six sites in Case Study 1 and the three sites in Case Study 2. These site-specific and logistical considerations may limit the extent to which the results accurately reflect the diversity of smallholder production practices across all provinces and farming typologies within the country. Seasonal coverage in Case Studies 1 and 2 was also restricted, potentially underestimating temporal variability in contamination patterns.
2. In Case Study 3, the absence of farm-level produce and soil sampling limited the ability to trace contamination pathways along the full production continuum. Although the

uMsunduzi River was confirmed as an irrigation source, the gap between water monitoring at the farm and microbiological testing of produce at the point of sale prevents direct attribution of pathogen transfer from irrigation water to fresh vegetables sold by street vendors.

3. Culture-dependent detection methods were primarily used in the overall project, which may under-represent viable but non-culturable (VBNC) organisms and emerging pathogens not typically included in standard guidelines.
4. Furthermore, the cost of sequencing and downstream analyses resulted in only a subset of isolates being subjected to WGS. This limited the broader interpretation of antimicrobial resistance and the dissemination of virulence genes across farms.
5. While water physicochemical parameters were evaluated, other environmental factors, such as chemical pollutants, hydrology, manure management practices, and wildlife intrusion that influence overall farming practices, could not be entirely controlled or quantitatively assessed.
6. Although earlier work (WRC 2021/2022-C2706) provided broader insights into consumer-facing contamination pathways across various retail settings, the current study evaluated produce from informal street vendors in only one urban area. As a result, variability across wider retail systems was not captured, limiting the extent to which findings can be extrapolated to other market contexts nationally.
7. Observed practices were documented during field visits to the nine farms; however, budget constraints of the solicited project did not allow for questionnaires or a broader analysis of the knowledge, attitudes, and practices of the smallholder farmers visited for sample collection and the wider farming community. A complementary study, funded by the European Commission under the HORIZON-CL6-2023-FARM2FORK-01-20 Horizon Europe Call titled: *Food Safety for Africa (FS4A)*, grant number 101136916, included an analysis of the knowledge, attitudes, and practices of smallholder farmers regarding food safety. Subsequently, 106 smallholder tomato farmers within the Gauteng region completed questionnaires. The current report does not provide a detailed account of the results; however, selected findings and analyses of the questionnaires have contributed to the formulation of the final guidelines and conclusions for this study.

Despite these limitations, the consistency of contamination trends observed across various farms and sample types underscores the robustness and significance of the findings. This highlights crucial priorities for implementing One Health-aligned intervention and surveillance strategies within smallholder fresh-produce supply chains.

Additionally, the results emphasise that the informal sector, which is vital for ensuring food security in low-income communities, remains insufficiently integrated into national food safety frameworks. To effectively enhance food safety in these systems, efforts must extend beyond individual behavioural changes and include structural reforms such as improving market infrastructure, upgrading water and sanitation facilities, and developing traceability systems for informal produce supply chains. In addition to fit-for-purpose guidelines for managing microbiological water quality risks for policymakers and regulatory agencies in fresh-produce

chains, an AMR monitoring and whole-genome sequencing-based surveillance programme will be adopted and supported by farmer training and community engagement, providing a constructive approach going forward.

### 6.3 Recommendations

The collective evidence generated in this project demonstrates that contamination risks in informal and smallholder fresh-produce systems are driven by structurally compromised water sources, mixed production practices, and limited risk-mitigation capacity. Effective intervention, therefore, requires fit-for-purpose guidelines for managing microbiological water-quality risks in irrigation water, tailored to the socio-economic and infrastructural realities of smallholder farming systems. Such guidance should move beyond universal compliance thresholds and adopt context-responsive, multi-barrier risk management frameworks that integrate water source classification, microbial indicators, practical treatment and mitigation options, and farmer-centred capacity building.

International evidence confirms that multiple water treatment technologies, such as electrolysis, ozonation, ultraviolet disinfection, and photocatalytic systems, can significantly reduce microbial loads in irrigation water. However, their applicability in smallholder contexts is constrained by cost, technical complexity, and maintenance requirements. Low-cost alternatives, including modified zeolite-based sorbents, filtration, sedimentation, and basic disinfection methods, show promise for decentralised use but require adaptation, field validation, and institutional support for sustainable uptake. Complementary evidence further indicates that river water consistently presents the highest microbiological risk, whereas reclaimed and stored pond water may meet regulatory standards with limited mitigation. These findings underscore the need for source-specific risk stratification, combined with practical agronomic controls, including preferential use of drip irrigation for leafy vegetables, minimising direct water-leaf contact, and allowing adequate die-off periods between irrigation and harvest.

To ensure operational relevance in South African informal food systems, the development of microbiological criteria for irrigation water should be grounded in qualitative, risk-based assessment tools aligned with international best practice and local empirical evidence. Decision-tree frameworks that guide farmers through water-source identification, irrigation-method selection, crop susceptibility, and seasonal dynamics provide a feasible mechanism for defining context-specific microbial targets without reliance on advanced laboratory infrastructure. This approach supports fitness-for-use models, in which water suitability is assessed relative to crop type, environmental context, and management practices rather than fixed compliance thresholds, thereby reflecting the realities of smallholder production and informal supply chains.

When stringent water quality standards cannot be achieved solely through treatment, multi-barrier risk-reduction strategies should be prioritised across the water-soil-produce continuum. Combining feasible, low-cost interventions, such as crop substitution towards lower-risk

commodities, reduced-contact irrigation methods, safe composting, improved animal management, and post-harvest washing with clean water or decentralised solar-powered heat treatment, can collectively achieve meaningful reductions in pathogen levels. These strategies complement microbiological monitoring, contamination source tracking, and genomic characterisation, and are essential for disrupting environmentally mediated transmission of pathogens and antimicrobial resistance.

The integration of these approaches provides the foundation for a fit-for-purpose guide that is scientifically rigorous, socio-economically feasible, and tailored to informal and resource-limited production contexts. Successful implementation will depend on bridging persistent knowledge-practice gaps, strengthening farmer capacity, and embedding socio-economic realities, including resource constraints, limited infrastructure, and gendered barriers, into guideline design and dissemination. Uptake should be supported through coordinated training programmes, extension services, stakeholder engagement, and accessible communication platforms to ensure translation of scientific evidence into measurable improvements in water safety, produce quality, market access, and public health protection.

## 6.4 Key Recommendations for a Fit-for-Purpose Smallholder Guidance Framework

### 6.4.1 Establish risk-based tiers and simplified water-source categories

Develop practical microbial risk tiers aligned with crop susceptibility (e.g., stricter *E. coli* thresholds for raw-eaten leafy vegetables) and water-source profiles (low-risk borehole/municipal vs. high-risk surface waters). Embed simple, field-appropriate testing protocols, treatment options (e.g., filtration, chlorination, sedimentation), and decision-tree tools to enable on-farm risk assessment without laboratory dependence.

### 6.4.2 Close knowledge-practice gaps through applied training and accessible tools

Implement farmer-centred training modules focused on contamination pathways and irrigation water risks, supported by low-cost testing kits, visual tools, and behaviourally informed nudges. Link training outcomes to incentives such as improved access to higher-value markets and certification pathways to reinforce practice change.

### 6.4.3 Integrate resource constraints and gender responsiveness into risk management

Prioritise affordable, scalable interventions (e.g., rainwater harvesting, safe composting, basic disinfection) and strengthen gender-responsive extension services to address documented disparities in knowledge-practice translation among female farmers.

### 6.4.4 Promote multi-barrier, farm-to-fork risk reduction strategies

Adopt an integrated approach combining safer water use, improved soil fertility management, adapted irrigation methods, and post-harvest hygiene controls to achieve cumulative reductions in pathogen levels. Leverage shared infrastructure (e.g., AgriParks and cooperative facilities) to expand access to improved water quality and handling capacity.

### 6.4.5 Strengthen market, policy, and sustainability linkages

Align fit-for-purpose guidelines for managing microbiological water quality risks with national food safety frameworks, certification schemes, and formal market requirements. Integrate soil health indicators and emerging hazards, including antimicrobial resistance, to ensure long-term environmental sustainability and public health protection.

### 6.4.6 Emphasise capacity building and flexible indicator selection

Support farmers through community outreach and training to improve understanding of contamination dynamics and appropriate hygiene indicators (e.g., *E. coli* and faecal coliforms), while setting realistic compliance baselines that reflect diverse agro-ecologies and informal market conditions. Ensure compatibility between field indicators and downstream microbiological and genomic surveillance.

### 6.4.7 Develop and disseminate guidelines in local languages

Produce the fit-for-purpose guide and all training materials in locally relevant South African languages and accessible formats (plain-language booklets, pictorial guides, mobile-friendly content). This will improve comprehension, inclusivity, and practical uptake, particularly among farmers with limited formal education, and strengthen knowledge translation across diverse smallholder communities.

# CHAPTER 7: FIT-FOR-PURPOSE GUIDANCE FOR FRESH PRODUCE IRRIGATION WATER ON SMALL-SCALE FARMS

## 7.1 Imperative for Context-Specific Water Quality Guideline

The development of fit-for-purpose guidelines for managing microbiological water quality criteria for irrigation water in informal (smallholder) fresh-produce farming represents a crucial advancement in ensuring food safety and public health, especially in diverse socioeconomic contexts. The cumulative results of the current project have reiterated that contaminated irrigation water can introduce a myriad of microbial pathogens onto crops, directly impacting food safety and public health throughout the supply chains. The implications go beyond the initial human health illnesses, as the presence of antibiotic-resistant bacteria in irrigation water can complicate the treatment of foodborne illnesses and significantly elevate health risks for consumers. This highlights a crucial need to address water quality, not just to prevent contamination but also to mitigate the spread of AMR.

The current study further highlighted how smallholder farmers operate under unique and often challenging circumstances that exacerbate their vulnerability to water quality issues. These farmers typically have limited financial and technical resources, which frequently restricts their access to formal, regulated irrigation systems (Beharielal, Thamaga-Chitja and Schmidt, 2018, 2022; Viviers et al., 2024). Consequently, the smallholder farmers are often compelled to rely on readily available water sources for crop production, including surface water, borehole water (groundwater), and, less commonly, potable or rainwater. Unfortunately, these readily accessible water sources, particularly surface waters such as rivers and dams, often exhibit poor microbiological quality due to widespread pollution and the declining condition of wastewater treatment infrastructure in South Africa (du Plessis et al., 2021; Duze et al., 2025). A significant concern is the prevalent use of overhead irrigation among small-scale farmers, as this method involves directly spraying water onto plant surfaces. This practice increases the risk of contamination of edible crop parts by microbial pathogens present in water of insufficient quality. Furthermore, the challenging environmental conditions, coupled with inherent resource constraints, severely complicate the establishment and maintenance of consistent, effective surveillance systems to monitor water and produce quality in these informal farming environments.

## 7.2 Need for Specific, Achievable Guidelines

A significant challenge confronting growers globally is accurately determining whether the quality of their available water sources is suitable for their intended agricultural uses (Drechsel, Marjani Sadeh and Pedro, 2023). This necessitates a deep understanding of the potential microbiological risks associated with agricultural water, which is often difficult given the complex interplay among agricultural practices, crop types, and prevailing climatic conditions (Drechsel, Marjani Sadeh and Pedro, 2023). While international guidelines and regulations frequently mandate measures such as using potable or clean water to reduce microbial

contamination risks, a more productive approach is to assess the water's fitness for its *intended purpose* rather than rigidly adhering to potable water standards for all uses (Drechsel, Marjani Sadeh and Pedro, 2023). Specifically, the EU (2017) has taken this direction by specifying different irrigation quality criteria (EC, 2017) for various irrigation scenarios. This shift towards a fit-for-purpose approach is increasingly supported by competent authorities who advocate for risk management strategies (Drechsel, Marjani Sadeh and Pedro, 2023). Current frameworks often fall short in achieving true fit-for-purpose adaptability because they tend to overemphasise human health considerations while neglecting integrated pollutant management, thereby hindering sustainable water reuse and public acceptance.

Given the complex interplay of high demand for fresh produce, prevalent water scarcity, and the unique challenges faced by smallholder farmers, there is an urgent and undeniable need to develop practical, fit-for-purpose guidelines for managing microbiological water quality standards for irrigation water. The limitations of applying broad, hypothetical water quality standards become acutely apparent in the highly diverse, resource-constrained contexts characteristic of informal farming. Generic standards often fail to account for local environmental conditions, socio-economic realities, and the specific farming practices employed, rendering them impractical and unachievable for smallholder farmers. For instance, contaminated irrigation water is a significant vector for pathogens like *Salmonella* spp. in produce, potentially amplifying food safety outbreaks in vulnerable regions. In many instances, the microbiological thresholds stipulated by current guidelines are simply unattainable given the quality of the readily available water sources, posing a significant practical hurdle for farmers. Additionally, surface water quality monitoring information is currently not readily available to small-scale farmers, even though municipalities and water companies monitor rivers.

It is recommended that the localised guidelines be meticulously aligned with the internationally recognised FAO risk-based water quality guidelines (FAO and WHO, 2021), ensuring they are both scientifically sound and practically implementable within the specific South African context. This localised approach is essential for establishing standards that are both effective and sustainable for the communities they serve. Furthermore, the threefold objectives of the criteria should be considered: to effectively protect public health, to actively prevent environmental microbial contamination, and to ensure the microbiological safety of fresh produce throughout the entire supply chain.

### 7.3 Monitoring and Management Strategies

Effective implementation of fit-for-purpose guidelines requires robust monitoring and proactive management. It is therefore crucial to consistently emphasise the importance of continuous monitoring of irrigation water quality and the urgent need to enhance existing water-quality control methods, particularly for smallholder farmers who often depend on limited, potentially compromised water resources. Such monitoring should not be a once-off event but an integral, routine component of farming operations, as reiterated by the recent FAO meeting report on

water quality in agriculture (Drechsel, Marjani Sadeh and Pedro, 2023). Furthermore, the data generated should be available to small-scale farmers who rely on local surface water bodies for irrigation. A key recommendation for enhancing food safety is the careful implementation of safer irrigation techniques, such as drip irrigation, whenever technically and financially feasible (Drechsel, Marjani Sadeh and Pedro, 2023). Additionally, simple and effective on-site water pre-treatment systems (e.g., sand filters) would improve the safety of surface water intended for full-contact irrigation of produce.

## 7.4 Key Components of Fit-for-Purpose Guidelines for Smallholder Farmers

### 7.4.1 Source water assessment and characterisation

A fundamental component of establishing effective guidelines involves a comprehensive evaluation and characterisation of the irrigation water source. This includes identifying the type of water source, such as surface water, borehole water, or collected rainwater, and understanding its typical quality parameters. For instance, in South Africa, surface water used for irrigation by farmers is frequently of poor microbiological quality, necessitating careful consideration (Viviers et al., 2024). The results from the overall project indicated that the general pH levels of surface water used for irrigation in South Africa fluctuate around neutral, meeting safe irrigation requirements. However, understanding the chemical quality characteristics of the water source is paramount for predicting potential contamination outcomes and identifying suitable preventive measures to avoid chemical hazards, in line with current good agricultural practices (Drechsel, Marjani Sadeh and Pedro, 2023; FAO and WHO, 2025).

### 7.4.2 Microbiological criteria and indicators

The core of fit-for-purpose guidelines lies in establishing relevant microbiological criteria. While the enumeration of *E. coli* is routinely used globally as an indicator of faecal contamination in irrigation water for fresh-produce production, established, validated, and internationally recognised methods (e.g., ISO standards) are readily available. Nonetheless, there is a need to develop and adopt standardised global guidelines that ensure data reliability and are adaptable to resource-limited settings. The FAO and WHO (2021) have also contributed to the microbiological risk assessment series regarding the safety and quality of water used with fresh fruits and vegetables (JEMRA expert panel, 2021). The most cited FAO health protection recommendations, developed based on the WHO 1989 guidelines, defined three water quality categories depending on the risk of contact, using faecal coliforms as a primary indicator (Drechsel, Marjani Sadeh and Pedro, 2023). These categories included irrigation for crops eaten uncooked, cereal/industrial crops, and localised irrigation (Drechsel, Marjani Sadeh and Pedro, 2023). Therefore, the development of suitable water quality guidelines necessitates harmonising international standards and incorporating modern risk assessment methodologies to better safeguard public health in the irrigation of fresh produce.

### 7.4.3 Risk mitigation strategies and good agricultural practices (GAPS)

Effective fit-for-purpose guidelines should incorporate comprehensive risk mitigation strategies and support the adoption of good agricultural practices (GAPs) to reduce the risk of microbial contamination.

#### On-farm interventions

On-farm interventions are crucial for reducing the risk of contamination. These can include adopting safer irrigation methods, such as drip irrigation, wherever feasible, which can significantly contribute to overall food safety by minimising direct contact between contaminated water and edible parts of crops. The European Commission (2017) provides guidance on addressing microbiological risks in fresh fruits and vegetables at primary production through good hygiene, including a matrix to help growers decide on risk levels (EC, 2017; WHO, 2019). The FAO and WHO (2019) have also addressed the qualitative effectiveness of selected control measures specifically for produce, with a strong focus on small-scale production contexts (EC, 2017; WHO, 2019). These measures offer growers a range of alternatives for risk reduction, detailing the possible effectiveness ratings of various water application and treatment methods in reducing microorganism levels (Drechsel, Marjani Sadeh and Pedro, 2023). Current GAPs should empower growers with practical knowledge to anticipate contamination outcomes and implement appropriate preventive measures (Drechsel, Marjani Sadeh and Pedro, 2023).

#### Technological and financial support

For resource-limited smallholders, the successful implementation of fit-for-purpose guidelines and associated risk mitigation strategies is heavily dependent on external support. Financial and technical assistance from governmental bodies or other external stakeholders is essential to facilitate the adoption of safer practices and technologies. This support can enable investments in improved irrigation infrastructure, water treatment solutions, and training programmes that enhance farmers' capacity to manage water quality effectively.

## 7.5 For Smallholder Farmers: Fit-For-Purpose Guidelines for Managing Microbiological Water Quality Risks

A 10-page guide titled *Pathways of microbiological contamination in South African smallholder fresh produce*, based on the conclusions of the current project, is included in Appendix 1. The guide addresses contamination pathways of faecal bacteria and AMR organisms in South African smallholder agriculture, from water sources to market. Key sections cover high-risk water sources (e.g., polluted rivers), farm risks (e.g., animal proximity, manure, soil splash), irrigation methods, harvest and market recontamination, practical solutions (e.g., drip irrigation, composting), and a One Health approach for stakeholders like farmers and extension officers. The guide content uses simple, evidence-based advice to promote safe

practices and reduce health risks in fresh-produce chains. All images used in the guide was generated using the Gamma Artificial Intelligence (AI) tool (https://gamma.app/).

This guide can be formatted and printed as compact, accessible A5 booklets for easy distribution among small-holder farmers (Figure 32). Future versions could also include translations in different languages and AI-generated voice narration to improve accessibility and broaden audience engagement.

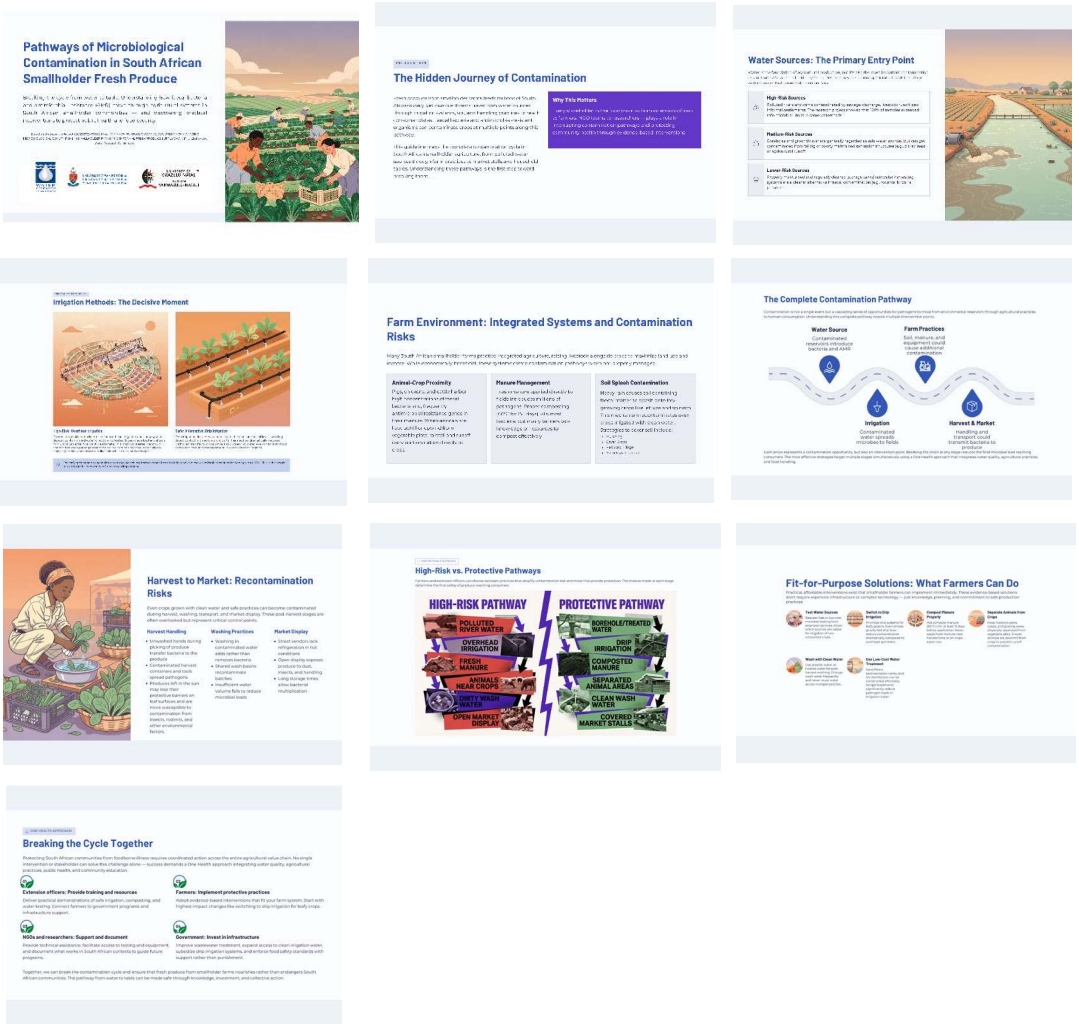



**Figure 32:** Screenshots of the guide that contains fit-for-purpose guidelines for managing microbiological water quality risks for smallholder farmers.

## 7.6 For Informal Fresh Produce Vendors: Fit-For-Purpose Guidelines for Managing Microbiological Water Quality Risks

A ten-page guide titled *Water safety for street vendors: Protect your customers*, based on conclusions from the current project, is included in Appendix 1. The guide provides insights and information for informal market vendors in South Africa on preventing bacterial contamination (e.g., pathogenic *E. coli*, *Salmonella* spp., and antibiotic-resistant germs) in fresh produce by using safe water for washing. Key topics include hidden dangers of

contaminated water, high-risk sources (like rivers or shared buckets), safer alternatives (e.g., municipal tap or boiled water), a three-step washing process (test, treat, wash), best practices, a daily checklist, and benefits for health and business. The content uses simple language, illustrations, and practical tips aimed at vendors to reduce recontamination risks in markets. All images used in the guide was generated using the Gamma Artificial Intelligence (AI) tool (https://gamma.app/).

This guide can be formatted and printed as compact, accessible A5 booklets for easy distribution among vendors (Figure 33). Future versions could also include translations in different languages and AI-generated voice narration to improve accessibility and broaden audience engagement.

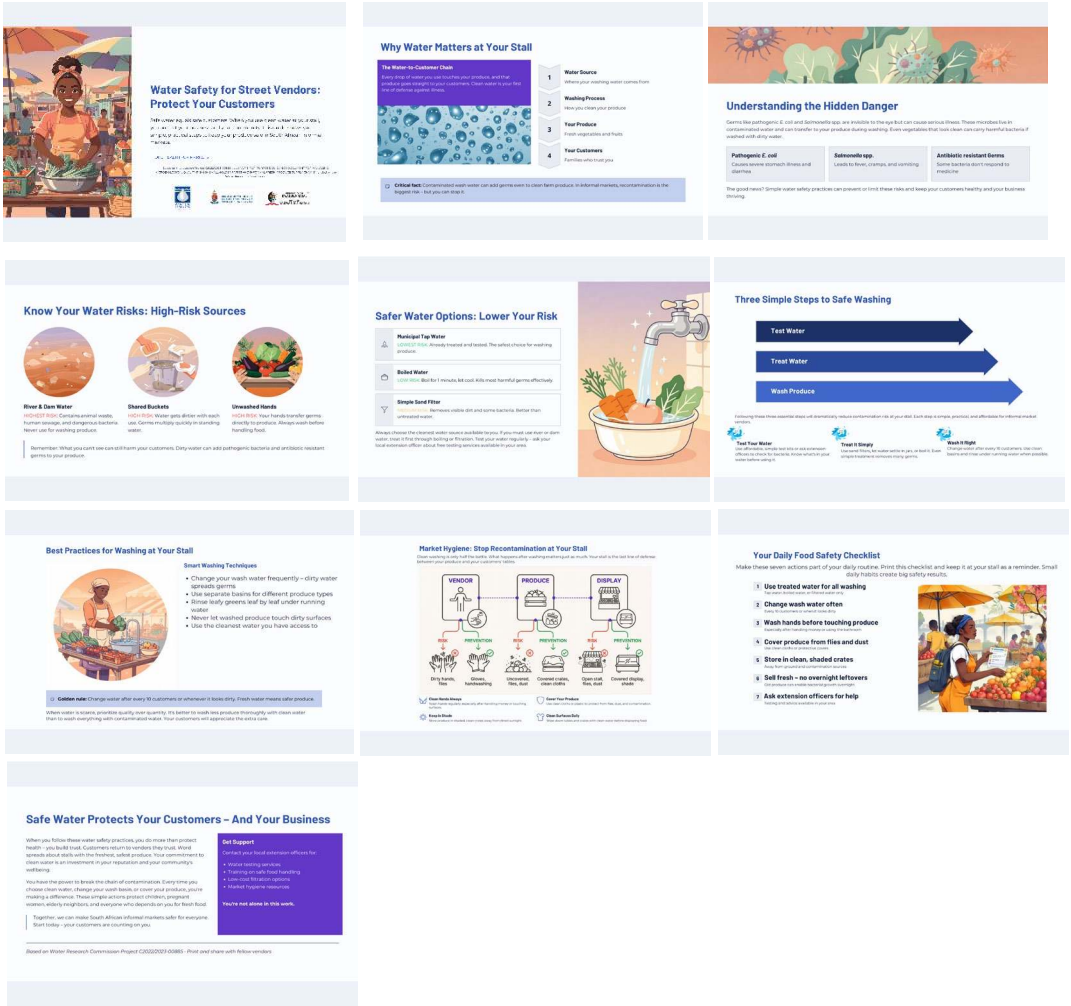



**Figure 33:** Screenshots of the guide that contains fit-for-purpose guidelines for managing microbiological water quality risks for street vendors.

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## APPENDICES

### Appendix 1: Fit-for-Purpose Water Quality Guides

Appendix 1A: Fit-for-purpose water quality guideline for smallholder farmers titled: *Pathways of microbiological contamination in South African smallholder fresh produce*.



Smallholder farmer guideline.pdf

Appendix 1B: Fit-for-purpose water quality guideline for street vendors titled: *Water safety for street vendors: Protect your customers*.



Street Vendor guideline.pdf

## Appendix 2: Post-Graduate Training of Students and Human Capacity Building (Theses and Dissertations Awarded and in Progress)

Six postgraduate students, four women and two men, were trained during the course of WRC project 2022/2023-00885 from 2012 to 2025 (Table 1). This included one postdoctoral student, three MSc students, and two doctoral students (all South African citizens). Dr Loandi Richter was trained in all project coordination matters and subsequently took over all research project management, reporting, and coordination responsibilities following the retirement of Dr Erika du Plessis in 2023. Two master's students have completed their studies.

**Table 1:** Students registered between 2022 and 2025

| | Student name and surname | Gender | Race | Degree | University | Country of origin | Student e-mail address |
|---|---|---|---|---|---|---|---|
| 1 | Dr Loandi Richter | Female | White | PostDoc | UP* | South Africa | loandi.richter@up.ac.za |
| 2 | Manana Dlangalala | Female | Black | PhD Biotechnology | UP* | South Africa | u18284818@tuks.co.za |
| 3 | Sheldon Viviers | Male | White | MSc Biotechnology | UP | South Africa | u18077367@tuks.co.za |
| 4 | Nkosinathi Ndlazi | Male | Black | MSc Microbiology | UKZN** | South Africa | 217019510@stu.ukzn.ac.za |
| 5 | PhilaL. Mbambo | Female | Black | MSc Microbiology | UKZN ** | South Africa | 217002085@stu.ukzn.ac.za |
| 6 | Thembeka Kwela | Female | Black | MSc Microbiology | UKZN** | South Africa | 219003364@stu.ukzn.ac.za |
| UP* University of Pretoria<br>UKZN** University of KwaZulu-Natal | | | | | | | |

## Appendix 3: Overview of Knowledge Dissemination through Conference Papers Presented, Scientific and Popular Articles

### Overview of knowledge dissemination

Publications in peer-reviewed scientific journals for the project period from 2022/2023, 2023/2024, and 2024/2024 EW are summarised in Table 2. Three papers have been published, and one is in preparation. In addition, a popular paper has been published in *The Conversation* and has also been adapted and featured in *Food Safety Magazine*. The additional knowledge dissemination activities, including webinars, conferences, and workshop presentations, are summarised in Table 3.

**Table 2:** List of scientific publications

| Authors and Titles | Journal | Publication status |
|---|---|---|
| **Publications in scientific journals** | | |
| **Sheldon A Viviers, Loandi Richter, Erika M du Plessis, Lise Korsten (2024)** Microbiological quality of irrigation water on highly diverse fresh- produce smallholder farms: elucidating environmental routes of contamination | *Journal of Applied Microbiology*, Volume 135, Issue 4, April 2024, lxae091, https://doi.org/10.1093/jambio/lxae091 | Published |
| **Sheldon Viviers; Loandi Richter-Mouton; Jonathan Featherston and Lise Korsten. (2025)** Occurrence and Genotypic Characterisation of Selected Multidrug-resistant ESKAPE-E Pathogens Isolated from Integrated Smallholder Fresh Produce Farms. | *Journal of Food Protection*, Volume 88, Issue 7, June 2025, https://doi.org/10.1016/j.jfp.2025.100543 | Published |
| **Nkosinathi D. Ndlazi, Maike Claussen, Stefan Schmidt. (2025)** Draft genome of an extended-spectrum β-lactamase-producing multidrug-resistant *Escherichia coli* sequence type 131 strain isolated from the Msunduzi River in South Africa. | *Microbiology Resource Announcement.* Volume 14:e00136-25 https://doi.org/10.1128/mra.00136-25 | Published |
| **Popular papers** | | |
| **M Dlangalala, S. Schmidt, L Richter-Mouton andL. Korsten.** Microbiological characterisation of small-scale integrated crop and pig production ecosystems. | To be determined In preparation | |
| **Loandi Richter-Mouton and Lise Korsten (2025)** Decades of South African research reveal key strategies to reduce water contamination in fruit and veg | *The Conversation* https://theconversation.com/decades-of-south-african-research-reveal-key-strategies-to-reduce-water-contamination-in-fruit-and-veg-258311 | Published |
| **Loandi Richter-Mouton and Lise Korsten (YEAR)** South African Scientists: Better Standards, Surveillance Needed to Address Contaminated Produce Irrigation Water | *Food Safety Magazine* https://www.food-safety.com/articles/10500-south-african-scientists-better-standards-surveillance-needed-to-address-contaminated-produce-irrigation-water | Published |

**Table 3:** List of presentations at webinars/ podcasts, radio interviews, conferences and workshops

| Type | Presentation |
|---|---|
| **2025** | |
| **Radio interview** | Radio 768 – Interview with Loandi Richter-Mouton. Sound bite: Impact of irrigation water quality on fresh produce. 18 July 2025 |
| **Workshops or presentation** | Food Safety Research@UP. Dr Loandi Richter-Mouton, 25 June 2025. Farmer Study Group Presentation/Dialogue with smallholder farmers from the Gauteng North region |
| **2024** | |
| **Poster presentations** | Manana Dlangalala, Erika du Plessis, Stefan Schmidt and Lise Korsten. Prevalence and characterisation of antibiotic-resistant bacterial pathogens in small-scale integrated vegetable-pig production ecosystems. |
| | Sheldon Viviers, Loandi Richter and Lise Korsten. Occurrence of antimicrobial-resistant potential pathogenic bacteria throughout the water-soil-plant nexus in smallholder fresh-produce farms. |
| | 8th World One Health Congress. Cape Town, South Africa, 20–23 September 2024 |
| | 8th World One Health Congress. Cape Town, South Africa, 20–23 September 2024 |
| | Ndlazi, N.D., M. Claussen, S. Schmidt. The microbiological quality of river water used for full-contact irrigation of ready-to-eat fresh produce – a one health scenario for a river in Pietermaritzburg, South Africa. |
| | 8th World One Health Congress, Cape Town, South Africa. 20–23 September 2024 |
| **2023** | |
| **Workshops or presentation** | A collaborative workshop that focused on Foodborne pathogens and AMR from farm-to-plate was held in Pretoria (during Dr Stacey Duvenage's visit from the University of Greenwich to South Africa. |
| | During Dr Stacey Duvenage's visit from the University of Greenwich to South Africa, a metadata analysis workshop was held (Seminar Room 2-7, Agricultural Sciences Building, Hatfield Campus, UP) with Dr Judy Bettrige, where the team focused on conceptualising and summarising the results for a Hygiene indicator bacteria metadata analysis publication |
| | One Health Workshop: Internal workshop with University of Pretoria students' project presentations. 22 March 2023 University of Pretoria |
| **Podcasts** | 7 August 2023 |
| | 8 August 2023 |
| | 22 March 2023 |
| | Ep. 156. Lise Korsten: Food Safety Considerations, From Post-Harvest Interventions to Future Challenges. https://www.food-safety.com/articles/8977-ep-156-lise-korsten-food-safety-considerations-from-post-harvest-interventions-to-future-challenges |
| **2022** | |
| **Online webinar or dialogue** | World Food Safety Day dialogue: Safer food, better health. Antimicrobial resistance in the water-plant-food-public health interface. Erika du Plessis and Lise Korsten |

## Appendix 4: Student Theses and Dissertations

**Nkosinathi Ndlazi** completed his MSc in Microbiology at the University of KwaZulu-Natal and will graduate in April 2026
Supervisor: Prof Stefan Schmidt
Co-Supervisor: Dr Maike Claussen

### Microbial quality of the Msunduzi River water used for irrigating fresh produce by local smallholder farmers in Pietermaritzburg, South Africa

### Abstract

The microbiological quality of river water used to irrigate ready-to-eat (RTE) fresh produce, often consumed raw or minimally processed, is critical to food safety. Microbially contaminated water used for overhead irrigation can introduce pathogenic bacteria to produce, posing potential health risks to consumers. This study evaluated the microbiological quality of the Msunduzi River used as an irrigation source by smallholder farmers in the Sobantu Area of Pietermaritzburg, South Africa. River water samples (n=18) were collected across four seasons from September 2022 to September 2023. Microbial quality was assessed by quantifying the general bacterial burden (aerobic plate count, total coliforms) and specific hygiene indicators (faecal coliforms, *Escherichia coli*, and intestinal enterococci). The presence of the representative foodborne pathogen *Salmonella* spp. was also verified. Antibiotic resistance (ABR) profiles were established for selected isolates of *E. coli* (n=80) and *Enterococcus* spp. (n=40) using the EUCAST disk diffusion method. Whole-genome sequencing (WGS) was performed for one MDR *E. coli* isolate, which was then subjected to further antibiotic resistance profiling. The biofilm-forming capabilities of selected *E. coli* and *Enterococcus* spp. isolates with varying resistance profiles were evaluated. Results indicated that the river water persistently surpassed the safety thresholds set for full-contact irrigation, including the South African standard (<1 *E. coli*/100mL), the European Union standard (<10 *E. coli*/100mL), and the World Health Organization standard (<1 000 *E. coli*/100mL). The water quality was generally poor, with aerobic plate counts ranging from log 4.60 to log 6.18CFU/mL, total coliform levels from log 3.85 to log 5.81 MPN/100mL, faecal coliforms from log 3.69 to log 5.66 MPN/100mL, and *E. coli* from log 3.34 to log 5.23 MPN/100mL. The counts for intestinal enterococci ranged from log 2.95 to log 4.59CFU/100mL. Additionally, *Salmonella* spp. was detected in 33% of the samples analysed. Notably, 30% of *E. coli* and 27.5% of *Enterococcus* spp. isolates exhibited antibiotic resistance to at least one clinically relevant antibiotic. The one multidrug-resistant (MDR) *E. coli* isolate detected demonstrated extended-spectrum β-lactamase (ESBL) activity and was identified as an extraintestinal pathogenic *E. coli* (ExPEC) of sequence type 131. Biofilm formation was observed in both antibiotic-resistant and susceptible strains, including the MDR ST131 *E. coli* isolate. These findings highlight the potential health risks associated with using water from the Msunduzi River for irrigation, as well as for domestic and recreational purposes. They underscore the importance of regular microbial monitoring, antibiotic resistance surveillance, and policy interventions to mitigate river contamination and protect public health.

**Sheldon Viviers** completed his MSc in Biotechnology at the University of Pretoria and graduated in September 2025
Supervisor: Prof Lise Korsten
Co-supervisor: Dr Loandi Richter-Mouton

**Abstract/Project Summary**

Subsistence farming in rural and peri-urban areas of South Africa has driven the establishment of smallholder farms that produce fresh produce for household consumption and generate income through community sales. These farms play a crucial role in household food security, supplying approximately 80% of fresh produce sold in informal markets across the country. However, knowledge of the microbial quality of produce, hygiene practices, contamination sources, the prevalence of foodborne pathogens, and antimicrobial resistance (AMR) in smallholder farming systems is limited. The environment, particularly water sources, significantly influences the transmission of human pathogens and AMR within the food supply chain system. Smallholder farmers often rely on contaminated water sources for irrigation, with limited infrastructure or financial resources to mitigate microbial contamination.

The roles of Enterobacterales and pseudomonads in agriculture and clinical settings are well documented. Species within the Enterobacterales order naturally inhabit the environment, especially across the water-soil-plant nexus, and include critical foodborne pathogens such as *Escherichia coli* and *Salmonella* spp. These organisms have developed robust mechanisms to resist clinically important antimicrobials, including the production of extended-spectrum β-lactamases (ESBLs), which confer resistance to third-generation cephalosporins. This study aimed to evaluate the microbiological quality, contamination sources, and presence of foodborne pathogens within the water-soil-plant nexus of six smallholder fresh-produce farms in South Africa. Additionally, it investigated the bacterial characteristics of selected ESKAPE-E strains, including their phenotypic AMR profiles and genotypic traits derived from genomic data, using standard microbiological and molecular techniques.

Irrigation water *E. coli* levels ranged from 0–3.45 log MPN/100mL, with five farms meeting the World Health Organization's (WHO) acceptable limit of 3 log MPN/100mL. However, one water sample (specifically from the municipal water source on Farm F) exceeded this standard. Fresh produce samples collected from four farms (n=65) generally harboured *E. coli* at low levels (<1 log CFU/g). Exceptions included single samples of kale, spring onion, bell pepper, and onion, and two tomato samples, all of which exceeded the international acceptable limit of 100CFU/g. Additionally, only one baby carrot sample tested positive for *Salmonella* spp., which was further identified as *Salmonella enterica*. Of the 224 samples, *E. coli* was detected in 40% (n=90) of water, soil, and fresh-produce samples after enrichment. DNA fingerprinting also revealed that *E. coli* isolates from the water-soil-plant nexus on each farm clustered together with high similarity values (>90%), with all phenotypically characterised as multidrug-resistant (MDR). This clustering strongly implicated irrigation water as a key source of contamination in fresh produce.

Chromogenic media and matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF MS) identified ESBL/AmpC-producing isolates in 17% (38/224) of the samples. These isolates were most frequently found in fresh produce (54%), followed by soil (31%) and water (15%) samples. Further phenotypic profiling using the double-disk synergy test (DDST) identified 47% of the isolates as ESBL producers, 43% as AmpC producers, and 18% as producers of both. Moreover, phenotypic resistance profiles, assessed via disc diffusion against 13 antimicrobial classes, revealed that all isolates were MDR, displaying high resistance to aminoglycosides, β-lactams and glycylcyclines. The selected isolates (n=20) subjected to whole-genome sequencing (WGS) belonged to six bacterial genera of clinical significance: namely *Enterobacter, Escherichia, Klebsiella, Pseudomonas, Salmonella* and *Serratia.* Most of the isolates were also derived from fresh produce (n=12), followed by water (n=5) and soil (n=3) samples across five of the six farms.

The WGS data identified common antibiotic resistance genes (ARGs) shared amongst the Enterobacterales and *P. aeruginosa* isolates, conferring resistance to important antimicrobial classes such as β-lactams (*bla*$_{\text{CTX-M-14}}$*;* *bla*$_{\text{CTX-M-15}}$*;* *bla*$_{\text{CTX-M-27}}$*;* *bla*$_{\text{SHV-11}}$, and *bla*$_{\text{TEM-176}}$), quinolones (*OqxA, OqxB, qnrD1_1* and *qnrS1_1*), and tetracycline (*tet(A)* and *tet(A)_6*). Moreover, a clinically significant *Serratia marcescens* strain from borehole water on the organic farm carried the colistin-resistance gene *mcr-9,* which was associated with the insertion sequence IS903. Notably, WGS analysis revealed the presence of clinically significant resistance and virulence genes among environmental isolates, particularly those associated with mobile genetic elements (MGEs).

Bacterial characteristics, including multilocus sequence typing (MLST) and serotyping, were determined from the WGS data. The three MDR *E. coli* strains exhibited distinct sequence types (STs): ST48, ST254, and ST1141, with serotypes O45:H9, O113:H4, and O156:H4. The MLST analysis of three MDR *K. pneumoniae* strains revealed two STs and associated K-loci: ST147 (K113) and ST36 (K178), while the two *E. cloacae* strains shared ST514. Lastly, the 10 *P. aeruginosa* isolates were grouped into four STs (ST313, ST514, ST564, and ST3017) and three serotype groups (O1, O3, and O9).

The presence of identical STs across various environments highlights the potential for these bacteria to spread and establish in diverse environments. Moreover, these findings emphasise the role of MGEs in spreading AMR genes across diverse environments, underscoring the need for robust surveillance systems to curb the transmission of resistant pathogens within a One Health framework. The study demonstrated that the prevalence and characteristics of MDR Enterobacterales and *P. aeruginosa* strains are consistent with global trends, emphasising the need for integrated surveillance and intervention strategies to curb their spread. In addition, the study underscores the importance of continuous monitoring and targeted control efforts in food production environments to limit the spread and establishment of these potentially pathogenic-resistant organisms. Lastly, the study provides important information that can inform the One Food approach to hazard identification and risk assessment.

**Manana Dlangalala** is in the writing phase of her doctoral thesis titled: Microbiological characterisation of small-scale and commercial integrated crop and pig production ecosystems.
Supervisor: Prof Lise Korsten
Co-Supervisor: Prof Stefan Schmidt
A thesis abstract will only be established as part of a thesis when she submits it for examination.

**Thembeka Khwela** is in the writing phase of her MSc dissertation with a preliminary title: Microbiological assessment of the uMsunduzi River water quality
Supervisor: Prof Stefan Schmidt
A thesis abstract will only be established as part of a thesis when she submits it for examination.

## Appendix 5: Supplementary Information – Ethical Clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Natural and Agricultural Sciences
Ethics Committee
E-mail: ethics.nas@up.ac.za

15 August 2022

ETHICS SUBMISSION: LETTER OF APPROVAL

Prof L Korsten
Department of Department of Plant and Soil Sciences
Faculty of Natural and Agricultural Science
University of Pretoria

**Reference number: NAS055/2022**
**Project title: Impact of water pollution on the microbiological safety and biome of fresh produce from farming to retail \ (predominantly informal) for fresh vegetables and to development of a fit-for-purpose water microbiological quality guideline for smallholder farmers and retailers and evaluate microbiological risk reduction strategies/technology to ensure food safety in the supply chains**

Dear Prof L Korsten,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Natural and Agricultural Sciences Research Ethics Committee.

Please note the following about your ethics approval:

- Please use your reference number (NAS055/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
- Please note that ethical approval is granted for the duration of the research (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
- The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
- **If Applications using GM permits**: If the GM permit expires before the end of the study, please make an amendment to the application with the new GM permit before the old one expires
- **If Applications using Animals**: NAS ethics recommendation does not imply that Animal Ethics Committee (AEC) approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Comments:

Please upload MSDS documents for hazardous laboratory chemicals that the project will be using in the laboratory.

Post approval submissions including application for ethics extension and amendments to the approved application should be submitted online via the Ethics work centre.

We wish you the best with your research.

Yours sincerely,

