Enumeration of phyllo-epiphytic and endophytic pathogens on leafy greens from farms and retails as affected by production parameters in the Free State, South Africa.

by

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DECLARATION OF OWN WORK

I, Dineo Attela Mohapi declare that this dissertation, **Enumeration of phyllo-epiphytic and endophytic pathogens on leafy greens from farms and retails as affected by production parameters in Free State, South Africa,** submitted to the Central University of Technology, Free State, in fulfilment of the requirements of the degree Master of Health Sciences: Environmental Health is my independent work has not been submitted to any other institution or organization for any reason whatsoever. Where other people's work has been used (either from a printed source, internet, or any other source), this has been properly acknowledged and referenced accordingly.

Signature……..……..…….. **Date: 26/ 06/2025**

DEDICATION

"*This work is dedicated to my dearest Mother Lisebo Merriam Mohapi, Ntebo.*"

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ABSTRACT

Leafy vegetables are a highly variable group of perishable foods that broadly can be defined as vegetables grown for their edible leaves. This study was conducted to investigate the production parameters at the farms and how they influences the end product. The study characterises opportunistic pathogens from various farms and retails and identify the aetiology and how these organisms affect human health and also emphasise the significance of sources that are regarded as microbial hazards or act as resevoirs for pathogenic organisms. Considering one health perspective it is also imperative to pay more attention to the presence of zoonotic pathogens that are resistance to certain antbiotics as it is a public health threat and a challenge that need to be highlighted due to high level of infectious illness caused by zoonotic and opportunistic pathogens. Since various agronomic activities leads to contamination of leafy greens in various stages of minimal processing including distribution, it is thus crucial to address such concerns at small-scale farms to mitigate cross-contaminate to the retails and provide an insight on hygiene and sanitation in both spheres. Minimal process includes many stages which introduces the disruption of tissue including cell integrity resulting in product harbouring of opportunistic pathogens. Spinach (*Spinacia oleracea* L.) and cabbage (*Brassica oleracea* var. *capitata* L.) are considered staple food in South Africa and are consumed daily, they were chosen due to their minimal processing in production, demand, purchase and their difference compared to intact vegetables regarding their physiology, processing, handling including storage. The study profiles and characterise opportunistic and zoonotic pathogens from both regimens due to processing parameters, hygiene practises and possible succession of pathogens form one niche to the other. The first survey was on the enumeration of microbiota on spinach and cabbage isolates from various farms including their storage crates. Secondly, the objective was to enumerate microbiota from spinach and cabbage from retails and further investigate the extent of these pathogens, the succession at the retail level, observe the proliferation of pathogens introduced along the way due to amplification. Fresh leafy spinach, crates and cabbage samples were analysed for each of the following microorganisms: total aerobic mesophilic bacteria, total coliforms, coagulase-positive *Staphylococci, Listeria and Bacillus* and further identification of isolates was done by utilisng Analytic Profile Index. Lastly, conduct antimicrobial susceptibility profile of identified opportunistics bacteria from farms and retails, the isolates were evaluated utilising Kirby-Bauer disk diffusion method against nine antibiotics from nine categories*: penicillin* (P; 10_µg*)*, *ampicillin* (AMP; 10_µg), *gentamicin* (CN; 10_µg), *ceftazidime* (CAZ; 30_µg), *chloramphenicol* (C; 30_ µg), *tetracycline* (TE; 30_µg), *vancomycin*

(VA; 30_µg), *erythromycin* (E; 15_ µg) and *ciprofloxacin* (CIP; 5_ µg). In this study, three farms had the highest number of opportunistic pathogens identified for cabbage with the least number of pathogens observed in the other two farms. With regards to spinach, two farms had the highest number of identified pathogens including *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Serratia* species and *Listeria* species and the lowest number was observed in the other two farms. Concerning spinach crates, two farms had the highest number of pathogens identified and the least numbers were observed in the other two farms. This indicate contamination mostly from livestock manure utilised on the crops including insufficient hygiene from workplace. Secondly, three retails had the highest number of pathogens identified for cabbage compared to the other two retails. With regard to spinach, three retails had the highest number of identified pathogens including *Bacillus* spp., *Enterobacter cloacae*, *Listeria ivanovii* and *Listeria monocytogenes* species compared to the other two retails. The study also highlights additional bacterial species enumerated from both commodities in two different environments. The additional species characterized are a result of proliferation introduced due to agronomic parameters, cold chain supply, and retail activities. The combined result of total coliforms and opportunistic pathogens found on the end-products indicates poor minimal processing, poor hygiene and sanitation standards. Lastly, *Enterobacter cloacae, Staphylococcus aureus, Micrococcus luteus, Staphylococcus sciuri, Acinetobacter haemolyticus, Burkholderia cepacia, Psuedomona luteola, Escherichia coli, Citrobacter freundii and Serratia marcescens* were tested again nine antibiotics. Multidrug resistance was observed with 79% resistance, this is a public health threat that points out challenegs regarding treatment of infections caused by these opportunistic and zoonotic pathogens. Rapid identification of antibiotics is essential and crucial for the development of effective antimicrobial compounds as well as prevention of antibiotic resistance. Necessary measures should be taken to reduce the level of contamination from small-scale farms to reduce antibiotic consumption in humans.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background and motivation

A myriad of fresh leafy green vegetables have been documented to provide ideal conditions for foodborne pathogens. Leafy green vegetables are perishable commodities and their phylloplane topography and natural apertures influence the diversity of microbial communities (Quadri *et al.,* 2015). Minimally processed fresh vegetables are generally defined as any vegetable that has been subjected to different processing stages, these include trimming, cutting, washing and disinfection, rinsing and storage (De Corato, 2020). The term phyllosphere has been utilised to describe the above-ground part of the plant environment and phylloplane is described as the leaf blade surface (Liu *et al*., 2023). Additionally, the term phylloplane has also been utilised, either instead of or in addition to the term phyllosphere.

The stability or homeostasis results from a dynamic balance of microbial-to-microbial and microbial-host interactions and under some circumstances the homeostasis may break down, predisposing a site to diseases (Li & Tian, 2016). Thus, it is likely that many pathogens did not initially evolve as pathogens but simply take on this role because of a lack of ability of the host to maintain homeostasis (Ehrlich *et al.,* 2008). This can happen in any state between microbial-to-microbial takeover resulting in more virulent pathogen or microbial-to-host interactions and consequently resulting in disease (Vonaesch *et al*., 2018). It is evident that when these opportunistic pathogens invade the human system, they disturb the immune system's stability by taking advantage and leading to breakdown causing illness. What makes pathogen a pathogen is therefore, the addition or deletion of metabolic capabilities in the symbiome that results in a disruption of homeostasis including the species relative abundance and its virulence factors (Ehrlich *et al*., 2008).

Vegetables, including Cabbage (*Brassica oleracea* var. *capitata*) and spinach (*Spinacia oleracea*), are not subjected to any lethal process which is mostly employed to effectively kill pathogenic organisms (Ampim *et al*., 2022). Precise identification of a potential source of contamination for produce is often difficult because contamination can occur anywhere in the farm-to-fork continuum (Whitney *et al*., 2021). However, the home is referred to as the primary location for food poisoning outbreaks (Byrd-Bredbenner *et al*., 2013). A leafy vegetable-associated outbreak is defined as an event in which two or more people become ill due to the ingestion of a common contaminated food (Herman *et al*., 2015).

Since minimal operations comprise of several units, they are likely to provide opportunities for potential cross-contamination. For example, a small quantity of contaminated commodities may be responsible for the contamination of a large quantity (Gil *et al*., 2015). Due to complex components of a commodity such as moisture, protein, high water activity, pH level and nutritional elements, their tissues can sustain bacterial growth with various bacterial symbiome (Abdul *et al*., 2021). Furthermore, contaminated leafy green vegetables may cause pathogen progression to the next portion of processed commodities leading to proliferation and succession of pathogens (Castro-Ibanez *et al*., 2016). Succession is defined as an orderly and anticipated manner by which microbial communities change over time due to microbial abundance and flexibility following the colonization of a new niche (Frierer *et al*., 2010).

Mesophilic foodborne pathogens such *as Salmonella* sp*., Shigella* sp*., Campylobacter* sp*., Staphylococcus aureus* and *Escherichia coli* O157:H7 can internalise and thrive on spinach and cabbage for weeks causing diarrhoea, Salmonellosis and Shigellosis (Mritunjay & Kumar, 2015). The environment also influences and changes the physiological and microbiological functions of minimally processed vegetables causing microbial proliferation of communities on various species (Artes & Allende, 2014). The European Food Safety Authority (EFSA) and European legislation, including the World Health Organization (WHO) placed leafy green vegetables, particularly lettuce, cabbage, and spinach, as the highest priority group due to microorganism interactions and the highly diverse microbiome including food poisoning outbreaks (Mehrotra *et al*., 2023).

Opportunistic pathogens can cross geographical barriers by attaching to natural apertures and thrive in new and different environments due to cross-contamination where they can multiply (Al-Kharousi *et al.,* 2016). Jackson *et al*. (2015) reported highly diverse members of the *Proteobacteria* and *Bacteroidetes* in the leafy green phyllosphere group on ready-to-eat salads including spinach. The increased ratios of *Firmicutes* to *Bacteroidetes* species have been correlated with obesity and Type II diabetes as they are bad gut microbes (Kagele, 2015). Notable genera of *Firmicutes* include *Bacilli* such as *Bacillus, Listeria*, *Staphylococcus*, *Clostridia* such as *Clostridium,* including *Acinetobacter* (Galperin, 2013).

The characteristics of the most common pathogenic bacteria causing foodborne diseases in cabbage and vegetables together with other outbreaks have been reviewed (Chen *et al.,* 2018; Johnson, 2019, Gordon, 2019). Between 1996 and 2006, leafy vegetable consumption increased by 9%, while foodborne outbreaks from leafy greens increased by 39% (Lynch *et al.,*

2006). In the United States, between 1998 and 2007, fresh produce was involved in 684 outbreaks, resulting in 26, 735 cases of illness, while fresh salads, fruits and vegetables were linked to 345, 228 cases resulting in 111 outbreaks (Uyttendaele *et al.,* 2015). Between 1998 and 2016, a subset of the leafy vegetable category accounted for between 20% and 40% of these reported outbreaks and 10% to 40% of all produce-related illnesses (Johnson, 2019). Approximately 46% of yearly foodborne illnesses were attributed to these fresh produce (Carstens *et al*., 2019). According to Herman *et al*. (2015), 606 leafy vegetable-associated outbreaks, with 20, 003 associated illnesses, 1030 hospitalizations, and 19 deaths were reported where leafy green vegetables-associated outbreaks were larger than those attributed to other food types in the United States (US) From 2015 to 2016, an outbreak was linked to leafy green vegetable salad with consequences of a healthy child developing meningitis after consuming packaged leafy green salad (Chen *et al*., 2017).

Between 2009 and 2018, the Food and Drug Association (FDA) and Centres for Disease Control and Prevention (CDC) identified 40 foodborne outbreaks of Shiga toxin-producing *E. coli* (STEC) infections in the United States with a confirmed case linked to leafy green vegetables (CDC, 2020). From 2014 to 2018, 51 foodborne disease outbreaks were linked to leafy greens, with 406 illnesses reported to the CDC, and five were multistate outbreaks that led the centre to issue warnings to the public (CDC, 2020). Turner *et al*. (2019) reported that approximately 2, 240 cases of illness were confirmed in the US with 298 cases in California resulting in 50 hospitalizations from spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*). McDaniel and Jadeja (2019) reported that up to 47.8 million cases of foodborne illness occur annually in the US and 9.4 million cases, 55, 961 hospitalizations and 1,351 deaths can be attributed to a select group of 31 foodborne pathogens of which bacteria account for 39%. The data further indicates that leafy green vegetables are among 85% of the implicated commodities associated with the outbreak (McDaniel & Jadeja, 2019). The 2028 illnesses, 477 hospitalizations, and 18 deaths associated with outbreaks linked to leafy greens reported to CDC in 2014-2021 represent only a part of illnesses caused by contaminated leafy greens during those years (CDC, 2020). Mehrotra *et al*. (2023) shared foodborne outbreaks associated with consumption of leafy greens contaminated with opportunistic pathogens from 2005 until 2021.

In South Africa, most of the farmers depend on surface water sources to irrigate their produce (Du Plessis & Korsten, 2015). Furthermore, it is highlighted that potential risks include

the proximity of growing informal settlements without adequate sanitation and stormwater services, ill-functioning wastewater treatment plants, and intensified urbanisation. Another challenge regarding small-scale farmers producing fresh leafy vegetables is livestock production and the usage of animal manure as fertiliser. Pathogen contamination may also originate from antibiotic residue from soil fertilizing manure (Van Pelt *et al*., 2018). Antibiotic contamination may cause deleterious potential effects in agroecosystems including the proliferation of various antibiotic-resistant bacteria and the spreading of antibiotic-resistant genes (ARGs) in the environment and even to human health (Hanna *et al*., 2018), contributing to the pool of ARG in the human gut and imposing a risk for resistance to therapeutic measures (Thanner *et al*., 2016). The presence of antibiotic resistance in both epiphytic and endophytic pathogenic microorganisms in vegetables contributes to the horizontal spreading of resistance among bacterial populations (Gekenedis *et al*., 2018).

Jongman and Korsten (2017) reported that several sources of surface water in South Africa are polluted, yet are still utilised for irrigation by commercial, small-scale, and homestead production systems. This is considered a potential risk factor exacerbating the prevalence of microorganisms and simply means that the viable count at the time of consumption may be elevated. It is reported that foodborne diseases are under-reported and poorly investigated in South Africa due to poor surveillance systems and poor integrated management (Shonhiwa *et al.,* 2019). In addition to the statement above, several policies have been drafted with no integrated system to curb foodborne diseases or monitor the outbreak of foodborne diseases and prevent them from spreading, which indicates the necessity for a good surveillance system in South Africa (Bisholo *et al*., 2018).

According to Gomba *et al*. (2016), increases in foodborne disease outbreaks associated with fresh produce have necessitated the need to identify potential sources of microbial contamination in produce and agricultural environments. The Metro Health District in the Eastern Cape province in South Africa reported that more than 65% of informal traders and food outlets in rural areas do not adhere to health standards, nor possess certificates of acceptability as a compulsory measure required by the South African legislation (Bisholo *et al.,* 2018). The confirmed report shows that primary horticulture production environments are still challenges in South Africa and need to be tackled regarding potential risk factors and critical control points for integrated health standards. For example, some highly infectious bacterial genera like *Corynebacterium, Streptococcus* and *Staphylococcus* were found to be

dominantly present on the outer surface of spinach (*Spinacia oleracea*) collected from the market, due to their leaf topography (Mostafidi *et al*., 2020). Du Plessis *et al*. (2017) highlighted the microbiological quality of spinach from retails and concluded that continued surveillance on a larger scale is required, particularly in the informal sectors. An effective, good epidemiological surveillance for foodborne diseases leads to better management and control of foodborne incidents (Bisholo *et al*., 2018).

1.2 Problem statement

Food hygiene errors, either from production or distribution, resulting in cross-contamination of potential pathogens between commodities, are a major contributing factor for pathogen proliferation leading to foodborne poisoning. The high prevalence of microbiota represents a potential hazard to food safety, the economy, industry as well as human health. Inadequate hygiene practices, poor food safety assurance and management systems, poor monitoring systems, and inadequate transportation contribute to contamination and proliferation of microorganisms. These include inadequate hazard analysis and risk assessment such as analysis of critical points including improper processing hygiene. Over and above the concerns mentioned, farm infrastructure and personal vehicles for produce transportation can present a hazard and contamination of various microorganisms.

This research is important as small-scale Free State farmers face challenges such as poor manufacturing structures, improper infrastructure, and poor general hygiene. The influence of pre-harvest and post-harvest potential risk factors on the microbial community of leafy green vegetables, the interactions of the opportunistic foodborne pathogen with the native microorganism to pathogen transition, and the effect of antimicrobial resistance spread and pathogen succession on leafy green vegetables and human health are increasingly becoming a concern. The main concern stems from the fact that most of the leafy green vegetables are sold directly from the farm to consumers, farm-to-farm exchange, informal traders, and some retailers. In light of the aforementioned, it is therefore crucial to understand how these pathogenic microorganisms can survive, thrive and establish themselves on the phyllosphere including their progression to the next niche.

1.3 Rationale of the study

The availability of food, utilisation and stability are holistically important as they also affect food security status. Stability and sustainability of agriculture are required to achieve food security. The burden of food poisoning, food spoilage and major food recall incidents

affects economic loss due to the disposal of contaminated fresh vegetables and affects public health (Birke & Zawide, 2019). Food security is a global challenge, and the stunting still affects most of the developing countries.

Thus, research is important regarding improving food safety management systems, reducing food poisoning, recall incidents and outbreaks by controlling critical points and food security in primary horticulture production environments, even at retail, and determining which antimicrobials will inhibit the growth of specific bacteria causing a certain infection. Leafy green vegetables are sensitive and perishable, and their production has shown to be a public risk. It is therefore imperative to emphasise microbial quality since fresh produce is minimally processed. Hygiene and sanitation are imperatives as these perishable commodities are moved straight from the fields to consumers for consumption. The pick-your-own method is a very perilous method when there is no proper general hygiene because the chances of contamination are high. Contamination can also spread between commodities when purchased in bulk.

The study contributes to the scholarly research on the primary production and retail food safety of fresh leafy green vegetables including resistance and sensitivity of antibiotics for certain infections. It also contributes to the understanding of contamination and potential risk parameters that could influence the predominance of leafy green vegetables. The data may be utilised to influence further educational efforts and future research gaps designed to provide risk mitigation for small-scale Free State farms and retails regarding spinach and cabbage production. Insufficient knowledge is available on leafy green vegetable production in the Free State except for their nutrition contents as these are staple foods. The aim was to identify, characterise and assess the prevalence of microbiota on spinach, cabbage and crates at small-scale farms including their proliferation leading to succession at retail establishments and evaluate the antimicrobial susceptibility of the identified microbial pathogens in the Free State, South Africa.

1.4 Aim

The aim of this study was to characterise opportunistic pathogens that are prevalent in small-scale farms due to agronomic parameters and their succession due to possible proliferation and amplification along the cold chain during distribution to the retail level, compare both spheres by assessing epidemiological data such as aetiology and risk to human health, and profile pharmaceutical antibiotics that will be utilised to treat infections caused by these pathogens. The following specific objectives were undertaken:

- To enumerate microbiota and identify microbial species isolated from spinach and cabbage on a small-scale farm level by analysing spinach and cabbage and storing crates before purchase and distribution to various destinations.
- To enumerate microbiota and identify microbial species isolated from packaged spinach and cabbage at retail establishments that are mainly supplied by these small-scale farms and determine the dominant species of different pathogens.
- To conduct antimicrobial susceptibility profile on pathogens isolated from spinach and cabbage.

1.5 Delineation of the Dissertation

Chapter One: This chapter focuses on the general overview of the background of minimally processed leafy green vegetables such as spinach and cabbage and associated microbiota, including outbreak incidents. It also covers the succession of microbial pathogens from farms to the retail point and antibiotic residue as contaminants. The threats associated with leafy green vegetables are illuminated and the study aims and objectives are presented.

Chapter Two: The chapter contains the literature review focusing on the minimal processing of microbial hazards that contribute to the contamination of leafy green vegetables. It covers the farm-to-fork continuum including microbe-microbe survival strategies and microbe transition.

Chapter Three: The chapter focuses on the enumeration and characterisation of phyllo-epiphytic and endophytic pathogens at the farms as affected by agronomic parameters.

Chapter Four: The chapter focuses on the proliferation and succession of opportunistic pathogens at retails.

Chapter Five: The chapter focuses on the antimicrobial susceptibility of enumerated microbiota isolates associated with spinach and cabbage vegetables from farms and retails due to commonly utilised antibiotics in livestock and the on-agriculture industry including their contamination of fresh produce.

Chapter Six: Summary**,** conclusion, and recommendations of the study including the recommendations for food safety management systems applicable to fresh produce.

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CHAPTER TWO

LITERATURE REVIEW

Published: Mohapi, D.A., Nkhebenyane, S.J., Thekisoe, O. and Khetsha, Z.P., **2024.** Insights on the prevalence of phyllo-epiphytic and endophytic pathogens on leafy vegetables from farms and retails in South Africa: A review**.** ***Journal of Applied Horticulture***, *26*(3), pp.284-291. https://doi.org/10.37855/jah.2024.v26i03.54**.**

2.1 Introduction

Each province in South Africa is unique in terms of suitable agricultural commodities that can be produced. Additionally, the Free State Agricultural Union reports that the province has 7.515 farming units, the highest in the country. Furthermore, it accounts for 26.4% of South Africa's field crops and 15.9% of all its livestock. Moreover, the Free State province is responsible for 15% of South Africa's gross agricultural income. The sector contributes approximately 7% to the provincial gross domestic product. Consumer demand puts pressure on the fresh fruit and vegetable industries for year-round supply. South Africa has successfully supported small-scale production, often as partial contributors to household food baskets and livelihoods (Nyathi and Ndlovu *et al*., 2022) although food safety knowledge and proper food handling practices were found to be inadequate in some areas (Mkhungo *et al*., 2018). A study in Ghana illucidated the need for appropriate control measures to mitigate contamination of fresh vegetables throughout farm-to-fork (Balali *et al*., 2020)

Supermarkets have been spreading rapidly in developing countries for the past decade. The rise in supermarkets is most significant in South Africa, Kenya, and Nigeria (Reardon *et al*., 2003). The participation of urban consumers in the informal vegetable market plays a vital part in the urban economy as it offers easy access to food supplies offered by informal traders (Marumo & Mabuza, 2018). Vermeulen (2010) reported that 79% to 99.5% of consumers purchase more perishable vegetables from informal traders mainly due to convenient location. Many retails obtain their vegetables from small-scale farms, due to accessibility and good discounts, particularly when purchased in bulk and as farms also permit pick-your-own.

In South Africa, most available studies on vegetables are based on indigenous leafy vegetables which are cultivated from home gardens and small plots in low-income rural villages (Maseko *et al.,* 2017). Additionally, other studies have extensively investigated leafy greens' nutrient content, potential contribution to dietary reference intakes and antioxidant levels (Van Jaarsveld *et al.,* 2014). Furthermore, their role in combating hunger and malnutrition is discussed in terms of their contribution to food security and nutritional status (Mavenghama, 2013). A study in Gauteng, South Africa reported that spinach (*Spinacia oleracea*), cabbage (*Brassica oleracea* (L.) var. *capitata*) and tomatoes (*Lycopersicon esculentum* L.) are considered popular vegetables, particularly for low-income families as part of a sustainable daily diet (Methvin, 2015; Pontsho *et al*., 2024).

2.2 Minimal processing of leafy greens: Pre-harvest and post-harvest prevalence

Pre-harvest and post-harvest sources are considered potential hazardous that influence the survival and growth of pathogenic microorganisms on fresh vegetables. Holvoet *et al.* (2012) reported that *Escherichia coli* can be found on weighing surfaces and conveyor belts in Belgium and these objects were highlighted as potential sources for cross-contamination due to poor manufacturing practices.

Minimal operations are known to cause the onset of many physiological changes, reducing the quality of a product (Ragaert *et al.,* 2007; Al-Dairi *et al*., 2023). Spinach, cabbage, and lettuce are the kinds of fresh produce that were identified as the commodity group of highest concern from a microbiological safety perspective because they are minimally processed with complex methods, which contributes to the increment of foodborne pathogens (WHO, 2008). Vegetables are minimally processed with the idea of improving quality and extending shelf-life, including fulfilling consumers expectations (Al-Dairi *et al*., 2023). Figure 2.1 below depicts various stages of minimal processing (Singh, 2008).

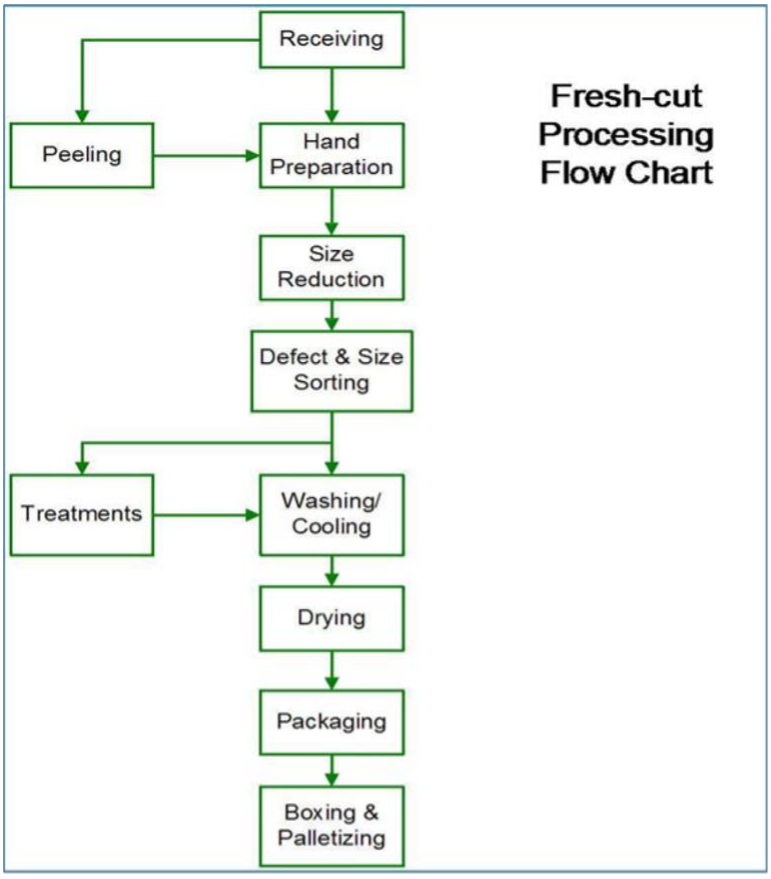

Figure 2.1: Minimal processing operations

Harvesting of produce may be done through mechanical harvesters in large operational plants and by hand in small operational plants before being taken to the receiving point at the processing facility (Wang *et al*., 2023). The preparation procedure generally includes peeling, manual preparation, size reduction, defect and size sorting using different objects, washing, packaging, and refrigeration but the first essential step is the removal of the outer leaf layer, especially in leafy greens such as cabbage (Siddiqui *et al.,* 2011). Washing the produce utilising potable water after harvest is an essential step to remove dirt and damaged tissues (Qadri *et al.,* 2015). Due to favourable conditions and sufficient potential resources, pathogenic microbes are well able to thrive, contaminate and shift to the next phase, form a new niche, and cause food poisoning (Redford *et al*., 2009).

2.3. Microbiota persistent and colonisation on the phyllosphere

Attachment is the first step in the establishment of bacteria in the phyllosphere (Brandl, 2006). They modify their habitat, divide, and increase in number utilising various mechanisms such as enhancing nutrients released from the plant phyllosphere and producing a polymer matrix for protection (Beattie & Lindow, 1999; Mandal *et al*., 2023). The presence, attachment, colonisation, growth, and survival of microorganisms on produce depend on nutrients, the heterogeneity traits of microflora, environmental conditions and plant phenology (Kramer *et al*., 2024. Colonisation and attachment of foodborne pathogens on a produce-forming biofilm is a major cause of foodborne infections including nosocomial infections (Jamal *et al*., 2018).

The composition and behaviour of bacteria diversity on the phyllosphere can be influenced by farming and storage conditions (Lopez-Velasco *et al.,* 2011). Handling of leafy vegetables should be considered as a critical control point to avoid cross-contamination, pathogen colonisation and attachment on leafy greens as their interaction and subsequent internalisation can be incorporated into biofilm (Jamal *et al*., 2018). Microbes' aggregates are associated with nutrients' availability, interaction with other bacterial populations to more virulent pathogens, survival from environmental stress, and chances of infiltration into the plant as a mode of protection from disinfectant (Siddiqui *et al.,* 2011). Another study referenced the sub-Saharan Africa fresh produce supply chain to improve harvest handling practises, develop regulations which will in turn improve fresh produce supply chain (Aworth *et al*., 2021).

Bacterial colonization strategies include modification of the phylloplane habitat including aggregation, ingression, and egression colonization, forming external and internal microbial populations (Beattie & Lindow, 1999; Mandal *et al*., 2023). Produce has natural

apertures such as stomata, including veins, and cell wall junctions and often have punctures, cuts, splits, and cracks due to injury during pre-harvesting and post-harvesting handling, and bacteria can attach and assemble at the injury sites to form biofilms (Burnett *et al.,* 2000; Mandal *et al*., 2023). A healthy phyllosphere may also support different numbers of epiphytic bacteria by passively leaking small amounts of metabolites such as carbohydrates, amino acids and organic acids to the leaf surface and assimilating atmospheric carbon dioxide into sugars (Julius *et al*., 2017).

Bacteria can modify and manipulate the environment to enhance colonization and internalization, the interaction in the phyllosphere can affect the physico-chemical properties which can then affect the safety of crops for human consumption (Sohrabi *et al*., 2023). Figure 2.2 below depicts ingression and egression colonization forming external and internal microbial populations.

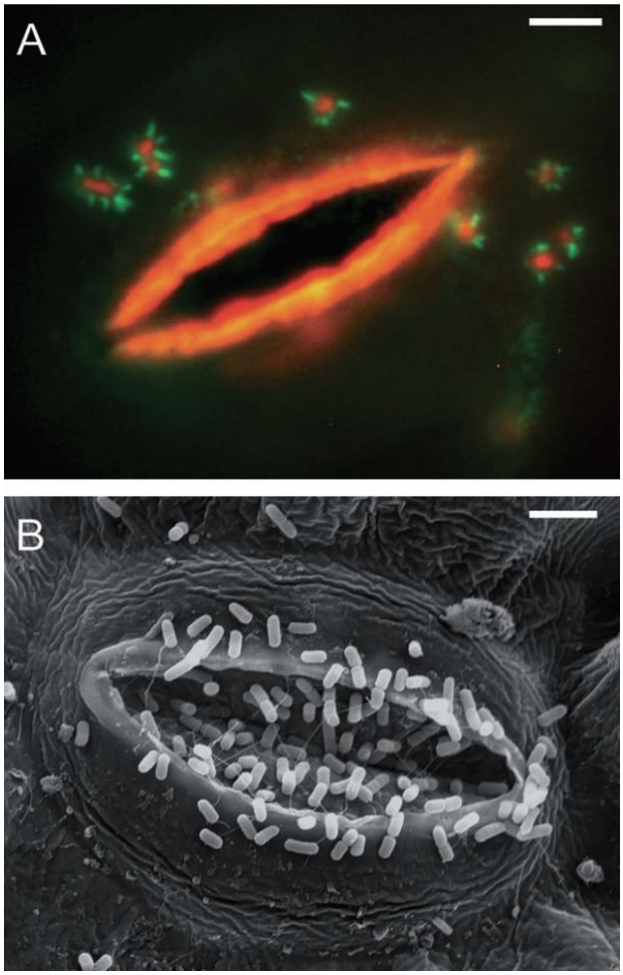

Figure 2.2: *E. coli* on guard cells of the leaf stomatae and bacteria reaching the stomatae cavity (A. Immunofluorescence staining, B. Scanning electron micrograph) (Berger *et al*., 2010).

However, phyllo-epiphytic bacteria can survive strenuous conditions and rapid fluctuations of various conditions that occur on phyllosphere by utilising two major strategies:

(i) Tolerance strategies to survive under direct exposure to environmental stresses,

(ii) Avoidance strategy through sites that are protected from those stresses

An efflux pump is another mechanism which allows the microorganisms to regulate their internal environment through toxic substance removal, including antimicrobial agents while quorum sensing is for communication and coordination of the bacterial population, also enhancing access to nutrient-rich niches, and motility and plays a role in biofilm formation (Gaurav *et al*., 2023). Different pathogens broadly utilise three strategies to acquire the necessary nutrients for survival and replication from plant cells:

(i) Manipulating host proteins to export nutrients to the apoplast where they reside (biotrophic),

(ii) Causing the host cell to undergo programmed cell death and feed on the remaining nutrients (necrotrophic), or

(iii) A combination of the two approaches (hemi biotrophic)

2.3.1 Persistence of opportunistic pathogens in soil and soil amendments

Many environmental and management factors that are employed introduce enteric pathogens to soil. These include raw manure, wastewater, human biosolids, compost, wild and domestic animal intrusion, and other anthropogenic activities (Gutierrez-Rodriguez and Adhikari, 2018). Garrec *et al.* (2003) and Manyi-Loh *et al* (2023) investigated the occurrence of *Listeria* spp. and *L. monocytogenes* in different mediums such as sewage sludge and reported that *L. monocytogenes* in particular was present in all stages of the treatment, 95% of activated sludge, 73% of dewatered sludges, and 80% of stored sludges. Physicochemical properties found within soil as well as soil texture, pH, organic matter content, cation exchange capacity, porosity, and organic and inorganic nutrient sources influence the microbial ecology of all soilborne bacteria and enteric pathogens that are found in soil (Griffiths & Phillippot, 2013). Microbes capable of completing their entire life cycle in soil are capable of infecting humans through food consumption (Gutierrez-Rodriguez & Adhikari, 2018). *E. coli* O157:H7 strains can survive on manure-amended soil for more than two months (Limoges et al., 2022). Other studies also enlightened on the application of manure, the appropriate times for harvest after applying manure, and the internalisation of microbes leading to biofilm (Myszka & Czaczyk, 2011).

2.3.2 Prevalence and persistence of enteric bacteria in irrigation water

Irrigation water is thought to be the leading pre-harvest source of contamination of fresh produce (Ijabadeniyi & Buys, 2012). In South Africa, few studies have highlighted and reported on the microbial contamination of surface water sources (Chigor *et al.,* 2013; Viviers *et al*., 2024). The study highlighted the clustering of *E. coli* isolated throughout the water-soil-plant nexus, implicated irrigation water in fresh produce contamination. Highlighting the importance of complying with irrigation water microbiological quality guidelines to limit the spread of potential foodborne pathogens throughout the fresh produce supply chain (Viviers *et al*., 2024). The concern is that this contaminated surface water utilised for irrigation may contaminate fresh vegetables which may also negatively affect the trade of vegetables to the EU and USA due to phytosanitary measures (Ijabadeniyi & Buys, 2012). Another study assessed metals/metalloids in water and fresh vegetables along South Africa and Mozambique river catchment and Metal concentrations were mostly higher in South African samples compared to Mozambican samples of both water and vegetables signifying carcinogenic health risks (Genthe et al., 2018). The deduction is that there is a correlation between increased numbers of pathogens on irrigated vegetables as a direct consequence of poor water quality utilised for irrigation (Decol *et al.,* 2017).

2.3.3 Infiltration and internalization of endophytic bacteria

Internalization of foodborne bacteria into edible parts of fresh produce plants represents a serious health risk (Wright *et al*., 2017). Hirneisen *et al*. (2012) define internalization as the uptake of human enteric pathogens through wound space, vascular tissue, and damaged roots of the plant into the intracellular spaces. Type of plant, bacteria serovar, contamination route, effect of environmental stress, age and mechanism greatly influence the likelihood of internalization of a human pathogenic bacterium within a plant (Hirneisen *et al*., 2012). Temperature difference between produce, humidity and effectiveness of disinfectant water plays a role in infiltration resulting in internalization through hydrostatic pressure differential, drawing bacteria into produce (Buchanan *et al*., 2017). The colonisation of bacteria following infiltration and internalisation is a dynamic process where various factors are included to promote contact, attachment, cell-cell interactions, defence from biocidal wash treatment and protection against stresses until the final stage in which bacteria disperse new colonisation (Srey *et al*., 2013). The schematic representation is outlined as follows.

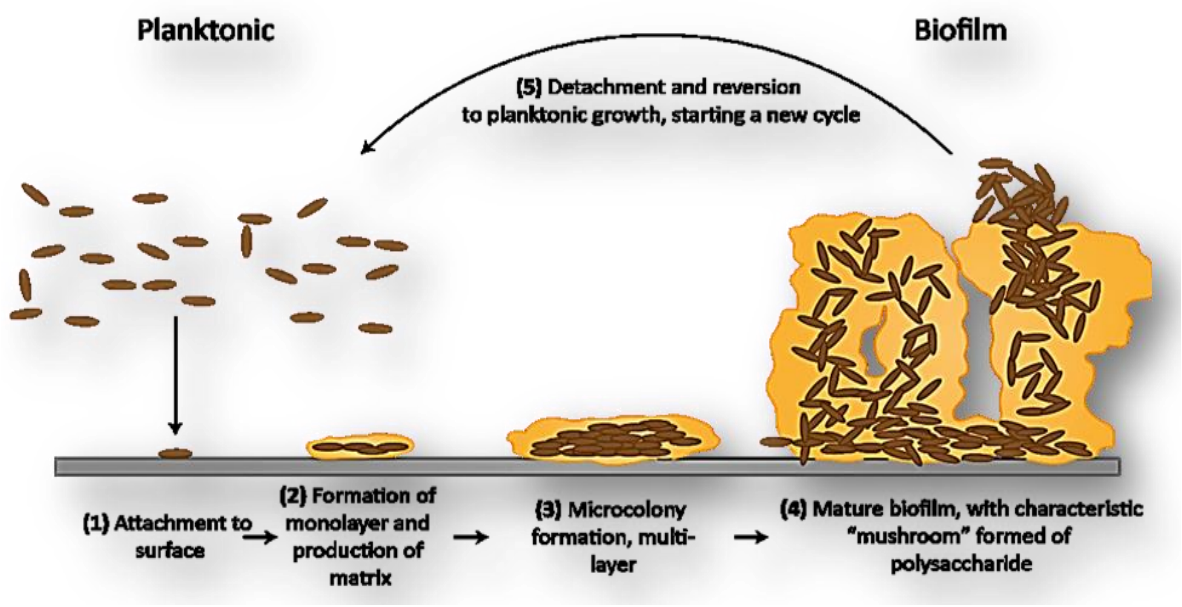

Figure 2.3: Biofilm formation on a crop (Vasudevan, 2014).

Microorganisms protect themselves from outside disturbance by an effective internal balance of physiological processes, a mechanism called homeostasis (Kumar *et al.,* 2017). The waxy cuticles, internal leaf tissue of the phyllosphere and other polysaccharides serve as protective factors for pathogenic bacteria by keeping disinfectants and other environmental stresses away (Kramer *et al*., 2024). Bacterial cells found in stomata, epidermal cell wall junctions, near hydathodes, depressions in the cuticle, trichomes, calyx, stems, and damaged and cut edges of produce due to internalization might be inaccessible to disinfectants (Olmez & Temur, 2010). Colonization of pathogens on damaged apertures and internal tissues may create a strategy by which Shiga-toxigenic *E. coli* (STEC) O157:H7 survives in a nutrient-rich habitat protected from external stresses (Saldana *et al*., 2011). Damaged or natural openings have high wettability which promotes water availability and nutrient leaching that in turn support microorganism growth (El Bouchtaoui *et al*., 2025). Damaged cells can induce antimicrobial resistance build-up and enhance the risk of resistance transfer (Verraes *et al*., 2013).

2.3.4 Processing facility parameters influencing the prevalence of pathogens

Post-harvest treatment of vegetables includes handling, workers, storage, washing, and transportation. Fresh produce's susceptibility to survival and growth of pathogens is caused by poor handling which provides opportunities for contamination, growth, and ingress into plant tissues (Esmael *et al*., 2023). Attachment of pathogens onto shredders and slicers re-introduced

L. monocytogenes contamination at a vegetable processing plant (Francis *et al.,* 2012). Pathogenic microorganisms such *as L. monocytogenes, Salmonella enterica,* and *E. coli* 0157:H7 have been noted to form biofilms in food processing facilities (Galie *et al*., 2018). The internal surface of the processing equipment which is difficult to clean can also cause heavy bacterial contamination on processed vegetables (Møretrø *et al*., 2017).

Cross-contamination can result from pathogen transfer from a contaminated leafy green vegetable to an uncontaminated leafy green vegetable through wash water (Gomba *et al*., 2017). The heaviest contamination was observed in the nets for soaking vegetables processed in cold water (Kaneko *et al.,* 1999; Santos *et al*., 2023). Ramos *et al*. (2013) detailed the advantages, limitations, and effectiveness of chemical disinfection of fresh produce to improve quality and safety. In addition to limitations of chemical disinfection, bacteria such as *Salmonella* and *E. coli* 0157:H7 are unable to survive at low temperatures but can proliferate at ambient temperatures as they are mesophilic. However, fresh vegetable storage at 4°C reduces the growth of psychrotrophic bacteria but organisms such as *L. monocytogenes* are capable of surviving at low temperatures (Jideani *et al*., 2017).

2.3.5 Storage facility area for cooling of leafy greens

The preservation and transportation of perishable foods are managed through a cold chain to slow down biological decay processes and deliver safe and high-quality foods to consumers (Mercier *et al*., 2017). The composition of bacterial diversity and microbial quality of fresh produce can be influenced by storage conditions (Lopez-Velasco *et al*., 2011). Many intrinsic factors such as water activity, pH, and nutrient content of fresh produce, including extrinsic factors such as relative humidity and temperature, can affect the stability of microorganisms affecting the quality and shelf life (Wang *et al*., 2023).

Refrigeration temperatures will not completely retard microbial spoilage as these are favourable conditions for some microorganisms such as *Pseudomonas* spp. and *Listeria* spp. which rapidly grow under such temperatures (Mercier *et al*., 2017). Failing to keep perishable food in the desired temperature range because of insufficient refrigeration can stimulate the growth of potential pathogens (Coorey *et al*., 2018). The very O_2-depleted atmospheres (0.25%) utilised to control enzymatic browning enhanced *Listeria* growth in fresh-cut Iceberg lettuce stored at mild temperatures (O'Beirne *et al*., 2015).

2.4 Succession of pathogens to retails

Small-scale growers who sell their produce locally to consumers mostly utilise their vehicles for all farm purposes (Sinkel, 2016). Holm *et al.* (2017) highlighted the lack of adequate water and sanitation infrastructure in the market as a contributing factor to bacteriological contamination of fresh vegetables with the prevalence of *E. coli* being the highest on leafy greens found in 74 (87 %) of the 85 samples. In contrast, Sagoo *et al*. (2001) reported on the microbiological quality of vegetables collected from various retailers and farms and noted that 99, 5% of samples were found to be of satisfactory or acceptable quality whilst only 15 (0,5%) were unsatisfactory quality. The 0,5% unsatisfactory results were due to *E. coli* and *Listeria* spp. (not *L. monocytogenes*) but the overall agricultural, hygiene, harvesting and production practices were good.

Insufficient precooling from the farm level can have a lasting effect on product temperature along the cold chain even if the subsequent steps are achieved at the correct ambient temperature at other stages of the cold chain (Nunes *et al*., 2014). The microbiological quality of samples of fresh leafy greens and fruits collected from supermarkets in Istanbul was analysed. In the study, 261 foods were sampled, 10 (3.83%) *Salmonella* sp. and 17 (6.51%) thermotolerant *Campylobacter* sp. were detected with the highest count highlighting strict temperature control of the cold chain (Buyukunal *et al*., 2015). Storage and farming operational practices influence the composition of bacterial groups on fresh cabbage, the microbiomes are highly diverse and complex and change dynamically during storage at refrigeration temperatures with the establishment of a dominant population (Lopez-Velasco *et al.,* 2011). Transportation, distribution practices, and storage conditions determine product quality and safety for future use and can influence the diversity and composition of produce-associated microbial communities (He *et al*., 2018).

At retail, fresh leafy vegetables must be refrigerated upon arrival to prevent temperatures from fluctuating. Food workers have been identified as the primary sources of infection in numerous outbreaks, either because they were carriers who did not exhibit symptoms or because they were in the prodromic phase of the illness before symptoms appeared (Sumner *et al*., 2011). Other outbreaks have been related specifically to retails either through a contaminated foodstuff being the source or an infected food handler where food handlers are asymptomatic (Rumble *et al*., 2017). This is a critical stage as workers are involved

in the handling of fresh vegetables such as off-loading and packing of leafy greens from storage to racks to display for consumers to purchase (Faour-Klingbeil *et al*., 2016).

In the Middle East, Faour-Klingbeil *et al*. (2016) reported that out of 90 commodity samples of raw salad vegetables, lettuce (*Lactuca sativa*), and parsley (*Petroselium cryspum*), 14% of *L. monocytogenes* and 45.5% of *S. aureus* were detected from harvest to retail establishments. In addition to the above-mentioned, the study also highlighted the shortfalls in hygienic farming due to the utilisation of inappropriately treated manure and broilers' (*Gallus gallus domesticus*) litter to fertilise the growing crops in the fields and as a source contributed to the high levels of *S. aureus* in the products at the retail establishments. The retail microbiome is regarded to be a mixture of various microbes due to various sources which include human oral skin-associated bacteria, soil bacteria, the outdoor environment, and the diverse indoor environment microbial community (Park *et al*., 2025).

2.4.1 Microbe to microbe transition in the phyllosphere to form a new niche

Other cultivars possessing a larger topography area influence more diverse microbial communities with increased richness and larger populations. Commensal-to-pathogen or pathogen-to-more virulent pathogen transition is multifactorial and depends both on the prevailing environmental conditions and specific gene-gene interactions placed within the context of the entire ecosystem (Ehrlich *et al*., 2008), which might be detrimental to other commensal and/or epiphytic bacteria on the leaves and affect their life circle by an ingression and egression continuum process (Dogan *et al*., 2023). Moreover, few studies highlighted that *Bacillus* strain can colonise cabbage endophytically and th further discovered from the roots that the transfer can be internal via the vascular system to the aerial parts of the plants which constitute the phyllosphere (Wulff *et al*., 2003; Dogan *et al*., 2023). The location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf were also discussed (Santoso *et al*., 2021).

Bacteria on the plant phyllosphere employ a range of strategies to colonise and survive on plant tissues, they produce a matrix which modifies the environment to promote cell growth and survival and also mimic plant hormones to facilitate their survival on the phylloplane (Radhika *et al.,* 2015). Natural apertures allow a ready passage for microorganisms as they contain a variety of nutrients, creating fluid channels for the internalization of bacterial pathogens and allowing microbial biofilm formation creating an ingression and egression continuum process with the rest of the plant microbes (Vacher *et al*., 2016).

2.5 Tolerated dose and infective dose

Different pathogenic species differ in the number of required cells or molecules to start an infection in a host. The number of cells that are required to infect a host and start an infection is termed infective dose, they vary intensely across pathogens with each species strain which also depends upon the mode of action (Lucy *et al*., 2014). In most instances, an infection can be caused by an intake of a small dose of pathogenic cells. The type of pathogens causing the disease is important in determining the dose level (Hara-Kudo &Takatori, 2011). The mode of infection and infection dose to cause a disease will then determine the level of risk and the severity of symptoms (Leggert *et al*., 2012). WHO reported that children under 5 years of age carry 40% of the foodborne disease burden, with 125,000 deaths every year (WHO, 2017).

The infective dose variation can be explained by the different biochemical mechanisms of the pathogens that are utilised to infect a host The correlation between disease and virulence that is more likely to overwhelm the host depends on the condition of the host cells when responding to the external molecules' condition that are invading their space internally. Table 2.1 below depicts bacteria species and the disease type of each species, including the infective dose from various foodstuff sources ingested.

Table 2.1 Pathogenic microorganisms associated with foodborne disease and their minimum infective dose (Leggert *et al*., 2017).

Bacteria sp.	Disease type	Tolerated dose	Foodstuff source	References
Bacillus cereus	Diarrheal toxin Intoxication-emetic	(>10^5/g)	Salads, vegetables	Granum (2017)
Escherichia coli 0157:H7	Infection	(10^1-10^2/g)	Contaminated fruits, vegetables	Schmid-Hempel &Frank (2007)
Listeria monocytogenes	Infection	(>10^2/g)	Raw vegetables	Farber & Peterkin (1991)
Staphylococcus aureus	Intoxication	(10^5/g)	Contaminated food source	CDC (2015)

2.6 Antibiotic resistant as a threat to human health

The discovery and utilisation of various antibiotics have contributed significantly to the control of infectious diseases, the reduction of the associated mortality, and the morbidity rate in both humans and animals (Rahman *et al*., 2022). The growing antimicrobial resistance (AMR) phenomenon is generally linked to selective pressure which is normally caused due to

improper use, overuse, or misuse of antimicrobials in humans and animals (Musoke *et al*., 2021).

More than two million people every year are affected by antibiotic-resistant infections, with at least 23,000 dying as a result of the infection (CDC, 2013). According to the reported data from 2009 from the European Centre for Disease Control, the European Union, Iceland, and Norway are estimated to have 25, 000 deaths that are caused by AMR strains each year leading to approximately 2.5 million extra hospital days (Spellberg *et al*., 2011). Additionally, CDC estimated 100, 000 and 80, 000 deaths caused by AMR strains were reported in the USA and China, respectively.

2.6.1 Antibiotic residue from soil amendment and animals as a contaminant to crops

Antibiotic resistance genes (ARGs) originate from microorganisms in the environment and are incorporated into mobile genetic elements (MGEs) such as plasmids, transposons integrons and are mobilised through various biomes using various mechanisms (Colavecchio *et al*., 2017). Integrons, plasmids and transposons are genetic elements found in bacterial genomes carrying ARGs and are present in the majority of gram-negative pathogens (Gilling *et al.,* 2014). Different environmental biomes house bacteriophages and antibiotic resistance bacteria which harbour ARGs that can be mobilized from commensal bacteria to human pathogenic bacteria (Gilling, 2014). Commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria (Santoso *et al*., 2021). The movement of genes from one organism to another through horizontal transfer can trigger a resistance phenotype (Sandberg & LaPara, 2016).

Soil has been investigated as a reservoir for bacteriophages carrying ARGs and at some point, regardless of the manure treatment used, bacteriophages have the potential for HGT in agricultural soil microbiomes (Ross & Topp, 2015). Mechanisms for antibiotic sorption to soil include amongst other things cation exchange, surface complexation, and hydrogen bonding (An *et al*., 2015). About 30-90% of a parent compound and its breakdown metabolites are excreted via urine or faeces, unchanged due to poorly absorbed antibiotics in an animal's gut (Du & Liu, 2012). An *et al*. (2015) reported that antibiotic contamination in animal manure, soil and sewage sludge had a higher concentration of antibiotic residue on vegetables.

Utilising manure and biosolids as a fertiliser is a common practice around the globe. Contamination of plants by antibiotic residue can also occur through different mediums

employed for making the soil fertile such as the utilisation of contaminated manure, sludge, and contaminated irrigation water (Lopez-Velasco *et al*., 2011). Feedlots are a potential source of antibiotics (Zalewska *et al*., 2021) and about 75% of antibiotics provided to feedlot animals could be excreted in the environment as manure waste (Kos *et al*., 2023). Bacteria are capable of surviving for long periods in the environment, their survival depends on their species and environmental conditions. However, their genetic elements can persist regardless of cell viability (Kos *et al*., 2023).

Animals given therapeutic and sub-therapeutic doses of antibiotics can generate manure containing elevated concentrations of antibiotic-resistant bacteria and ARGs (Marti *et al.,* 2014). After being used as a supplement in the field, this excreted antibiotic compound in animal manure can be adsorbed, bio-accumulated, or degraded through an abiotic/biotic process. After that, it will revert to the parent compound, harming the field and contaminating fresh produce (Sandberg & LaPara, 2016). Plant uptake and bioaccumulation of antibiotics have received considerable interest due to issues of food safety and human health. The majority of gram-negative pathogens now have integrons carrying resistance bacteria and are known for the dissemination of antibiotic resistance (Gilling, 2014).

2.6.2 Antibiotic residue uptake as a contaminant to the crop

Another study shared knowledge on plant uptake of most used antibiotics and their issues regarding food safety and human health (Yannarell *et al.,* 2012). The plant is organ dependent, and the absorption of antibiotic residue is first via the roots from the soil through different mechanisms and the absorbed residues can be found in tissues (Wang *et al*., 2015). Bioaccumulation and response of antibiotic residue from the plant vary depending on the type of species, the antibiotic class and the antibiotic concentration which occur through different mechanisms which include ionization, sorption properties, water solubility, and others (Minden *et al.,* 2017). Moreover, Yannarell *et al.* (2012) reported on the entry of antibiotics into the environment and the chemical characteristics of antibiotics, their behaviour and persistence in soil including their mechanism of degradation.

Other studies reported on antibiotic plant uptake from the environment and their concentration in different vegetable commodities (Dolliver *et al*., 2007; Bassil *et al*., 2013). One study evaluated the plant uptake of sulfamethazine antibiotic on manure and plants and concluded that the uptake concentration of antibiotic is dependent on the concentration in the

manure (Dolliver *et al*., 2007). Several studies investigated the effect of antibiotics on human microbiota, particularly intestinal microbiota which itself consists of several thousands of species that collectively maintain gut physiology, homeostasis (including metabolic energy balance), immune responses, and the resistance of bacteria to subsequent disease (Keeney *et al*., 2014). The human microbiome is an integral component of the human body and if altered bacterial communities can shift microbiomes from healthy states to disease-associated states (Pflughoeft & Versalovic, 2012).

2.7 Indicator organisms

Indicator organisms have been utilised extensively for a very long time to assess the microbiological status of food products which are often termed safety or index indicators depending on their purpose. They may indicate the potential presence of various pathogens, a gap in adequate sanitation and improper hygiene practices and/or good hygiene practices or a process failure reflecting quality attributes that may influence consumer acceptability of a product (Costell *et al*., 2010). Frequently, the presence of indicator organisms is a concern, but in most instances, it is the quantity that is significant as the count may reflect the time and conditions of storage (Njuguna *et al*., 2025). Since native microflora are a component of produce products, contamination can lead to opportunistic species becoming established in a niche. This occurs when homeostasis is disrupted and the host mechanism fails, turning normal microflora pathogenic. Characteristics such as species abundance and spatiotemporal presence can also contribute to this transition (Ehrlich et al., 2008). Furthermore, the majority of infections are opportunistic, competing with the native microflora and capable of transforming the majority of the community into pathogens that expand or form new communities. Gram-positive bacteria are the culprits behind almost 50% of bloodstream infections and foodborne illnesses (Khaleque *et al*., 2024).

2.8 Conclusion

The biggest challenges faced by small-scale farmers are farm infrastructure, crop production, and livestock breeding proximity to the crops due to shortage of space. Successional patterns exhibited by microbial communities in leafy greens have received relatively no attention in South Africa. Microbial assessments of all the pathogenic organisms that are a threat to the human species are significant in reducing unnecessary illnesses. Food safety and hygienic measures are important in minimizing the prevalence of microorganisms. A growing population leads to growing food production, therefore, food safety and quality are

critical components in all areas of food production. Food safety and food quality are required in all processes of production to avoid foodborne poisoning and outbreaks. Implementing a complete sanitation program that encompasses the entire processing and hygiene programme is essential to minimize the risk of contamination by pathogens and to assure consumers' safety.

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CHAPTER THREE

PHYLLO-EPIPHYTIC AND ENDOPHYTIC PATHOGENS ON *Brassica oleracea* var. *capitata* (L.) and *Spinacia oleracea* (L.) AS AFFECTED BY SMALL-SCALE FARM PRODUCTION SYSTEMS

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Abstract

The phyllosphere hosts a considerable number of microorganisms, providing a vast habitat for naturally associated phyllobacteria due to its topography, as it offers various colonisation and infiltration sites. Contamination of vegetables may occur through pre-harvest and post-harvest activities and includes cross-contamination from infected personnel. The study aimed to examine the prevalence of microbial contamination for spinach (*Spinacia oleracea* L.) and cabbage (*Brassica oleracea* var. *capitata* L.) at various farms, based on the production and agronomic systems in the Free State, South Africa. In addition, the study further demonstrated that several potentially pathogenic microorganisms are present in common fresh leafy greens such as spinach and cabbage. Almost all the analysed and identified microorganisms were reported to be opportunistic pathogens. The spinach and cabbage phyllospheres were contaminated mostly with *Staphylococcaceae, Pseudomonadaceae, Morganellaceae, Caulobacteraceae, Moraxellaceae, Burkholderiaceae, Yersiniaceae, Xanthomonadaceae, Listeriaceae* and total coliform species. The predominant genera were *Staphylococcaceae, Morganellaceae* and *Pseudomonadaceae* in spinach and cabbage isolates. The analysed isolates revealed a high level of contamination by opportunistic pathogens such as total coliform, *Morganellaceae* and *Staphylococcaceae,* reflecting a deficit in agricultural production systems and hygiene practices. From the study, the authors could also demonstrate the rapid rate at which these pathogens can spread through the food chain and cause food poisoning. It was concluded that the frequency and degree of bacterial contamination in this present study were quite substantial and it was recommended that these vegetables be thoroughly washed before consumption, especially when consumed uncooked, specifically in a green salad. Moreover, preparing cooked meals using these leafy vegetables would be better.

Keywords: Spinach, cabbage, consumption, contamination, farm activities, foodborne illness, food safety.

3.1 Introduction

The phyllosphere is a natural environment that includes nutrient-rich leaf surfaces and a complex and diverse population of microorganisms, the most common of which are bacteria (Bashir *et al*., 2022). Epiphytes are microorganisms that are easily removed from their environment by either disinfectant or wash treatment, and endophytes are those that remain and internalise (Micci *et al*., 2022). Microorganisms are common residents of the phyllosphere

and are termed native microflora (epiphytes) and endophytic bacteria (Dees *et al*., 2015). However, their interaction with other microorganisms can be pathogenic, synergistic or antagonistic. Pathogenic or non-pathogenic bacteria have several opportunistic strategies which they utilise to contaminate fresh vegetables in the field or at the time of consumption (Nithya & Babu, 2017). Environment microbe-microbe interactions with the crop microbe, including their mechanisms, are pivotal for the establishment of opportunistic pathogenic bacteria on the crop (Kiselev, 2022). Various strategies include contamination capability, pathogen interaction, microbial dominance, biofilm, and transition, including endophytic survival utilising various defence mechanisms.

Biofilms may represent about 80% of the total microbial population on the phylloplane (Moitinho *et al*., 2020). The sources that contribute to the habitation of microorganisms in the phyllosphere are the atmosphere, insects, seed, or animal-borne sources including nutrients (Whipps, 2008). The bacterial interactions on the phyllosphere can have a significant effect on the fitness of plants by either promoting plant growth or suppressing or stimulating the colonization of plant and human pathogens (Moitinho *et al*., 2020).

Minimal processing generally includes peeling, manual preparation, size reduction, defect and size sorting using different objects, washing and packaging. Produce can be harvested manually in small-scale operational plants or mechanically in large-scale operations from the receiving point to the packaging and distribution. It is well recognized that minimal processing marks the onset of a physiological shift in produce since cutting and trimming expose sites conducive to providing nutrients to toxic microorganisms.

Food poisoning occurs when contaminated food is consumed, the food may be infected by toxins from toxic microorganisms such as *Staphylococcus aureus, E. coli* or *Listeria* species (Hernández-Cortez *et al*., 2017). Furthermore, contamination may be through anthropogenic activities such as poor sanitation and poor hygiene practices, including improperly treated irrigation water or poor agricultural practices, either as a result of personnel or agronomic devices utilised during minimal processing. Toxins are toxic elements within bacteria that inflict pathogenic traits depending on the type of toxin. When ingested through contaminated food they manipulate the human immune system resulting in gastrointestinal infection and other severe complications (Ghazaei, 2022). Bacteria utilise virulence mechanisms such as toxin production to cause a microbial infection or disease condition. Toxins and virulent factors are responsible for the pathogenesis of opportunistic pathogens, they cause human infections

which are characterised by severe complications and symptoms including vomiting, diarrhoea, and abdominal cramps leading to illness (Abebe *et al*., 2020). The transfer of pathogens from one composition to the other is always the cause of cross-contamination. If conducive conditions exist some of these pathogens can grow, colonise and form a biofilm. If the food is ingested, the toxin is released, and possible food poisoning can occur. Risk assessment in minimal processing is crucial since biohazards pose a threat to human health.

The fresh vegetable phyllosphere typically contains natural non-pathogenic epiphytic microorganisms (Mulaosmanovic, 2021; Motshabi *et al*., 2021). Furthermore, microbial communities on the phyllosphere differ in species, composition, dominance and nutrition required. A particular study utilised 16S rRNA gene-directed PCR-DGGE to compare the phyllosphere communities of seven different plant species and the major finding was that microbial phyllosphere communities were more complex than previously thought (Laforest-Lapointe & Whitaker, 2019).

Microbes that flourish and thrive on the phyllosphere interact with the host which in turn shapes the niche allowing growth of the microbial population (Dees *et al*., 2015). Phyllosphere bacteria may include those bacteria that are pathogenic to the plant (Moitinho *et al*., 2020). Antagonist bacteria work against pathogens by preventing their growth while biological control agents or biocontrol agents help to promote plant health and reduce the severity of disease (Beattie, 2006). Native microflora are naturally present in the phyllosphere and are assumed to play an important role against phytopathogens by activating a defence mechanism (Iqbal *et al*., 2023). Their potential as an antagonist agent against enteropathogens serves as the foundation for this defence mechanism. Plant health depends on these phyllosphere bacteria as they have a potential effect against human pathogenic microorganisms which are a major threat to food production, including ecosystem stability (Adomako & Yu, 2023).

The phyllosphere is dominated by gram-negative microbiota, with *Pseudomonas* spp. accounting for 50–80% of the total microbial population. This increases the chance of pathogens persisting on the phyllosphere (Sohrabi *et al*., 2023). Between 30% and 50% of the human population carry *S. aureus* as commensal bacteria and contamination from this pathogen can occur through improper handling (Le Loirs *et al*., 2003; Tigabu & Getaneh, 2021). Furthermore, laboratory experiments with various cultures have revealed many active mechanisms by which bacteria can impair or kill other microbes. Pathogens can occasionally

be outcompeted by native bacteria, but the adaptation and interaction depend on specific needs between the plant and bacteria. Pathogenic microorganisms such as bacteria and viruses are the most common cause of food poisoning (Australian Institute of Food Safety, 2021). The battle against bacterial foodborne diseases is facing new challenges because of rapidly changing patterns of human consumption, the globalization of the food market, including climate change (Argaw & Addis, 2015).

Cabbage and spinach are highly susceptible to microbial contamination and farm operations comprise several units which are likely to provide opportunities for potential cross-contamination. In light of this, leafy green vegetables are not subjected to any lethal process which is typically employed to effectively kill pathogenic organisms. It is hypothesised that pre-harvest and post-harvest parameters contribute to the amplification of pathogenic microorganisms. The absence of appropriate transportation and inefficient agronomic and hygiene practices, including inadequate storage and cooling, compromise market quality and food safety. The objective of the study was to enumerate microbiota and identify microbial species isolated from spinach and cabbage at the small-scale farm level by analysing spinach and cabbage and storing crates before purchase and distribution to various destinations.

3.2 Materials and Methods

3.2.1 Study area and sampling technique

Sample collection

The present study was conducted by procuring sixty samples of raw unpackaged spinach phyllospheres from four different farms and seventy-five samples of cabbage heads from five different farms, respectively, in different local municipal districts within the Free State province, South Africa. The selected farms represented the major small-scale farms which supply the most leafy greens to various buyers making the results of the study representative. Spinach and cabbage were chosen due to their minimal processing, production, demand and purchase. The farms selected were small-scale farms that supply small villages, black markets such as street vendors, informal markets or traders, guest houses and local supermarkets, and farm-to-farm exchange which is termed intra-farm exchange and includes some privately owned retails, and other neighbouring districts.

Samples were collected in the following towns in the Free State Province, South Africa: Motheo District - Mangaung Metropolitan (CUT farm 1 - 29.1217°S, 26.2128°E), Lejweleputwa District - Matjhabeng Local Municipality (Confido farm 2 - 28.9784°S, 27.0264°E), Thabo Mofutsanyana District - Setsoto Municipality (Maokodi farm 3 - 28.9093°S, 27.5555° E [Spinach samples were not available during sampling for farm 3]), Fezile Dabi District - Moqhaka Local Municipality (Meadows farm 4 - 27.6373°S, 27.2323°E), and Thabo Mofutsanyana - Dihlabeng Local Municipality (Naledi farm 5 - 28.2423°S, 28.3111°E). All farms were selected based on the centralised market in Bloemfontein. The market requirement is based on Good Agricultural Practices (GAP), and at most, the selected farmers followed a similar production system to meet the market specifications (Mahlangu *et al*., 2020). A random sampling study design was conducted on spinach and cabbage samples from three different sections, the middle part and two sides of the stored samples ready for purchase. To ensure sample collection was random and representative, at least five areas were assessed for sampling. The samples collected were selected based on the random sampling method, a sampling technique in which each sample has an equal probability of being chosen. A sample chosen randomly is meant to be an unbiased representation of the total population.

Fresh leafy spinach and cabbage samples were analysed for each of the following microorganisms or microbial species: total aerobic mesophilic bacteria, total coliforms, coagulase-positive *Staphylococci* and *Listeria*. All samples were collected aseptically and subsequently transported to the laboratory where they were prepared, plated on various pre-solidified agars from the homogenate of the samples prepared, and incubated within 12 hours on the same day of collection.

3.2.2 Microbiological analysis

Sample preparation

Cabbage samples were cut into quarters, and one-quarter of each batch was taken for processing and bacterial identification. The quarters were coarsely chopped and combined in a sterile hood to avoid contamination. Two opposing segments were taken and the other two were discarded. The remaining segments were mixed and further reduced in the same way to be representative of the whole (Annor, 2009; Moloantoa *et al*., 2023). The ready-to-be-purchased spinach phyllospheres were washed, chopped, roughly mixed and weighed (Annor, 2009).

In this present study, a total of 25 g of each collected sample was added to 90ml of sterile buffered peptone water solution (Merck, South Africa) and homogenized in a stomacher (Stomacher® 400 circulation Seward, Lasec, South Africa) for 260 rpm for 1 min. Thereafter, the mashed samples were filtered through a sterile folded paper filter (Lasec, South Africa). The sequential dilutions were prepared using filtrated samples for plate count analyses. Subsequently, serial dilutions of up to 10^5 folds of the homogenate were prepared for each sample and utilised for bacterial analysis. Serial dilutions of the samples were made in 0.1% buffered peptone water; 0.1ml from each dilution (10^1 to 10^5) was pipetted and spread plated in duplicates on a standard pre-solidified agar medium and incubated at 32°C for 72 hours. After incubation, plates with microbial colonies from 30 to 300 were counted.

The aerobic mesophilic count, *Enterobacteriaceae* (total coliform), *Staphylococci*, and *Listeria* were enumerated from the homogenate of the samples prepared. Plate count agar, including selective media such as MacConkey with crystal violet and salt, MacConkey without crystal violet and salt, Baird-Parker (BP) supplemented with egg yolk (Merck, Republic of South Africa), and Brilliance chromogenic *Listeria* (ThermoFisher, Scientific, Republic of South Africa) were selected. The isolated colonies were counted using an 80 Scan 1200® Automated Colony Counter (Interscience). The mean number of colonies counted for all count types was expressed in log colony forming units (CFUs). Isolates were further characterized biochemically utilising Analytical Profile Index (API) 20E for *Enterobacteriaceae* and related genera whilst API 20NE was utilised for the identification of non-fastidious and non-enteric gram-negative rods. API STAPH was utilised for *Staphylococci*, *micrococci* and related genera and API *Listeria* for identification of *Listeria* spp. The tests were performed according to the manufacturer's instructions (bioMérieux, France).

Total aerobic mesophilic

The enumeration of the total viable aerobic mesophilic count was determined by plate count using the standard plate count agar (PCA) medium. Samples were serially diluted in buffered peptone water (BPW), and then aliquots of 0.1ml were inoculated using the plate count utilising spread-plate technique, following incubation at 37ºC for 48 h (Shalini, 2010).

Enterobacteriaceae (total coliform)

To count the members of *Enterobacteriaceae*, 0.1 ml of 10^{1}–10^{5} serial dilution of the leafy green vegetable samples was spread plated on MacConkey with crystal violet and salt and MacConkey without crystal violet and salt. Plates were incubated at 32°C for 24 hours after spreading. Colonies were counted as members of *Enterobacteriaceae* (Spencer & Spencer, 2001).

Isolation of *Staphylococcus* spp.

The enumeration of coagulase-positive *Staphylococci* was performed using Baird-Parker agar (BPA) plus egg yolk and potassium tellurite following serial dilution in BPW. BPA plates were incubated at 37ºC/48 h and checked for typical/atypical colonies (black, shiny, convex, and surrounded or not by clear zones, 2-5 mm). Between 5 and 10 typical and atypical colonies were purified on blood agar plates. Results were expressed based on the number of coagulase-positive *Staphylococci* on plates (Acco *et al*., 2003).

Colonies were streaked out on plate count agar plates for pure colonies before being analysed utilising API 20E, API 20NE and on blood agar for API STAPH identification of organisms (Biomerieux, Republic of South Africa). Briefly, 1- 4 colonies of identical morphology from young cultures (18-24 h) were picked and emulsified in 5 ml of sterile sodium chloride (0.85%) for API 20E, API STAPH and 20NE and the turbidity adjusted to the equivalent of the turbidity of a 0.5 McFarland standard. The standardized bacterial suspension was carefully distributed into the tubes of the test strip to avoid the formation of bubbles. Anaerobiosis was created by overlaying with sterile mineral oil and the strips were subsequently incubated in a humid atmosphere for 18–24 h at 37°C.

An additional oxidase test was performed for *Pseudomonadaceae* by adding 2-3 drops of reagent directly to suspect colonies on the nutrient agar plate. The colour change was observed within 10 seconds. When using Kovac's Oxidase reagent, microorganisms are oxidase-positive when the colour changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the colour changes to purple within 60 to 90 seconds. Microorganisms are oxidase-negative if the colour does not change, or it takes longer than 2 minutes.

Isolation of *Listeria* spp.

For the isolation of *Listeria* spp., approximately 25g of each sample was homogenized with *Listeria* broth and stomach for a minimum of 30 seconds to thoroughly mix the sample. The broth was incubated without agitation at 30ºC for 24 ± 2 hours. The bag was gently agitated and a microbiological loop was utilised to remove 0.1ml and inoculate it onto a *Brilliance Listeria* agar plate (chromogenic). The inoculum was carefully spread as soon as possible over the surface of the plate using a sterile spreader without touching the sides of the plate with the spread. The inoculated plates were inverted so that the bottom was uppermost and incubated at 37ºC for 24 ± 2 hours (ISO 16140 standard). The plates were examined for blue colonies with and without opaque white halos (ISO 16140 standard). As an additional test, the type of haemolysis was observed and recorded.

Colonies were streaked on blood agar for pure colonies before being confirmed through biochemical identification utilising the API *Listeria* system (Biomerieux, Republic of South Africa). For *Listeria,* after suspension with a turbidity of 1 McFarland, haemolysis was observed and recorded on the result sheet. After the distribution of suspension into the tube, incubation reagents were added and results were recorded again. A drop of ZYM B reagent was added to the test. Data interpretation was performed using the API database with the apiwebTM identification software to obtain the identification result for each strain tested.

3.3 Data analysis

The data on how growth medium influences pathogen prevalence and the mean microbial count were statistically analyzed using the general linear model of SAS software version 9.2 to determine the analysis of variance (ANOVA). Tukey's least significant difference (LSD_T), described by Steel and Tourie (1980), was utilised to determine the significant results between variants. The statistical difference between treatment means was determined at the ($p \leq 0.05$) probability level. The Shapiro-Wilks test was performed on standardised residuals to test for any deviations from normality (Shapiro & Wilk, 1965). The growth medium influencing the prevalence of pathogens and microbial mean count was subjected to multivariate data analysis, using principal component analysis (PCA-XLSTAT 2015) to identify and evaluate the groupings between the variables.

3.4 Results and Discussion

3.4.1 Cabbage phyllosphere microbial count concentrations

Analysis of Variance (ANOVA)

Significant interactions between the farms and concentrations were observed in all five concentrations of the microbial mean counts in different farms (Tables 3.1 and 3.2). All microbial concentrations (10^1, 10^2, 10^3, 10^4, and 10^5) had highly significant ($P<0.05$) microbial colony counts. The significantly high microbial mean count overall for 10^1 concentration was observed in PCA and WS followed by WOS with the least in BP growth media in all farms' growth media (Table 3.1). The highest microbial mean count observed for PCA was in Confido farm and Meadows farm, followed by Naledi farm and CUT farm, with the least in Maokodi farm, respectively. The highest microbial mean count observed in Confido farm was 113 $\log_{10}$ cfu/ml and the lowest was 68.50 $\log_{10}$ cfu/ml in Maokodi farm. The highest microbial mean count observed for WS was in Meadows farm followed by Naledi farm and Confido farm with the least in CUT farm and Maokodi farm, respectively. Meadows farm's microbial mean count was the highest and significantly different from the rest of the farms. In WOS the highest microbial mean count was observed in the Confido farm with 106.50 $\log_{10}$ cfu/ml and Naledi farm, followed by the CUT farm and Maokodi farm, with the lowest count in Naledi farm with 37.50 $\log_{10}$ cfu/ml. Table 3.1 and Table 3.2 below depict the mean logs for cabbage samples from different farms in different concentrations.

Meadows farm had the highest BP microbial mean count compared to Naledi farm, Maokodi farm and Confido farm, with the least observed in CUT farm. Meadows farm's microbial mean count was significantly different from Naledi farm, Maokodi farm and Confido farm. The highest microbial mean count from different farms in different concentrations was observed in Meadows farm with 113.00 $\log_{10}$ cfu/ml and the lowest microbial mean count was observed in CUT farm BP with 28.00 $\log_{10}$ cfu/ml.

The overall significant microbial mean count for concentration 10^2 was observed in PCA followed by WS and WOS with the BP being the least in all farms (Table 3.1). The highest microbial mean count observed for PCA was in Meadows farm 4 and Confido farm, followed by Naledi farm 5 and CUT farm 1, with the least in Maokodi farm, respectively. CUT farm microbial mean count was significantly different to the Maokodi farm microbial mean.

Meadows farm was observed to have the highest microbial mean count in WS, followed by Naledi farm and Confido farm, with the lowest count in CUT farm and Maokodi farm. Meadows farm was significantly different to Naledi farm, while Confido farm was significantly different from CUT farm. Confido farm and Meadows farm were observed to have the highest microbial mean count for WOS, followed by Naledi farm, with the lowest count in Maokodi farm and Cut farm. Confido farm and Naledi farm microbial mean counts were significantly different from CUT farm. The highest microbial count observed for BP was in Meadows farm and Naledi farm, followed by Maokodi farm and Confido farm, with the least in CUT farm.

The highest microbial mean count for PCA was observed in Meadows farm and Confido farm followed by CUT farm, Naledi farm and Maokodi farm, respectively (Table 3.1). Naledi farm's microbial mean count was significantly different to Maokodi farm's. In WS, the highest microbial mean count was observed in Naledi farm and Meadows farm, followed by Confido farm and Maokodi farm, with the least in CUT farm, respectively. In WOS, the highest microbial mean count was observed in Confido farm, followed by Meadows farm, Maokodi farm and CUT farm, with the least in Naledi farm, respectively. Confido farm's microbial mean count was significantly different to Naledi farm's. The highest microbial mean count observed for BP was in Meadows farm and Naledi farm, followed by Maokodi farm and Confido farm, with the least observed in CUT farm. A significant difference was observed between the Maokodi farm and Confido farm microbial mean count.

Confido farm and Maokodi farm had the highest microbial mean count for PCA, followed by CUT farm, with the least difference from Maokodi farm and Naledi farm, respectively (Table 3.2). The highest microbial mean count observed in WS was in Naledi farm, followed by Confido farm and Meadows farm, with the least in CUT farm and Maokodi farm. In WOS, Confido farm had the highest microbial count, followed by Maokodi farm, Meadows farm, and CUT farm, with the lowest count observed in Naledi farm, respectively. Confido farm's microbial mean count was significant compared to Maokodi farm's microbial mean count. Meadows farm had the highest microbial mean count for WS, followed by Maokodi farm, with the least in Naledi farm. Meadows farm and Confido farm had the highest microbial mean count for PCA, followed by Naledi farm and Maokodi farm, with the least in CUT farm, respectively (Table 3.2). Meadows farm had the highest WS microbial mean count (17.50 $\log_{10}$ cfu/ml), followed by Confido farm with 6.00 $\log_{10}$ cfu/ml. Naledi farm's microbial mean count was significantly different from Confido farm's. Confido farm was the only farm

with a microbial mean count of 14.00 $\log_{10}$ cfu/ml for WOS ans microbial mean count of 6.50 $\log_{10}$ cfu/ml for BP.

Table 3.1: Mean $\log_{10}$ cfu/ml of bacteria sampled from farm cabbage phyllosphere, 10^{1}-10^{3}.

	Concentrations ($\log_{10}$ cfu/ml)		
Farms	**10^{-1} Cons**	**10^{-2} Cons**	**10^{-3} Cons**
CUT	61.25 ± 26.09^{c,d}	45.00 ± 21.86^{d}	26.50 ± 14.70^{c}
Confido	84.62 ± 30.45^{b}	65.75 ± 26.03^{b}	44.12 ± 20.27^{a,b}
Maokodi	47.25 ± 17.24^{d}	39.12 ± 10.13^{d}	29.50 ± 6.84^{c}
Meadows	99.37 ± 13.31^{a}	80.00 ± 15.80^{a}	48.75 ± 22.24^{a}
Naledi	70.87 ± 24.23^{b,c}	59.25 ± 25.40^{c}	34.00 ± 15.56^{b,c}
LSD_T	*14.12	*6.18	*14.57
F-Value	48.01	65.71	28.29
P-Value	<.0001	<.0001	<.0001
Agar			
BP	52.70 ± 20.75^{c}	39.80 ± 15.20^{c}	23.60 ± 7.54^{b}
PCA	95.80 ± 16.26^{a}	79.80 ± 15.02^{a}	57.30 ± 18.82^{a}
WOS	69.00 ± 30.11^{b}	54.70 ± 26.85^{b}	31.30 ± 13.81^{b}
WS	73.20 ± 29.60^{b}	57.00 ± 23.22^{b}	34.10 ± 10.79^{b}
LSD_T	*12.63	*5.53	*13.03
F-Value	46.35	83.69	81.98
P-Value	<.0001	<.0001	<.0001
Farms x Agar			
CUT x BP	28.00 ± 5.56^{i}	20.50 ± 2.12^{g}	12.50 ± 0.70^{e}
CUT x PCA	96.00 ± 2.82^{a,b,c,b}	75.50 ± 4.94^{c,d}	48.50 ± 9.19^{b,c,d}
CUT x WOS	63.50 ± 7.77^{e,f,g}	49.00 ± 2.82^{e}	22.50 ± 2.12^{c,d,e}
CUT x WS	57.50 ± 0.70^{e,f,g,h}	35.00 ± 4.24^{f}	22.50 ± 0.70^{c,d,e}
Confido x BP	43.00 ± 5.56^{f,g,h,i}	33.00 ± 1.41^{f}	20.50 ± 2.12^{d,e}
Confido x PCA	113.00 ± 11.31^{a}	89.50 ± 9.19^{a,b}	70.50 ± 9.19^{a,b}
Confido x WOS	106.50 ± 12.02^{a,b}	87.50 ± 6.36^{a,b,c}	50.00 ± 7.07^{b,c}
Confido x WS	76.00 ± 2.82^{c,d,e}	53.00 ± 8.48^{e}	35.50 ± 3.53^{c,d,e}
Maokodi x BP	44.50 ± 6.36^{f,g,h,i}	34.00 ± 2.82^{f}	28.00 ± 1.41^{c,d,e}
Maokodi x PCA	68.50 ± 2.12^{d,e,f}	55.00 ± 5.65^{e}	38.00 ± 9.89^{c,d,e}
Maokodi x WOS	41.00 ± 4.24^{f,g,h,i}	33.50 ± 2.12^{f}	28.50 ± 0.70^{c,d,e}
Maokodi x WS	35.00 ± 26.87^{h,i}	34.00 ± 1.41^{f}	23.50 ± 2.12^{b,c,d,e}
Meadows x BP	83.00 ± 8.48^{b,c,d,e}	57.50 ± 10.60^{e}	31.00 ± 8.48^{c,d,e}
Meadows x PCA	104.00 ± 4.24^{a,b,c}	93.00 ± 2.82^{a}	82.00 ± 14.14^{a}
Meadows x WOS	96.00 ± 0.70^{a,b,c,d}	80.00 ± .48^{b,c,d}	40.50 ± 9.19^{c,d,e}
Meadows x WS	114.00 ± 11.31^{a}	89.50 ± 4.94^{a,b}	41.50 ±4.94^{b,c,d}
Naledi x BP	65.00 ± 4.24^{e,f,g}	54.00 ± 4.24^{e}	26.00 ± 2.82^{c,d,e}
Naledi x PCA	97.50 ± 0.70^{a,b,c}	86.00 ±1.41^{a,b,c}	47.50 ± 7.77^{b,c,d}
Naledi x WOS	37.50 ± 0.70^{g,h,i}	23.50 ± 4.94^{f,g}	15.00 ± 2.82^{e}
Naledi x WS	83.50 ± 7.77^{b,c,d,e}	73.50 ± 4.94^{d}	47.50 ± 6.36^{b,c,d}
LSD_T	*28.25	*12.37	*29.14
F-Value	9.45	16.13	8.81
P-Value	<.0001	<.0001	<.0001

Means followed by the same letter in the same column are statistically non-significant (P<0.05); ns = not significant; * = significant **Abbreviations**: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker

Table 3.2. Mean log_{10} cfu/ml of bacteria sampled from farm cabbage phyllosphere, 10^4-10^5

	Concentrations (log_{10} cfu/ml)	
Farms	**10^{-4} Cons**	**10^{-5} Cons**
CUT	13.50 ± 13.66^{b}	9.00 ± 2.82^{c}
Confido	28.50 ± 19.27^{a}	14.37 ± 10.92^{b}
Maokodi	11.00 ±4.81^{b}	9.00 ± 2.82^{c}
Meadows	21.85 ± 19.37^{a,b}	33.50 ± 3.53^{a}
Naledi	12.75 ± 9.26^{b}	14.00 ± 4.08^{b}
LSD_T	*11.14	*4.71
F-Value	14.53	45.43
P-Value	<.0001	0.0014
Agar		
BP	12.12 ± 3.83^{a}	6.50 ± 3.53^{c}
PCA	32.80 ± 19.77^{b}	18.60 ± 11.93^{a}
WOS	12.20 ± 12.04^{b}	14.00 ± 1.41^{b}
WS	12.70 ± 8.49^{b}	11.75 ± 6.70^{b}
LSD_T	*9.95	*4.47
F-Value	35.75	25.71
P-Value	<.0001	0.0045
Farms x Agar		
CUT x BP	ND	ND
CUT x PCA	29.50 ± 12.02^{b,c}	9.00 ± 2.82^{c,d}
CUT x WOS	3.50 ± 0.70^{e}	ND
CUT x WS	7.50 ± 2.12^{d,e}	ND
Confido x BP	13.50 ± 2.12^{b,c,d,e}	6.50 ± 3.53^{d}
Confido x PCA	54.50 ± 54.50^{a}	31.00 ± 1.41^{a}
Confido x WOS	33.50 ± 2.12^{a,b}	14.00 ± 1.41^{b,c}
Confido x WS	12.50 ± 12.02^{b,c,d,e}	6.00 ± 1.41^{d}
Maokodi x BP	12.00 ± 5.65^{b,c,d,e}	ND
Maokodi x PCA	14.50 ± 3.53^{b,c,d,e}	9.00 ±2.82^{c,d}
Maokodi x WOS	11.00 ± 7.07^{c,d,e}	ND
Maokodi x WS	6.50 ± 0.70^{d,e}	ND
Meadows x BP	14.50 ± 3.53^{b,c,d,e}	ND
Meadows x PCA	52.00 ± 12.72^{a}	33.50 ± 3.53^{a}
Meadows x WOS	10.00 ± 2.82^{c,d,e}	ND
Meadows x WS	11.00 ± 1.41^{c,d,e}	ND
Naledi x BP	8.50 ± 3.53^{c,d,e}	ND
Naledi x PCA	13.50 ± 0.70^{b,c,d,e}	10.50 ± 0.70^{c,d}
Naledi x WOS	3.00 ± 1.41^{e}	ND
Naledi x WS	26.00 ± 2.82^{b,c,d}	17.50 ± 0.70^{b}
LSD_T	*21.59	*5.44
F-Value	9.25	111.46
P-Value	0.0001	0.0005

Means followed by the same letter in the same column are statistically non-significant (P<0.05); ns = not significant; * = significant.

Abbreviations: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

Multivariate data analysis (MVDA)

Multivariate data analysis was applied using a PCA (MVDA) to group correlating microbial mean counts. Similar results from ANOVA were obtained from this method (Figure

3.1). The score plot and loading matrix, based on the first and second principal components (PC1 and PC2), accounted for 86.92% of the total variance. The biplot loading in PC 1 showed that the microbial mean counts for PCA and WS correlated in different concentration percentages. Plate count agar from Meadows farm, Confido farm and Naledi farm had the highest microbial mean count compared to the other farms and correlated in concentration percentages 2 and 5 including a WS microbial mean count from Naledi farm and Meadows farm. All 3 farms had the highest PCA microbial mean followed by WS microbial mean. Figure 3.1 below depicts variations of cabbage mean counts and their correlation in different concentrations and table 3.3 below outlines the factor loading and principal components (component 1 and component 2) for cabbage samples between farms and utilised mediums.

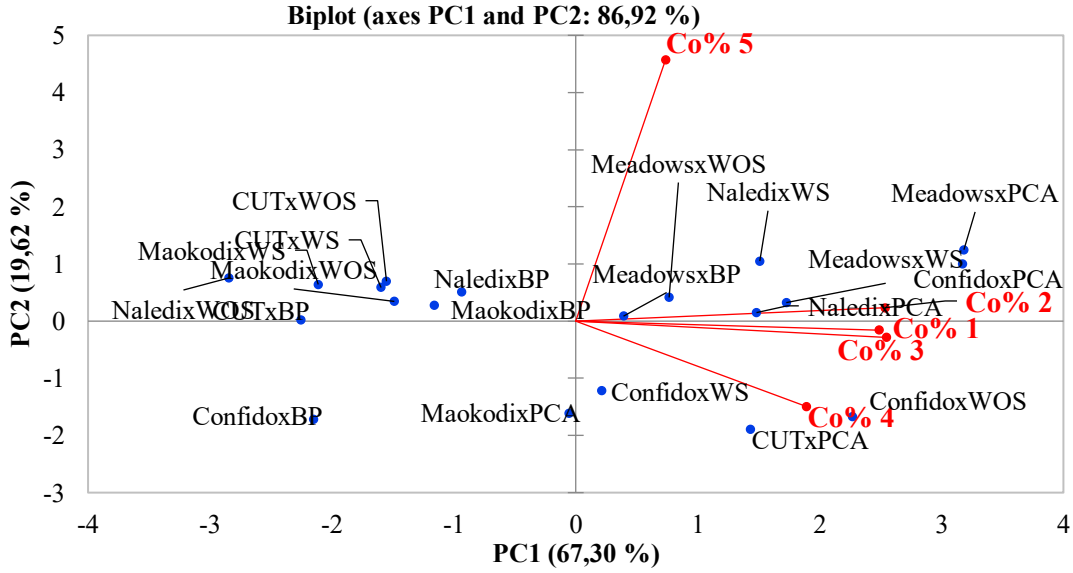

Figure 3.1. Principal component biplot illustrating the variations of cabbage microbial mean count correlations in different concentrations in different farms using different growth mediums **Abbreviations**: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

Table 3.3: Principal components analysed for cabbage microbial mean counts between different farms

Traits	Principal component 1	Principal component 2
Eigenvalue	3,365	0,981
Variability %	67,300	19,620
Cumulative %	67,300	86,920
Factor loading		
MaokodixWS	**0,846**	0,078
MaokodixWOS	**0,758**	0,041
MaokodixBP	**0,814**	0,046
MaokodixPCA	0,001	**0,973**

CUTxWS	**0,813**	0,110
CUTxWOS	**0,655**	0,132
CUTxBP	**0,576**	0,000
CUTxPCA	0,358	**0,623**
MeadowsxWS	**0,561**	0,020
MeadowsxWOS	0,285	0,084
MeadowsxBP	**0,508**	0,025
MeadowsxPCA	**0,821**	0,127
NaledixWS	**0,543**	0,263
NaledixWOS	**0,919**	0,065
NaledixBP	**0,604**	0,181
NaledixPCA	**0,842**	0,009
ConfidoxWS	0,029	**0,947**
ConfidoxWOS	**0,646**	0,352
ConfidoxBP	0,595	0,380
ConfidoxPCA	0,855	0,084

Values in bold correspond for each observation to the factor for which the squared cosine is the largest.

Abbreviations: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

3.4.2 Spinach phyllosphere microbial count concentrations

Analysis of Variance

As illustrated in Table 3.4, five concentrations were utilised to determine the microbial mean count for spinach from different farms. A significant interaction between farms and microbial mean counts in the different growth mediums was observed in all concentrations except for 10^2 and 10^5 concentrations (Table 3.4). Microbial concentrations 10^1, 10^3 and 10^4 had highly significant ($P<0.05$) microbial colony counts.

Table 3.4. Mean $\log_{10}$ cfu/ml of bacteria sampled from spinach phyllospheres from different farms

	Concentrations ($\log_{10}$ cfu/ml)				
	10^{-1} Cons	**10^{-2} Cons**	**10^{-3} Cons**	**10^{-4} Cons**	**10^{-5} Cons**
Farms					
CUT	67.00 ± 28.19^{b}	55.75 ± 16.88^{b}	32.62 ± 12.19^{b}	10.37 ± 6.90^{c}	5.00 ± 4.24^{b}
Confido	92.25 ± 22.68^{a}	$69.00 \pm 23.71^{a,b}$	49.50 ± 19.42^{a}	25.75 ± 15.60^{a}	13.75 ± 14.75^{b}
Meadows	103.50 ± 30.38^{a}	78.75 ± 24.35^{a}	$41.50 \pm 26.65^{a,b}$	19.37 ± 25.64^{b}	35.00 ± 1.41^{a}
Maokodi	ND	ND	ND	ND	ND
Naledi	92.37 ± 6.04^{a}	$67.12 \pm 20.44^{a,b}$	44.75 ± 13.54^{a}	16.87 ± 11.63^{b}	7.00 ± 5.45^{b}
LSD_T	*13.54	18.12^{ns}	*11.74	*3.62	*10.47
F-Value	49.45	2.55	6.60	16.48	28.14
P-Value	<.0001	0.10	0.0070	0.0001	
Agar					
WS	99.62 ± 25.17^{a}	76.12 ± 19.91^{a}	49.25 ± 18.17^{a}	17.25 ± 14.69^{a}	6.50 ± 4.65^{b}
WOS	97.62 ± 11.56^{a}	69.25 ± 15.71^{a}	43.75 ± 15.24^{a}	19.00 ± 10.43^{a}	9.00 ± 5.47^{b}

BP	57.75 ± 24.85[b]	48.37 ± 21.37[b]	24.25 ± 14.26[b]	5.12 ± 2.94[c]	3.75 ± 2.21[b]
PCA	100.12 ± 15.18[a]	76.87 ± 21.37[a]	51.12 ± 17.25[a]	30.62 ± 22.72[a]	21.12 ± 16.33[a]
LSD_T	*13.54	*18.12	*11.74	*3.62	*8.76
F-Value	88.91	5.08	19.71	44.31	16.47
P-Value	<.0001	0.01	<.0001	<.0001	<.0001
Farms x Agar					
LSD_T	*27.08	36.25[ns]	*23.48	*7.25	13.25[ns]
F-Value	13.09	1.94	9.76	26.60	15.80
P-Value	<.0001	0.14	0.0003	<.0001	0.30

Means followed by the same letter in the same column are statistically non-significant (P<0.05); ns = not significant; * = significant; ND = No data.

A significant interaction between the microbial count and growth medium was observed between Meadows farm, Confido farm, CUT farm, and Naledi farm (Table 3.4). The highest microbial mean count for PCA was observed in Meadows farm 4 and Confido farm. The highest microbial mean count for WS was observed in Meadows farm and Confido farm, followed by Naledi farm, with the least observed in CUT farm, respectively. In WOS, Meadows farm had the highest microbial mean count, followed by Naledi farm, with the lowest count observed in Confido farm and CUT farm, respectively. Meadows farm's microbial mean count was significantly different to Naledi farm's. The highest BP microbial mean count with a significant difference was observed in Naledi farm, followed by Confido farm and Meadows farm, with the least in CUT farm. The Naledi farm microbial mean count was significantly different to Confido farm, Meadows farm and CUT farm. A significant difference was observed between the Meadows farm and CUT farm microbial mean count.

Figure 3.2 below depicts the microbial mean counts for different farms including the concentration of microorganisms in different concentrations in different agars. Colonies for different microorganisms are calculated in log_{10}cfu/ml. The highest microbial mean count for plate count agar was observed in Meadows farm and Confido farm, followed by Naledi farm, with the lowest count observed in CUT farm, respectively (Table 3.4 and Figure 3.2). Confido farm had the highest microbial mean count for WS, followed by Meadows farm and Naledi farm, with the least observed in CUT farm, respectively. The Confido farm microbial mean count was significant compared to Meadows farm and Naledi farm. Naledi farm and CUT farm were significantly different from each other. In WOS, Naledi farm and CUT farm had the highest microbial mean count, followed by Meadows farm and Confido farm, respectively. Meadows farm had the highest microbial mean count for BP, followed by Naledi farm and Confido farm, with the least observed in CUT farm.

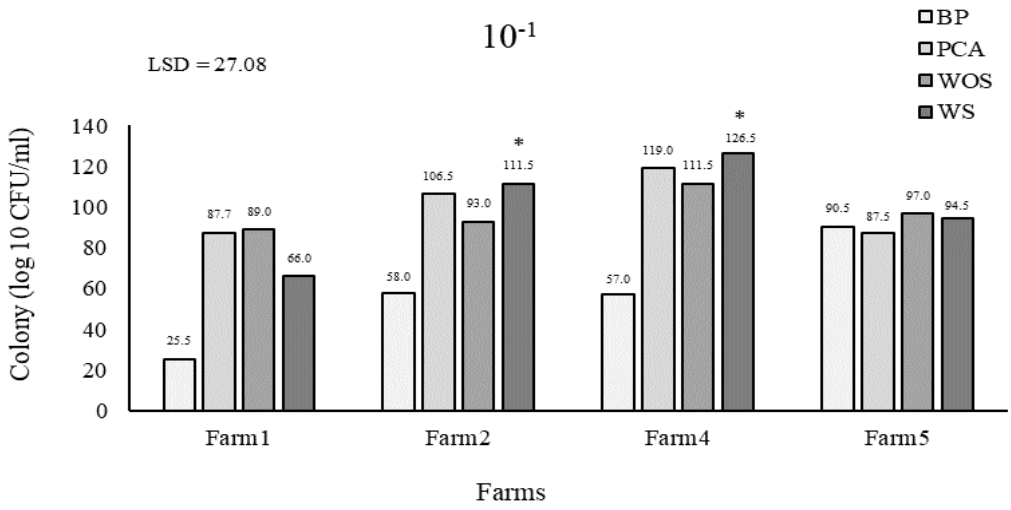

Figure 3.2. Microbial mean counts for spinach in this study are presented graphically as mean $\log_{10}$ cfu/ml (concentration 10^{1}). **Abbreviations**: Asterisk (*) = Interactions of interest for discussion. Farm1 - CUT; Farm2 - Confido; Farm4 - Meadows; Farm5- Naledi. Farm3 – Maokodi- had no data

The highest PCA microbial mean count was observed in Meadows farm, followed by Confido farm and CUT farm, with the least observed in Naledi farm, respectively (Table 3.4 and Figure 3.3). The Meadows farm mean count was significantly different to the Confido farm microbial mean count. Confido farm was observed with the highest microbial mean count in WS, followed by Meadows farm and Naledi farm with the least in CUT farm, respectively. The Confido farm microbial mean count was significantly different to the Meadows farm microbial mean count. Significant growth was also observed in WOS with the highest microbial mean count in Naledi farm, followed by CUT farm, with the lowest count observed in Meadows farm including Confido farm. Naledi farm was significantly different from CUT farm. In BP, Meadows farm had the highest microbial mean count, followed by Naledi farm, with the lowest counts observed in Confido farm and CUT farm.

Figure 3.3 below depicts microbial mean counts for different farms including the concentration of microorganisms in different concentrations in different agars. Colonies for different microorganisms are calculated in $\log_{10}$cfu/ml.

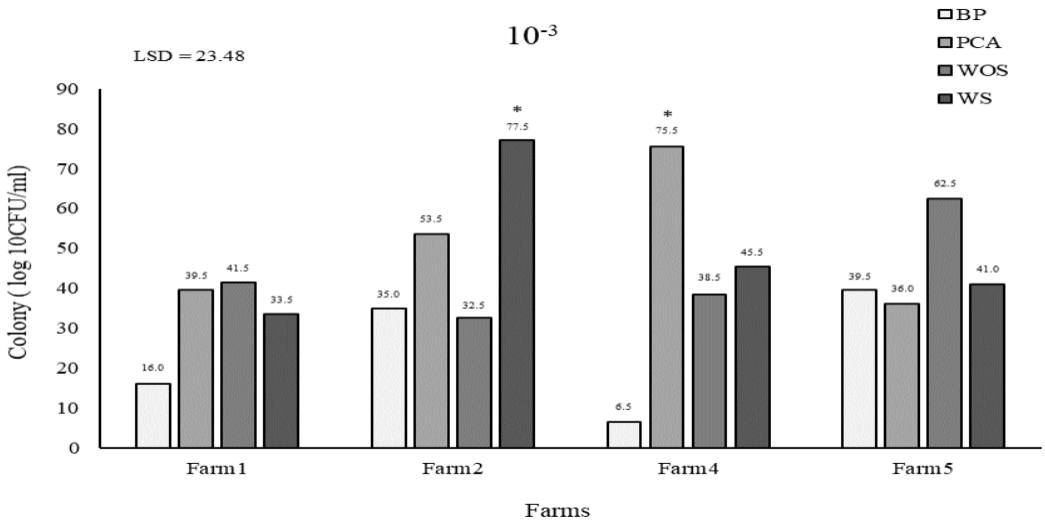

Figure 3.3. Microbial mean counts for spinach in this study are presented graphically as mean $\log_{10}$ cfu/ml (concentration 10^3) **Abbreviations**: Asterisk (*) = Interactions of interest for discussion Farm1- CUT; Farm2 - Confido; Farm4 - Meadows; Farm5- Naledi. Farm3 had no data.

Meadows farm had the highest microbial mean count for PCA, followed by Confido farm, with the lowest count observed in Naledi farm and CUT farm, respectively (Table 3.4 and Figure 3.4). The Meadows farm microbial mean count for PCA was significantly different to Confido farm. The Confido farm microbial mean count was significantly different to Naledi farm and CUT farm. Naledi farm had the highest microbial mean count in WOS, followed by CUT farm and Confido farm, with the lowest count observed in Meadows farm. The Naledi farm microbial mean count was significantly different to the CUT farm microbial mean count. Confido farm was observed with the highest microbial mean count in WS, followed by Meadows farm, with the lowest count in CUT farm and Naledi farm. The Meadows farm microbial mean count was significantly different from the CUT farm, Confido farm and Naledi farm microbial mean count. Naledi farm had the highest microbial mean count for BP, followed by Confido farm and CUT farm, with the lowest count observed in Meadows farm, respectively. The Naledi farm microbial mean count was significantly different from the CUT farm and Meadows farm. Figure 3.4 below depicts microbial mean counts for different farms including the concentration of microorganisms in different concentrations in different agars. Colonies for different microorganisms are calculated in $\log_{10}$cfu/ml.

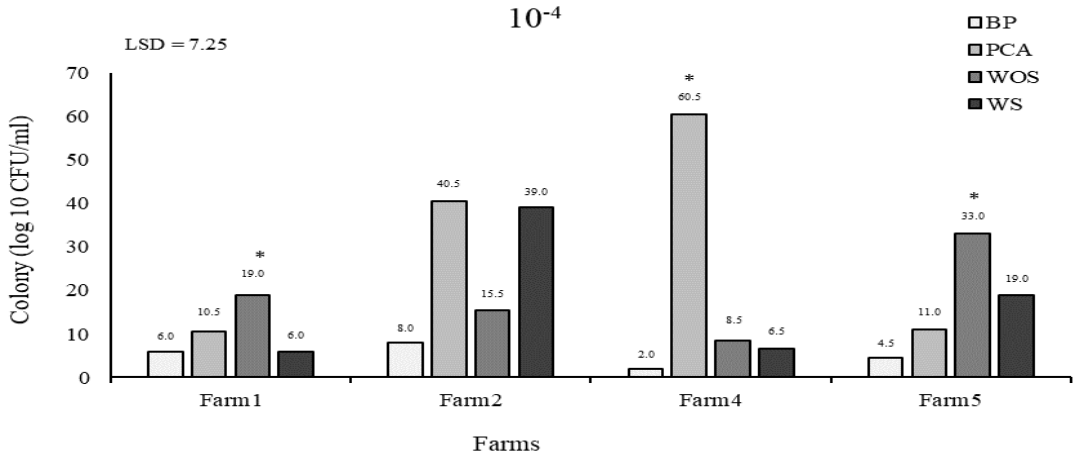

Figure 3.4. Microbial mean counts for spinach in this study are presented graphically as mean $\log_{10}$ cfu/ml (concentration 10^4). **Abbreviations**: Asterisk (*) = Interactions of interest for discussion. Farm1- CUT; Farm2 - Confido; Farm4 - Meadows; Farm5- Naledi. Farm3 had no data

In PCA, Confido farm and Meadows farm had the highest microbial mean count followed by CUT farm and Naledi farm, respectively (figure 3.4). The Meadows farm and CUT farm microbial mean counts were significantly different to each other as well as the Meadows farm and Confido farm microbial mean counts. In WS, Confido farm had the highest microbial mean count followed by Naledi farm and the least was observed in CUT farm and Meadows farm, respectively. Naledi farm had the highest microbial count for WOS, followed by CUT farm, with the least in Confido farm and Meadows farm, respectively. In BP, Confido farm had the highest followed by CUT farm, with the least in Meadows farm and Naledi farm, respectively.

Multivariate data analysis

Multivariate data analysis was applied using a PCA to group correlating microbial mean counts. Similar results from ANOVA were obtained from this method (Figure 3.5). The score plot and loading matrix, based on the first and second principal components (PC1 and PC2), accounted for 81.72 % of the total variance. The biplot loading in PC 1 (61.44%) showed that the microbial mean count for PCA and WOS correlated in concentration percentages of 2. 4 and 5. Plate count agar from Confido farm and the Meadows farm microbial mean count correlated and were significant as these two farms had the highest microbial mean count followed by Maokodi farm. The Maokodi farm and CUT farm WOS microbial mean counts were significant. On the other hand, the WS microbial mean counts for Confido farm, Meadows farm and Naledi farm showed a correlation in concentration

percentages 1 and 3 due to high microbial mean counts. Table 3.5 below outlines the factor loading and principal components for spinach samples between farms and concentrations of organisms in different agar.

Table 3.5: Principal components analysed for spinach microbial mean counts

Traits	Principal component 1	Principal component 2	Principal component 3
Eigenvalue	3,072	1,014	0,503
Variability (%)	61,437	20,280	10,070
Cumulative %	61,437	81,717	91,787
Factor loading			
MaokodixWS	0,143	0,489	0,165
MaokodixWOS	0,143	0,489	0,165
MaokodixBP	0,143	0,489	0,165
MaokodixPCA	0,143	0,489	0,165
CUTxWS	**0,785**	0,173	0,037
CUTxWOS	0,075	0,427	0,447
CUTxBP	**0,806**	0,177	0,000
CUTxPCA	0,407	**0,562**	0,029
MeadowsxWS	0,305	0,023	0,499
MeadowsxWOS	0,048	0,008	**0,809**
MeadowsxBP	0,449	0,243	0,009
MeadowsxPCA	**0,938**	0,031	0,021
NaledixWS	0,338	0,356	0,020
NaledixWOS	**0,804**	0,087	0,075
NaledixBP	**0,503**	0,416	0,020
NaledixPCA	**0,560**	0,331	0,007
ConfidoxWS	**0,640**	0,298	0,047
ConfidoxWOS	0,443	0,257	0,013
ConfidoxBP	**0,832**	0,076	0,084
ConfidoxPCA	**0,821**	0,120	0,019

Values in bold correspond for each observation to the factor for which the squared cosine is the largest.

Abbreviations: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

Figure 3.5 below depicts variations of cabbage mean counts from different farms and their concentration correlation in different agars.

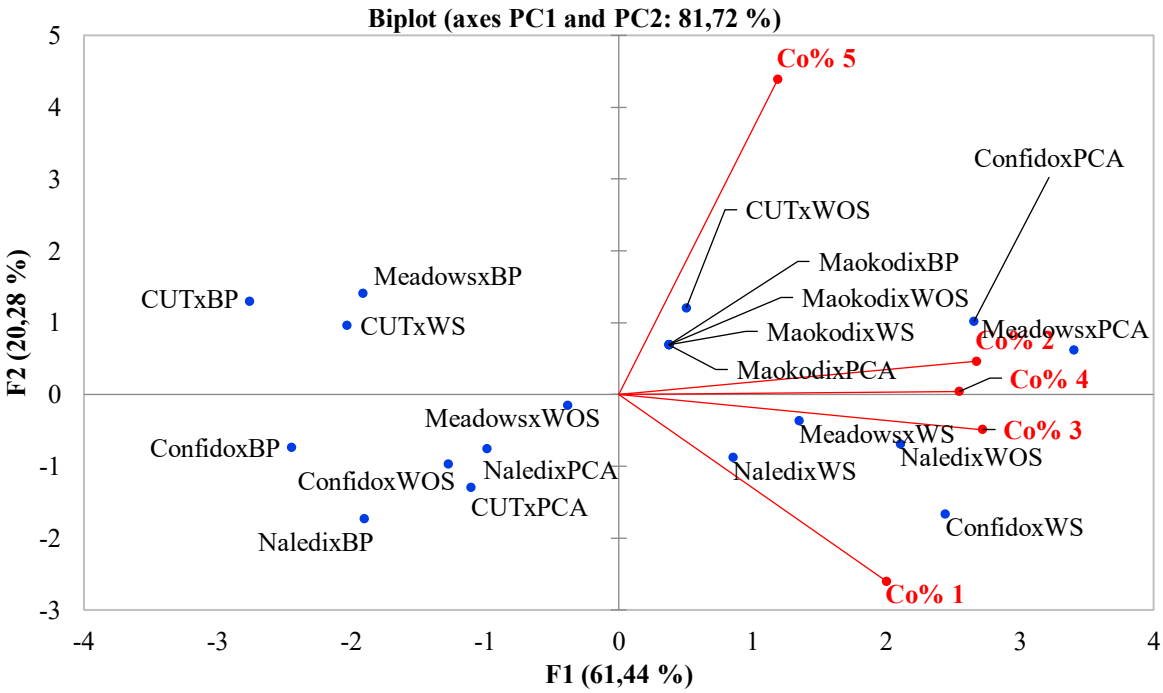

Figure 3.5. Principal component biplot illustrating the variations of spinach microbial mean count correlations in different concentrations in different farms using different growth media.

Abbreviations: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

3.4.3 Farm spinach crates microbial count concentrations

Multivariate data analysis

Multivariate data analysis was applied using a PC analysis to group correlating microbial mean counts. Similar results from ANOVA were obtained from this method (Table 3.7 and Figure 3.10). The score plot and loading matrix, based on the first and second principal components (PC1 and PC2), accounted for 83.22 % of the total variance. The biplot loading in PC 1 (67.36%) showed that the microbial mean count for PCA and WS correlated. Plate count agar (PCA) from Naledi, CUT, Confido and Meadows microbial mean count correlated in concentration percentages 4 and 5. These farms had the highest microbial mean counts for PCA than in any other growth media. On the other hand, the WS microbial mean count for Naledi and Meadows farm correlated. Naledi and Meadows had the highest microbial mean count for WS in concentration percentages 1, 2 and 3.

Table 3.6 below outlines the factor loading and principal components (component 1 and component 2) for spinach crate samples between farms and utilised medium concentrations.

Table 3.6: Principal components analysed for microbial mean counts of spinach crates between different farms

Traits	Principal component 1	Principal component 2
Eigenvalue	3,368	0,793
Variability %	67,364	15,860
Cumulative %	67,364	83,224
Factor Loading		
MaokodixWS	0,175	0,235
MaokodixWOS	0,175	0,235
MaokodixBP	0,175	0,235
MaokodixPCA	0,175	0,235
CUTxWS	**0,798**	0,088
CUTxWOS	**0,778**	0,126
CUTxBP	0,471	0,432
CUTxPCA	**0,954**	0,013
MeadowsxWS	0,062	0,070
MeadowsxWOS	0,390	0,177
MeadowsxBP	0,067	0,027
MeadowsxPCA	**0,990**	0,005
NaledixWS	0,109	**0,885**
NaledixWOS	0,043	0,001
NaledixBP	0,126	0,778
NaledixPCA	**0,949**	0,011
ConfidoxWS	0,425	0,428
ConfidoxWOS	**0,918**	0,075
ConfidoxBP	**0,706**	0,018
ConfidoxPCA	**0,980**	0,016

Values in bold correspond for each observation to the factor for which the squared cosine is the largest.

Abbreviations: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

Figure 3.6 below depicts variations of spinach crate mean counts and their correlation in different concentrations. Different farms are compartmentalised according to their concentrations in various agars.

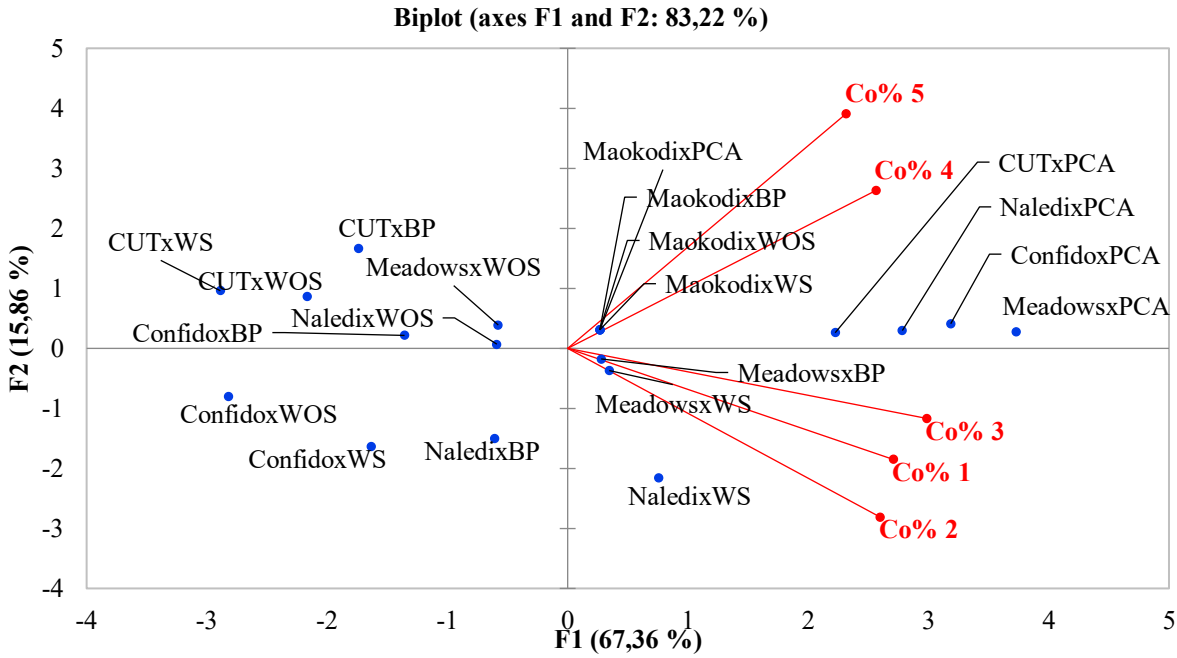

Figure 3.6. Principal component biplot illustrating the variations of spinach crate microbial mean count correlation in different concentrations in different farms using different growth media. **Abbreviations**: WS= MacConkey with salt; WOS= MacConkey without salt; PCA=Plate count agar; BP= Baird-Parker.

3.4.4 Bacterial contamination from various farms

Cabbage phyllosphere microorganisms

In this present study, Confido farm, Maokodi farm and Meadows farm had the highest number of pathogens identified for cabbage, followed by Naledi farm, with the least number observed in the CUT farm (Table 3.7). With regard to spinach, Confido farm and Meadows had the highest number of identified pathogens with the least number observed in CUT farm and Naledi farm. The *Listeria* spp. microbial count was considered insignificant as the count was less than thirty but above twenty-five. Table 3.7 below identifies pathogens enumerated from spinach, cabbage, and spinach crate samples.

The CUT farm cabbage was contaminated with *Pseudomonas luteola* and *Serratia* spp. These pathogens are versatile gram-negative bacteria that mainly emanate from the soil, water and living organisms including animals, insects, and humans. The possibility of contamination may be from personnel through inadequate personnel hygiene during the sorting of produce before packaging as it is manually sorted by workers. The farm possesses food safety programmes, including several produce safety guides which are in place. Concerning the revision of hygiene and sanitation standards, frequent monitoring is required to avoid

negligence of personnel regarding hygiene practices. There are no similar cases in the literature to support this hypothesis.

Table 3.7. Identification of pathogens identified from spinach and cabbage phyllosphere isolates

Cabbage phyllosphere	Farms	Pathogens identified
	CUT	*Pseudomonas luteola, Serratia marcescens, Serratia ficaria,*
	Confido	*Brevundimonas vesicularis, E. coli* (97.7%), *Chryseomonas luteola* (93.9%), *Staphylococcus lentus, Staphylococcus xylosus, Morganella morganii, Proteus mirabilis, Micrococcus luteus*
	Maokodi	*Staphylococcus sciuri* (76.1%) with second taxon *Staphylococcus xylosus* (23.8%) and third, *Staphylococcus lentus* (0.1%), *Serratia liquefaciens, E. coli, Pseudomona luteola*
	Meadows	*Acinetobacter Baumanni, Serratia marcescens, Staphylococcus aureus* (97.7%), *Staphylococcus epidermis* (79.4%), next taxon was *Staphylococcus aureus* with (18.4%), *Burkholderia cepacia, E. coli*
	Naledi	*Yersinia enterocolitica* (99.8%) next taxon *E.coli* (0.1%), *Staphylococcus aureus*
Spinach phyllosphere	CUT	*Staphylococcus aureus, Pseudomonas stutzeri, E. coli, Serratia ficaria* (97.0%)
	Confido	*Brevundimonas vesicularis, Burkholderia cepacia, Pseudomonas stutzeri, Staphylococcus aureus, Pseudomonas luteola, E. coli, Pseudomonas aeruginosa, Citrobacter freundii, Serratia marcescens, Morganella morganii, Listeria monocytogenes, Listeria ivanovii*
	Maokodi	No data
	Meadows	*Staphylococcus sciuri, Staphylococcus aureus, Proteus mirabilis, E. coli* (99.2%), *Yersinia enterocolitica, Morganella morganii, Proteus penneri* (99.6%), next taxon *Proteus Vulgaris group* (0.2%), *Providentia stuartii, Citrobacter freundii, Listeria ivanovii*
	Naledi	*Stenotrophomonas maltophilia, Proteus mirabilis, Staphylococcus heamolyticus* (85.9%), *Staphylococcus aureus*
Spinach crate	CUT	*Staphylococcus aureus, Pseudomonas stutzeri, E.coli* (78.6%)
	Confido	*Proteus mirabilis, Pseudomonas luteola, Citrobacter freundii, Morganelle morganii, Micrococcus luteus* (99.0%) *next taxon Staphylococcus capitis* (0.6%), *Staphylococcus lentus*
	Maokodi	No data
	Meadows	*Pseudomonas luteola, Staphylococccus xylosus* (99.7%) next taxon *Staphylococcus chromogenes* (0,1%), *Burkholderia cepacia*
	Naledi	*Serratia marcescens, Providentia stuartii* (84.3%), next taxon *Providentia rettgeri* (12.9%),

(%) – *viable species count shown by API Web*

In this present study, cabbage obtained from Confido farm had the highest number of microbes from various taxa than other farms followed by Meadows farm. A great possibility exists that microbial diversity from Confido farm was influenced by poor infrastructure including poor hygiene practices since it was observed that the farm conditions were not good,

from the field to the packaging, and then to the vehicle. *Staphylococcus* spp. and *E.coli* from Confido farm may be due to manual preparation from harvesting to storage without proper disinfection between processing. The grower's guide and food safety and quality assurance standards in place were neglected due to the high demand for the production of cabbage. Consequently, the farmer ended up focusing more on production rather than food safety. Strict supervision, including frequent production safety rules, coupled with critical control points is required. The present study concluded that the prevalence of *Proteus mirabilis* from Confido farm and Meadows farm was attributed to non-composted manure contaminating the vegetables, as livestock manure was utilised as fertiliser. Meadows farm not only produces leafy greens but also livestock. The timing of manure application as fertilizer and harvest time is crucial for avoiding crop contamination.

The presence of *Serratia liquefaciens* obtained in Maokodi farm indicated contamination from the environment and personnel as this species is considered a human pathogen. Maokodi farm utilises a conveyor belt where personnel sort out, peel and cut defects manually. Contamination can move from one batch of produce to the other. In addition, *Staphylococcus* species was utilised as a hygiene quality indicator where its presence indicated inadequate hygiene practices. Pasewu *et al*. (2014) highlighted contamination of cabbage in the following order, *Staphylococcus aureus* (51%), *E. coli* (28%) and *Pseudomonas aeruginosa* (4%). The bacterial load identified on leafy vegetables increased with time during storage (Söderqvist *et al*., 2017).

Meadows farm vegetable contamination could be through water and livestock present around the farm. Another possibility from Meadows farm can be the utilisation of animal-based fertilizer for the enrichment of soil which contributes to soil contamination leading to contamination of crops. Concerning Meadows farm, the microbial pathogens identified were mostly from the *Staphylococcaceae* family therefore suggesting poor hygiene practices from personnel followed by *E. coli* suggesting faecal contamination which might be due to various livestock on the farm. Slater *et al*. (2018) also emphasized that *S. aureus* isolated from the leafy green samples was an indication of poor hygienic practices by farmers.

Different *Acinetobacter* species are generally associated with various habitats such as soil, water, sewage, humans, foods, and animals and have been involved in a variety of nosocomial infections, including bacteraemia, urinary tract infection, and secondary meningitis (Almasaudi, 2018). Hamouda *et al.* (2011) isolated *Acinetobacter baumanii* species from faecal

specimens, skin, nostril and ear swabs from pigs and cattle slaughtered for human consumption, from a list of about 3111 farms. *Acinetobacter baumanii* was also isolated from Meadows farm and this was indicative of insufficient hygiene during processing and a possible cross-contamination from livestock, the possibility being contamination from livestock around the farm. It is therefore important that operating procedures should be followed strictly, particularly with regard to hand washing facilities to prevent contamination as these pathogens are present in environments where hygiene practices are poor and where faecal contamination poses a risk.

In this study, *Y. enterocolitica* was isolated from Naledi farm which indicated a possibility of crops growing closer to the soil leading to contamination of cabbage. Thorough washing of leafy greens is required to eliminate or reduce the microbial load from produce. Since the farm did not have any livestock present, the present study concluded that contamination came from the soil and possible cross-contamination during the washing of produce. *Yersinia enterocolitica* and *Serratia* spp. occur naturally in soil and water and *Yersinia* infections have overtaken *Shigella* and *Salmonella* species as the most common cause of bacterial gastroenteritis (Aziz & Yelamanchili, 2018). Additionally, the *Y. enterocolitica* pathogen has been isolated from a variety of animals with pigs being the most common source and the spread can be from one pig to another in a herd. *Yersinia enterocolitica* has also been found to infiltrate into plants such as cabbage, peas (*Pisum sativum* var. *saccharatum*) and oats (*Aneva sativa*) from infected soil and water as its survival is affected by moist environmental conditions (Vlu *et al*., 1991). *Yersinia enterocolitica* was more frequently detected in ready-to-eat vegetables with the highest prevalence observed in Finland where it was isolated from 33% of fresh leafy vegetables (Verbikova *et al*., 2018). Furthermore, between 2006 and 2009 approximately 7,600 and 9,000 cases of yersiniosis were reported in Europe annually and the WHO registered 340 deaths between 1994 and 2008.

Spinach phyllosphere microorganisms

At CUT farm, *Staphylococcus* spp. and *Pseudomonas* spp. were predominant which generally suggests poor hygiene which contributed to microbial load. Personnel hygiene and proper sanitation are important to eliminate *E. coli* contamiantion. Proliferation of these isolated organisms is possible with a possibility of contamination. Proper sanitation and hygiene of personnel can curb the contamination to avoid spread which may lead to an infection.

Listeria spp. was detected in Confido farm and Meadows farm. Contamination may emanate from poor agricultural management and inadequate hygiene during pre-harvest and post-harvest processes. At Meadows farm, *Listeria ivanovii* may be contamination from livestock as *Listeria* emanates from the environment and intestinal tract of domestic animals and livestock and is shed in faeces. In South-West Nigeria, *Pseudomonas* species were isolated from both irrigation water and vegetables, the data indicate that contamination of the vegetables was increased because of contaminated water utilised for irrigation (Akinde *et al.,* 2016). In this study*, Pseudomona stutzeri* was isolated from Confido farm and CUT farm. Radovanovic *et al*. (2020) demonstrated that the prevalence, persistence, and ability of *Pseudomonads* to form biofilm on surfaces of food processing plants enhance their resistance to adverse conditions including several antimicrobial treatments during washing. According to Mritujay and Kumar (2017), isolated spinach samples had the highest microbial mean count of 7.3 log cfu/g with a frequency of 6.1-9.6 log cfu/g, higher than cabbage and cucumber (*Cucumis sativus* L.) and thus indicating poor handling of spinach during storage. It is also shown that the prevalence of microorganisms particularly in sprouts (*Brassica oleracea* L. var. gemmifera) and fresh spinach can be significantly higher at the final post-harvest stages compared to the early stages of handling (Frank *et al*., 2011). This may be due to subsequent recontamination including pathogen amplification during post-harvest activities of minimal processing. Bacteria can retain or be trapped in plant parts even after vigorous disinfection (Solomon *et al*., 2002). This simply demonstrates the ability of a pathogen to utilise its ability to resist certain disinfectants and internalize during the washing of fresh leafy vegetables following recontamination.

Citrobacter spp. are facultatively anaerobic, motile, gram-negative bacilli in the *Enterobacteriaceae* family that are widely distributed in the environment and intestinal tracts of humans and animals (Murray *et al*., 2010; Adegun *et al.,* 2019). Sixty-six bacteria were isolated from 60 vegetable samples and of these isolates, *Salmonella* spp. recorded 43.3%, followed by *Citrobacter freundii* 18.3%, *Klebsiella* spp. 15.0%, *Enterobacter* spp. 11.7%, *Proteus* spp. and *Alcaligenes* spp. 5.0% each, *E. coli* and *Providencia* spp. 3.3% each, and *Vibrio* spp. 1.7% (Oluboyo *et al*., 2019). Another study isolated and characterised *Citrobacter* spp. in fruits and vegetables sold for consumption in ILE-IFE, Nigeria and concluded that *Citrobacter* spp. recovered from fruits and vegetables are not flora to fruits and vegetables but are frequently isolated from animals and as an opportunistic pathogen in humans. In this present study, the contamination of spinach likely emanates from faecal

contamination from animals or inadequate hygiene from handling of produce during cutting to a specific size before storage.

Proteus mirabilis was isolated from spinach and tomato from two different local vegetable agricultural fields (Shoket *et al*., 2014). *Proteus* spp. have growth potential even at low infectious doses and are a potential human health risk most commonly causing urinary tract infections and infection-related kidney stones with 50% risk fatality (Scherberich *et al*., 2021). In this study, *Proteus mirabilis* may indicate poor agricultural practices by utilising livestock manure and poor harvesting practices. *Proteus mirabilis* isn this study was identifies on farms that not only produce leafy greens but livestock, most of these farms utilised livestock manure as fertiliser to crops. *Proteus mirabilis* from livestock manure are able to survive in typical storage conditions can be detected in the soil and crops following land application of the manure. Regular surveillance or analysis of food safety and critical control points is necessary to avoid contamination from the field to the minimal processing facility.

At Meadows farm, *Staphylococcus* spp. were the most predominant organisms isolated from spinach. The presence of *Staphylococcus* spp. could be due to poor hygiene and sanitation around the farm. *Pseudomonas* spp. were also isolated and their presence could be due to their tolerance strategies to survive under certain temperatures and environments with conducive conditions of proliferation due to improper storage of spinach after sorting. Control of operations, maintenance and sanitation, including personal hygiene, must be maintained. Strict measures must be implemented about the storage of spinach and cabbage to avoid temperature abuse which enables organisms to utilise tolerance strategies, even forming biofilms to survive and thrive.

Providencia spp. have been commonly found in soil and sewage and have been broadly isolated from chickens (*Gallus domesticus* L.), cows (*Bos taurus* L.), and dogs (*Canis familiaris* L.) (Wie, 2015). *Providencia* infections include urinary tract infections, gastroenteritis, and bacteraemia, and infections are usually nosocomial. *Providencia* spp. represent an emerging problem because of the increasing prevalence of antibiotic resistance secondary to extended-spectrum beta-lactamase. The genus *Providencia* spp. found in Naledi farm likely emanates from contamination of soil, water utilised for irrigation and inadequately treated sludge utilised as fertilizer for the crops. The microbial status of water utilised for the irrigation of fresh leafy greens needs to be prioritised to avoid the uptake and contamination of bacteria into vegetables. Sludge or manure-rich soil influences microbiological and chemical

parts of soil and vegetables. The genus *Providencia* spp. found in Meadows farm likely emanates from contaminated soil, as animal manure is utilised as fertilizer. Infection and illness can be caused by an extremely low dosage of toxins. The farm also utilises basins to wash produce and for in-between washing. The water is not changed frequently and the knives for cutting and trimming produce are not disinfected to avoid cross-contamination, particularly when more batches of spinach are to be washed.

Stenotrophomonas maltophilia emanates from isolates from manure, chicken faeces, soil, plants, salads, water, and raw milk and has been implicated as the causative pathogen in respiratory tract infections, endocarditis, bacteraemia, meningitis, and urinary tract infections. Chlorinated water used to wash pre-packaged, ready-to-eat salads before sale proved to be insufficient to remove *S. maltophilia* from these items, possibly because the bacterium may exist in biofilms (Qureshi *et al*., 2005; Agri *et al*., 2022). In this present study, it was assumed that *Stenotrophomonas maltophilia* from spinach isolates may be contaminated by manure or possible contamination from the soil. For this reason, it is important to monitor the time for the application of manure and the time to harvest to avoid contamination.

Spinach crate microorganisms

Most farms utilise reusable plastic crates for various processes, including packaging, therefore bacterial contamination and transfer are possible between processes. Transmission of bacteria can occur between the surfaces of objects, due to the length of the contact, the type of surface, the temperature, and food items, including handling, and the type of food. The transfer of microorganisms including multiplication between fresh vegetables, crates and equipment surfaces is significant (Newman *et al.,* 2017). For example, a study by Cotter *et al*. (2012) found that *Salmonella* survival on plastic crates was enhanced by inadequate cleaning. Another study demonstrated a higher risk of cross-contamination of fresh produce from *Salmonella* by polypropylene (plastic crates) compared with cardboard and medium-density fibreboard (López-Gálvez *et al*., 2021).

According to Mritunjay *et al*. (2017), reusable plastic crates have been reported as potential sources for cross-contamination among various batches of foodstuffs. Proper maintenance of these reusable crates is of utmost importance to avoid contamination. In the present study, most of the organisms isolated from cabbage and spinach were also isolated on plastic crates. For example, in the Confido farm, cabbage, spinach and crates had almost the

same microorganisms, this might be due to the fact that spinach and cabbage were stored in one container as there are not enough resources available at that particular farm so there was cross-contamination between the two crops. The data indicate that cabbage and spinach might be contaminated by the crate or vice versa. It was also apparent that inadequate cleaning contributed to re-contamination between the vegetables and the containers. All the crates that are utilised for harvest, pick-up and storage should be labelled clearly for their sole purpose.

3.5 Conclusion

The incidence and predominance of microbial pathogens in spinach and cabbage in sampled farms was due to inadequate hygiene during processing. Bacteria more easily attach and colonize vegetable surfaces with grooves than those with smooth surfaces (Warning & Datta, 2017). Cabbage had the largest surface area compared to spinach, resulting in the prevalence of *Enterobacteriaceae*. In this present study, it was justifiable to conclude that cabbage has a large surface area compared to spinach, which enables ease of attachment, internalization, and colonization of various microorganisms. Variations of bacterial pathogens and their prevalence could be attributed to the differences in minimal processing infrastructure which included inadequate hygiene, poor agricultural practices, and negligence. In general, the presence of predominant microorganisms indicated inadequate processing and unhygienic conditions which contribute to a succession of microorganisms. Results in this present study further corroborated that cabbage from various farms harboured diverse bacterial communities, and the communities from each farm on each cabbage were significantly distinct from one another according to the bacterial family except for *Staphylococcus* species and *E. coli* predominance. Therefore, it is recommended that these vegetables be thoroughly washed before consumption, especially when consumed uncooked, specifically green salad.

Studies conducted in 1999 and 2010 reported that *L. ivanovii* was exclusively linked to ruminants but it was later highlighted that *L. ivanovii* infections occurred in humans after the ingestion of foodstuffs that were contaminated. It was concluded that a wide variety of foodstuffs are now a source of this pathogen and that, similarly to *L. monocytogenes, L. ivanovii* is capable of persistence in food production establishments (Rossi *et al*., 2022). *Listeria* pathogen is difficult to eliminate, particularly from the food chain, including ready-to-eat foods and vegetables. The persistence of *Listeria* strains is exacerbated by extrinsic factors, including poor hygiene, ineffective sanitisers, and the presence of specific genes responsible for biofilm formation (Lee *et al*., 2019). The frequency and degree of bacterial contamination

in this study were determined to be statistically significant. Knowledge of the composition and diversity of *Enterobacteriaceae* and *Staphylococcaceae* communities in cabbage and spinach may be useful in the establishment of control measures to mitigate the transmission of pathogens to consumers. Food handlers need to be repeatedly reminded of hygiene and food safety. Monitoring and regular supervision are essential to control and minimise microbial hazards that lead to contamination.

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CHAPTER FOUR

Proliferation and succession of opportunistic pathogens on leafy greens from retails in the Free State, South Africa

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Abstract

Agronomic parameters in small-scale farms, including the cold chain process contribute to the proliferation and succession of opportunistic and pathogenic microorganisms. Minimal processing operations influence the change in species composition and microbial-microbial exchange as they are not subjected to any lethal process which is typically employed to effectively kill pathogenic organisms. Except for their nutrition index that is available, there is little information available about leafy green vegetable production in South Africa. This study aimed to enumerate microbiota and identify microbial species isolated from spinach (*Spinacia oleracea* L.) and cabbage (*Brassica oleracea* var. *capitata* L.) at retails that are mainly supplied by small-scale farms in the Free State, South Africa. Analytical profile index (API) tests were conducted for further confirmation and identification. *Burkholderia cepacia* (99.7%), *Proteus mirabilis* (99.9%), *Enterobacter cloacae* (97.7 %), next taxon *Enterobacter amnigenus* with (1.5%), *Pseudomonas oryhabitants, Enterobacter aerogenes* (98.8%), *Acinetobacter lwoffii, E. coli* (99.9%), *Chryseomonas luteola* from cabbage. *Pseudomonas putida, Bacillus megaterium, Enterobacter aerogenes* (98.8%), *E. coli* (99.9%), *Brevibacillus laterosporus (97.7%),* next taxon *Bacillus pumilus* (0.8%), *Bacillus cereus, Bacillus lentus (98.5), Proteus mirabilis* (99.8%), next taxon *Proteus Vulgaris group* (0.2%), *Chryseomonas luteola*, *Listeria ivanovii* (96.4%), *Listeria monocytogenes* with 99.9% from spinach. The data may be utilised to influence further educational efforts and identify future research gaps to provide risk mitigation for small-scale farms and retails regarding leafy greens farm-to-fork continuum. Thus, this data is crucial for evaluating the pathogenic potential trend and its aetiology regarding cross-contamination, prevalence and proliferation due to progression of pathogenic microorganisms at retails.

Keywords: Cold chain, leafy greens, proliferation, succession, contamination

4.1 Introduction

The South African food sector is increasing and has the highest concentration of supermarkets in Africa, resulting in considerable expansion of the retail division (Nair, 2020). The retail sector in South Africa contributed about 9% to the overall GDP, reaching R491.60 billion in sales in 2016 (Ntloedibe, 2017). In 1999, the total fruits and vegetables consumption in South Africa was 3,600,000 tons, of which 55% of it was sold in fresh produce markets (Department of Agriculture, 2021). Additionally, South Africa accounts for 2.6 million tons which is 92. 5% of fresh vegetables and only 4% is exported. From 2019 to 2020 the

horticultural industry was highlighted as the second-largest agricultural sector following livestock production worth R141 billion and it contributed over R 92 billion which is 29% of the total agricultural gross value of R312 billion gross value during the production year 2019 to 2020 (Department of Agriculture, 2021). Thus, the informal markets, such as fresh fruits and vegetables produce markets, are responsible for the highest distribution of fresh produce. The food service industry receives about 60% of fresh produce and about 40% of the fresh produce is distributed to various retail markets (Gil *et al*., 2015). The viability and sustainability of informal vegetable markets will exist as long as there is substantial demand from consumers in urban areas (Marumo & Mabuza, 2018). Retail is the final arrival point for food before being purchased by consumers. The storage, handling, and display of fresh leafy vegetables in retail stores are important as certain pathogens, including psychrotrophs, can survive in cool environments. Farm activities can influence the progression of pathogens on the commodity, including retail microbial hazards. The assessment of microbial hazards causing the succession of opportunistic pathogens at retail establishments that are mainly supplied by these small-scale farms is imperative. Various extrinsic parameters contribute to the frequency of development of microbial populations or alter the frequency of development in microbes. Microbial succession mainly consists of three classifications, namely, autotrophic, endogenous heterotrophic, and exogenous heterotrophic, and each class occurs depending on the type of environment as bacterial traits differ (Kapinusova *et al*., 2023). For example, organic carbon elements derived from vegetables, in this instance, fuel endogenous heterotrophic succession with initial development being fast due to environmental factors allowing the progression of microbes. Green leafy substrate quality changes gradually due to microbial succession and population, meaning that the substrate is directly changed by the ingression and egression of microbes available on it (Poorter *et al*., 2024).

Washing and disinfecting as part of minimal processing prevent cross-contamination and inactivate pathogenic microorganisms that may be present by rinsing away exudates that would otherwise provide nutrients for microbes, thereby reducing microbial load. Due to favourable conditions and sufficient potential resources, pathogenic microbes are well able to thrive, contaminate and shift to the next phase and form a new niche (Poorter *et al*., 2024). The emergence and proliferation of dominant microbial populations are caused by the wide range of metabolic mechanisms found in the microbial pool (San *et al*., 2018). Studies have highlighted the potential health risks caused by washing fresh perishable vegetables with various disinfectants, including their limitations and alternatives (Yousuf *et al*., 2020;

Chinchkar *et al*., 2022; Piližota, 2023). For example, other studies showed how the depletion of free-chlorine disinfectants attributed to the dissemination of contamination of pathogens between different fresh vegetables and fruit commodity batches (Abnavi, 2021; Zhu *et al.,* 2024). Warriner and Namvar (2013) demonstrated that failure to maintain disinfectant levels in the washing of spinach resulted in cross-contamination between the batches leading to an outbreak with 200 confirmed cases of food poisoning and seven deaths. Abatcha *et al*. (2019) and Yang *et al.* (2020) also highlighted the statistics of foodborne poisoning cases including deaths caused by foodborne pathogens isolated from contaminated leafy greens. This demonstrates how microorganisms can overcome certain types of disinfectants and manage to cross barriers to the next phase causing outbreaks and even death.

The interaction that occurs between pathogens and fresh vegetables is multifaceted with the probability of contamination occurring at critical points such as harvesting, initial processing at the facility, transportation, and final preparation in the kitchen (Lynch *et al.,* 2009). This is one of the critical reasons to conduct comprehensive research on the interaction, microbial phyllosphere and microbial traits, including the transition, antagonist behaviour, resources, and dominance of these microbes on plants. A recent study assessed the microbiological quality of fresh vegetables including spinach and lettuce obtained from farms within Kaduna Metropolis, Nigeria (James, 2019). It was observed that almost all the examined, ready-to-eat vegetables had bacterial counts above the acceptable limit and were microbiologically unacceptable. If the primary production practices such as disinfection and other practices are not according to food safety regulations and guidelines, this will lead to the prevalence of microbial hazards and succession to retail points.

The objective of this study was to enumerate microbiota and identify microbial species isolated from spinach and cabbage at retails that are mainly supplied by small-scale farms. The influence of pre-harvest and post-harvest potential risk factors on the microbial community of leafy green vegetables and the interactions of the opportunistic foodborne pathogen with the native microorganism to pathogen transition is a great concern for pathogen succession on leafy green vegetables.

4.2 Materials and methods

4.2.1 Study area and sample collection

The present study was conducted by procuring ninety samples of raw unpackaged spinach phyllosphere and ninety samples of cabbage heads from five retails that are mostly supplied by the chosen small-scale farms in this study. The selected retails represent the major retails that supply the most leafy greens to consumers, making the results of the study representative. Samples were collected in the following towns in the Free State Province, South Africa: Motheo District - Mangaung Metropolitan (29.1217°S, 26.2128°E), Lejweleputwa District - Matjhabeng Local Municipality (28.9784°S, 27.0264°E), Thabo Mofutsanyana District – Setsoto Municipality (28.5225°S, 27.5241° E, Fezile Dabi District - Moqhaka Local Municipality (27.6373°S, 27.2323°E), and Thabo Mofutsanyana District – Mantsopa Local Municipality (29.1136°S, 27.2718°E).

4.2.2 Sampling technique

The study design used random sampling which was conducted on spinach and cabbage samples from three different sections of the stored samples ready for purchase. To ensure that sample collection was representative, at least six areas, the middle part and two sides of the stored samples were assessed. The samples collected were selected based on the sampling technique in which each sample has an equal probability of being chosen. A sample chosen is meant to be an unbiased representation of the total population.

Fresh leafy spinach and cabbage samples were analysed for each of the following microorganisms or microbial species: total aerobic mesophilic bacteria, total coliforms, coagulase-positive *Bacillus* and *Listeria*. All samples were collected aseptically, subsequently transported to the laboratory, and were prepared and plated on various pre-solidified agars from the samples' homogenates and incubated within 12 hours, on the same day of collection. The *Aerobic mesophilic* count, *Enterobacteriaceae* count (total coliform), *Bacillus* count, and *Listeria* count were enumerated from the homogenate of the samples prepared. Plate count agar, including selective media such as Violet red bile agar (VRBA), *Bacillus* chromoSelect agar (Merck, South Africa), and Brilliance chromogenic *Listeria* (ThermoFisher Scientific, South Africa) was utilised for the purpose. The isolated colonies were counted using an 80 Scan 1200® Automated Colony Counter (Interscience). The mean number of colonies counted for all count types was expressed in log colony forming units (CFUs). Isolates were further characterized biochemically using API 20E for *Enterobacteriaceae* and related genera whilst

API 20NE was utilised for the identification of non-fastidious and non-enteric Gram-negative rods. API 50 CHB/E Medium was utilised for the identification of *Bacillus* and related genera, as well as Gram-negative rods belonging to the *Enterobacteriaceae* and *Vibrionaceae* families and API *Listeria* for the identification of *Listeria* (bioMérieux, France). The tests were performed according to the manufacturer's instructions.

Total aerobic mesophilic

The enumeration of the total viable aerobic mesophilic count was determined utilising plate count agar (PCA) and nutrient agar medium. Samples were serially diluted in buffered peptone water (BPW), whereafter aliquots of 0.1ml were inoculated using the plate count spread-plate technique, and incubated at 37ºC/48 h (Shalini, 2010).

Enterobacteriaceae

To count the members of *Enterobacteriaceae*, 0.1 ml of a10^{1}–10^{5} serial dilution of the leafy green vegetable samples was spread plated on violet-red bile agar. Plates were incubated at 32°C for 24 hours after spreading. Red to pink colonies, surrounded by precipitated bile, were counted as coliforms (Spencer & Spencer, 2008).

Isolation of *Bacillus* spp.

For the enumeration of *Bacillus*, samples were serially diluted in BPW, inoculated on *Bacillus* ChromoSelect agar, and spread evenly onto the surface of each plate with a sterile glass spreading rod before being incubated at 37ºC for 48 hrs. Peacock blue colonies with blue zones were subjected to appropriate biochemical tests.

Isolation of *Listeria* spp.

For the isolation of *Listeria* spp., approximately 25g of each sample was homogenized with *Listeria* broth and stomach for a minimum of 30 seconds to thoroughly mix the sample. The broth was incubated without agitation at 30ºC for 24 ± 2 hours. The bag was gently agitated and a microbiological loop was utilised to remove 0.1ml and inoculate it onto a *Brilliance Listeria* agar plate (chromogenic). The inoculum was carefully spread as soon as possible over the surface of the plate using a sterile spreader without touching the sides of the plate with the spread. The inoculated plates were inverted so that the bottoms were uppermost and incubated at 37ºC for 24 ± 2 hours. The plates were examined for blue colonies with and

without opaque white halos (ISO 16140 standard). As an additional test, the type of haemolysis was observed and recorded.

Colonies were streaked out on plate count agar plates and blood agar for pure colonies before being analysed using API 20E, API 20NE, API 50CHB/E, and API *Listeria* for the identification of organisms (Biomerieux, Republic of South Africa). Briefly, 1-4 colonies of identical morphology from young cultures (18-24 h) were picked and emulsified in 5 ml of sterile sodium chloride (0.85%) for API 20E, 20NE, and 50CHB/E and the turbidity was adjusted to the equivalent of the turbidity of 0.5 McFarland standards. The standardized bacterial suspension was carefully distributed into the tubes of the test strip to avoid the formation of bubbles. Anaerobiosis was created by overlaying with sterile mineral oil and the strips were subsequently incubated in a humid atmosphere for 18–24 h at 37°C.

An additional oxidase test was performed for *Pseudomonadaceae* by directly adding 2-3 drops of reagent to suspect colonies on the nutrient agar plate. The colour change was observed within 10 seconds. When using Kovac's Oxidase reagent, microorganisms are oxidase-positive when the colour changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the colour changes to purple within 60 to 90 seconds. Microorganisms are oxidase-negative if the colour does not change, or it takes longer than 2 minutes.

For *Listeria,* after suspension with a turbidity of 1 McFarland, haemolysis was observed and recorded on the result sheet. After the distribution of the suspension into the tube, incubation reagents were added and results were recorded again. A drop of ZYM B reagent was added to the test. Data interpretation was performed using the API database with the apiwebTM identification software to obtain the identification result for each strain tested.

4.3 Data analysis

Statistical analysis of the data on growth medium influencing the prevalence of pathogens and microbial mean counts was performed using the general linear model of SAS software version 9.2 to determine the analysis of variance (ANOVA). Tukey's least significant difference (LSD_T), described by Steel and Tourie (1980), was utilised to determine the significant results between variants. The statistical difference between treatment means was determined at the ($p \leq 0.05$) probability level. The Shapiro-Wilks test was performed on standardised residuals to test for any deviations from normality (Shapiro & Wilk, 1965). The growth medium influencing the prevalence of pathogens and the microbial mean count was

subjected to multivariate data analysis, using principal component analysis (PC-XLSTAT 2015) to identify and evaluate the groupings between the variables.

4.4 Results and Discussion

4.4.1 Cabbage phyllosphere microbial count concentrations

Analysis of Variance (ANOVA)

A significant interaction between retails in different towns and microbial mean counts in different growth media was observed in all concentrations except for 10^2 concentrations (Table 4.1). Microbial concentrations 10^1, 10^3 and 10^4 had highly significant value of (P<0.05) microbial mean counts. The highest microbial mean count observed for PCA in 10^1 concentration was in Hennemann, Ficksburg, and Kroonstad followed by Bloemfontein, with the lowest mean count observed in Ladybrand, respectively. The Hennemann and Ficksburg microbial mean counts observed in PCA were significantly different from the rest of the retails. Kroonstad and Bloemfontein retails' microbial mean counts were significantly different to the Ladybrand retail. The highest *Bacillus* microbial mean count was observed in Ladybrand and Ficksburg retails, followed by Kroonstad, and Bloemfontein, with the lowest count observed in Hennemann. Ladybrand and Ficksburg were significantly different to Kroonstad.

Table 4.1: Mean $\log_{10}$ cfu/ml of bacteria sampled from cabbage phyllosphere from different retails

Factors	**10^1**	**10^2 Cons**	**10^3 Cons**	**10^4 Cons**
Supermarkets				
Bloemfontein	69.83 ± 37.08^{b}	53.00 ± 34.64^{a}	36.16 ± 28.39^{a}	46.00 ± 20.42^{a}
Ficksburg	82.50 ± 31.04^{a}	54.16 ± 24.29^{a}	33.00 ± 10.84^{a}	22.33 ± 11.48^{c}
Hennemann	67.50 ± 48.16^{b}	69.66 ± 65.10^{a}	39.00 ± 38.39^{a}	$39.33 \pm 11.15^{a,b}$
Kroonstad	72.00 ± 38.63^{b}	57.50 ± 32.98^{a}	32.50 ± 6.83^{a}	14.00 ± 8.24^{c}
Ladybrand	85.33 ± 14.73^{a}	58.33 ± 31.75^{a}	37.66 ± 18.28^{a}	$27.83 \pm 24.14^{b,c}$
F-value	8.76	2.05	0.38	7.67
P-value	0.0026	0.1796	0.8205	0.0153
Agar				
Bacillus	45.33 ± 18.92^{b}	26.60 ± 8.13^{b}	19.40 ± 10.09^{b}	10.66 ± 3.84^{b}
PCA	105.53 ± 9.15^{a}	90.46 ± 26.93^{a}	51.93 ± 18.44^{a}	36.33 ± 16.60^{a}
F-value	624.83	109.37	61.28	23.65
P-value	0.0001	0.0001	0.0001	0.0028
Sup x Agar				
F-value	19.90	3.14	7.14	5.87
P-value	0.0001	0.0649	0.0055	0.0386

As illustrated in Table 4.1, a significantly (P<0.05) high microbial mean count for 10^2 was observed for PCA in all retails. The highest microbial mean count observed for PCA was in Hennemann, Ficksburg, and Kroonstad followed by Bloemfontein, with the lowest count observed in Ladybrand, respectively. The Hennemann microbial mean count was significantly different from Ficksburg, Kroonstad, Bloemfontein and Ladybrand. Henneman had the highest microbial mean count for *Bacillus* in all retails. The Ficksburg and Ladybrand microbial mean counts were significantly different from one another. Table 4.1 below illustrates various cabbage concentrations from various retails.

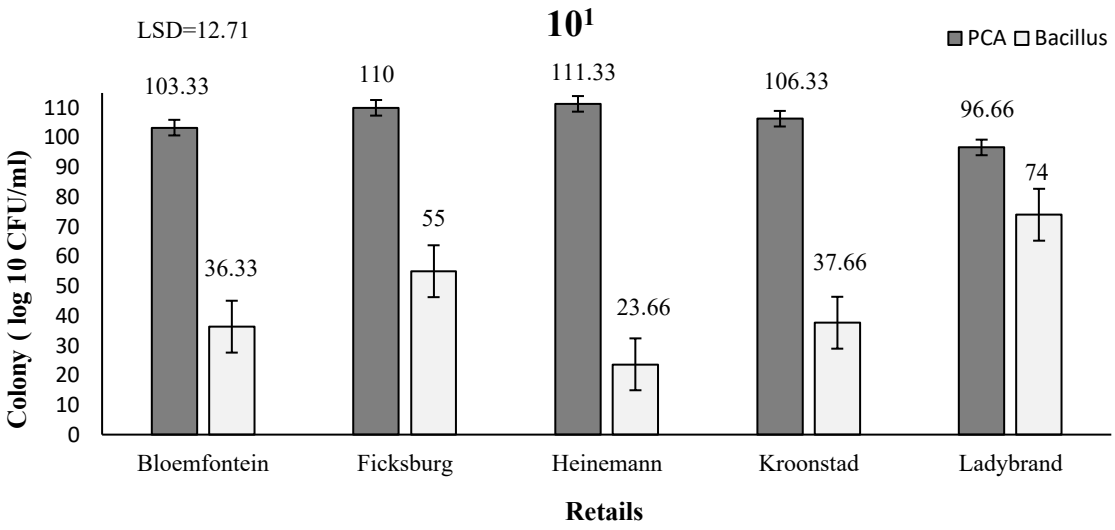

Figure 4.1: Microbial mean counts for cabbage in this study are presented graphically as mean $\log_{10}$ cfu/ml (concentration 10^1). **Abbreviations**: Asterisk (*) = Interactions of interest for discussion.

A significant microbial proliferation for 10^3 was observed in all retails for PCA microbial mean count (Figure 4.1). Hennemann and Bloemfontein were observed with the highest microbial mean count followed by Ladybrand and Ficksburg, with the least microbial mean count observed in Kroonstad, respectively. Hennemann and Bloemfontein were significantly different to Ladybrand, Ficksburg and Kroonstad. A significant growth was also observed in *Bacillus* with the highest microbial count in Ficksburg, Kroonstad and Ladybrand followed by Bloemfontein, with the least microbial mean count in Hennemann, respectively. Henneman had a low count and was significantly different to all the other retail microbial mean counts.

Figure 4.2 below depicts different cabbage concentrations from different retails and colonies from each concentration were calculated by utilising colony $log1_0$cfu/ml.

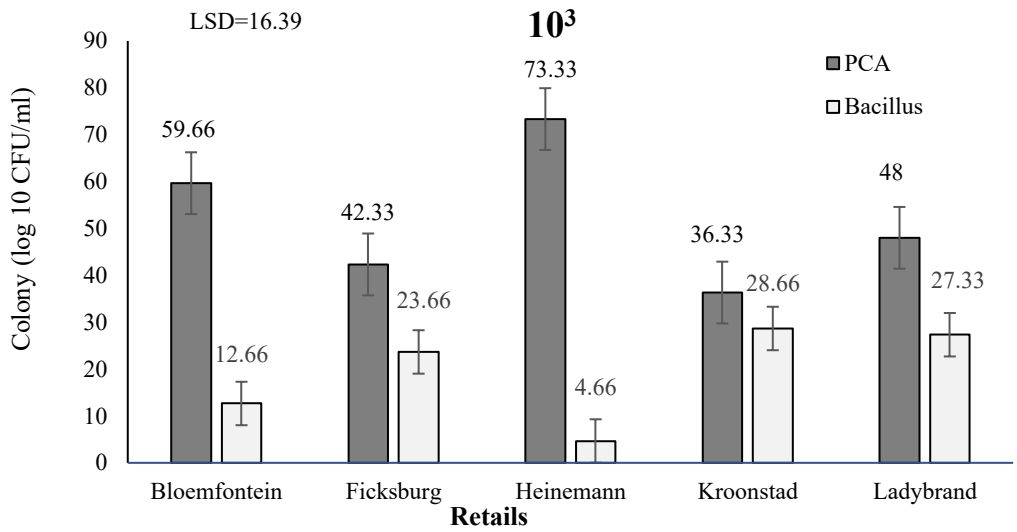

Figure 4.2: Microbial mean counts for cabbage in this study are presented graphically as mean log_{10} cfu/ml (concentration 10^3). **Abbreviations**: Asterisk (*) = Interactions of interest for discussion.

A significant interaction between retail and growth media with a higher microbial mean count of 10^4 was observed in PCA (Figure 4.3). Ladybrand, Bloemfontein, Hennemann and Ficksburg had higher microbial mean counts with the lowest count observed in Kroonstad, respectively. Kroonstad retail had the lowest microbial count and was significantly different to all other retails' microbial mean counts. Kroonstad's microbial mean count was significantly different to all the other retails. Kroonstad and Ficksburg had the highest microbial mean count for *Bacillus* with the least microbial mean count in Ladybrand retail. The Ladybrand microbial mean count was significantly different to Ficksburg and Ladybrand.

Figure 4.3 below depicts different cabbage concentrations from different retails and colonies from each concentration were calculated by utilising colony $log1_0$cfu/ml.

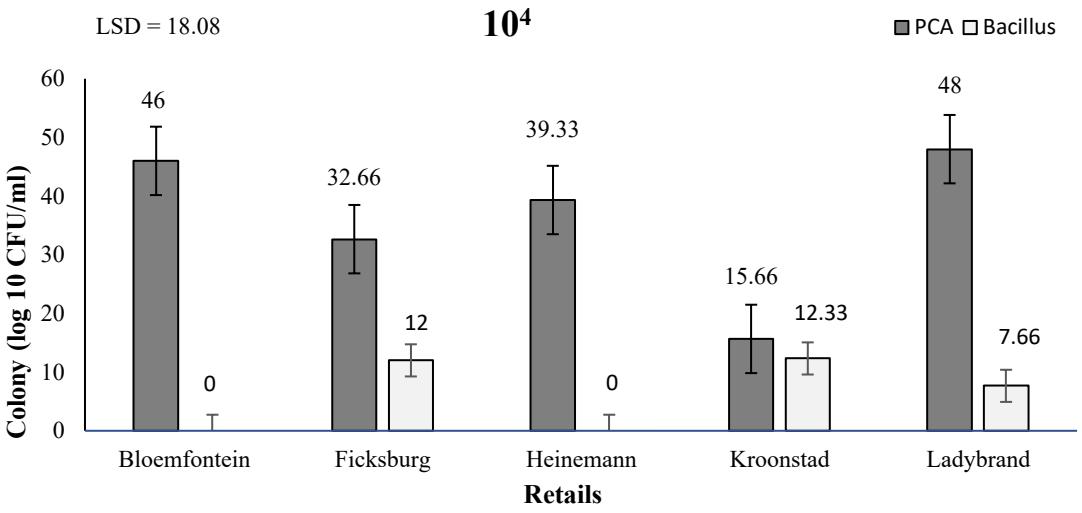

Figure 4.3: Microbial mean counts for cabbage in this study are presented graphically as mean log_{10} cfu/ml (concentration 10^4). **Abbreviations**: Asterisk (*) = Interactions of interest for discussion.

Multivariate data analysis

Multivariate data analysis was applied using a PC analysis to group correlating microbial mean counts. Results from this method were comparable to those of an ANOVA (Figure 4.4).

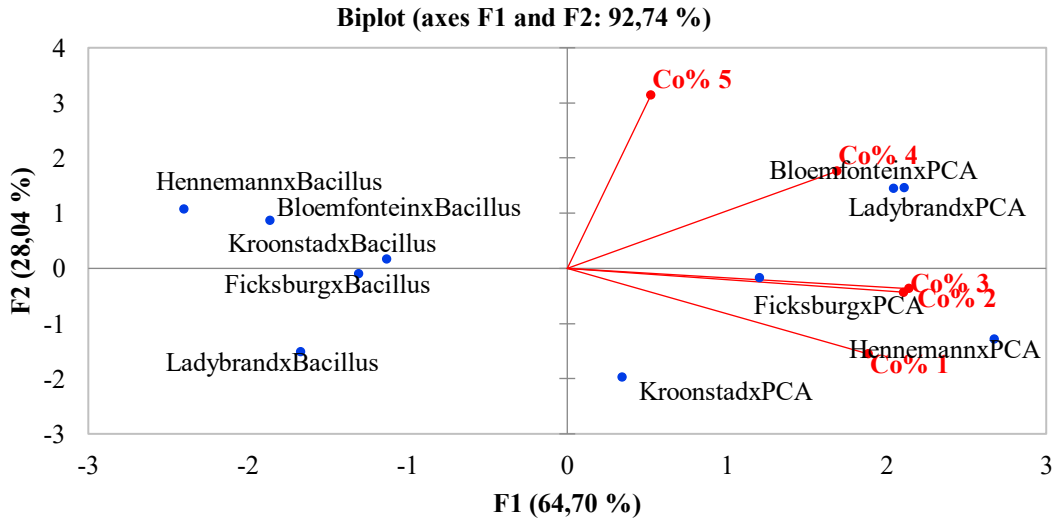

Figure 4.4: Principal component biplot illustrating the variations of cabbage microbial mean count correlation in different concentrations from farms in different growth media

Abbreviations: PCA=Plate count agar

The score plot and loading matrix, based on the first and second principal components (PC1 and PC2) accounted for 92.74 % of the total variance. The biplot loading in PC 1 (64.70) showed that the microbial mean counts for PC analysis from Ladybrand and Bloemfontein correlated with concentration percentages 4 and 5. Ficksburg, Henneman and Kroonstad microbial mean counts correlated with concentration percentages 1, 2 and 3.

4.4.2 Spinach phyllosphere microbial count concentrations

Analysis of Variance

Significant interactions between retail and concentrations were observed on spinach phyllosphere samples with concentrations 10^1 and 10^2, 10^3 and 10^4 (Table 4.2).

Table 4.2: Mean $\log_{10}$ cfu/ml of bacteria sampled from spinach phyllosphere from retails

Factors	**10^1 Cons**	**10^2 Cons**	**10^3 Cons**	**10^4 Cons**
Supermarkets				
Bloemfontein	56.66 ± 59.42^{c}	78.66 ± 16.07^{a}	67.00 ± 12.28^{a}	43.00 ± 10.53^{a}
Ficksburg	80.00 ± 40.65^{a,b}	69.50 ± 38.11^{a}	44.66 ± 22.88^{b}	27.83 ± 13.04^{b}
Hennemann	86.66 ± 37.21^{a}	66.50 ± 33.58^{a}	45.00 ± 12.99^{b}	28.50 ± 10.27^{b}
Kroonstad	78.83 ± 28.35^{a,b}	72.50 ± 39.09^{a}	50.00 ± 20.42^{b}	23.33 ± 7.52^{b,c}
Ladybrand	72.33 ± 36.95^{b}	41.16 ± 21.14^{b}	27.83 ± 13.93^{c}	14.6 ± 12.06^{c}
F-value	16.73	7.27	6.48	10.01
P-value	0.0002	0.0090	0.0125	0.0033
Agar				
Bacillus	38.40 ± 19.19^{b}	33.66 ± 8.38^{b}	28.83 ± 9.07^{b}	16.83 ± 9.37^{b}
PCA	111.40 ± 10.94^{a}	88.66 ± 22.01^{a}	57.33 ± 16.62^{a}	32.86 ± 10.90^{a}
F-value	858.90	130.40	31.42	25.21
P-value	0.0001	0.0001	0.0005	0.0010
Sup x Agar				
F-value	13.62	2.53	0.52	3.59
P-value	0.0005	0.1303	0.6811	0.0657

The highest microbial mean count difference for 10^1 was observed in Hennemann followed by Ficksburg, Bloemfontein and Ladybrand, with the lowest counts observed in Kroonstad, respectively. The highest microbial mean count for *Bacillus* was observed in Henneman with the lowest count observed in Bloemfontein, respectively. The Hennemann, Kroonstad, Ficksburg and Ladybrand microbial mean counts for *Bacillus* were significantly different to the Bloemfontein microbial mean count.

Figure 4.5 below depicts different spinach concentrations from different retails and colonies from each concentration were calculated by utilising colony $log1_0$cfu/ml.

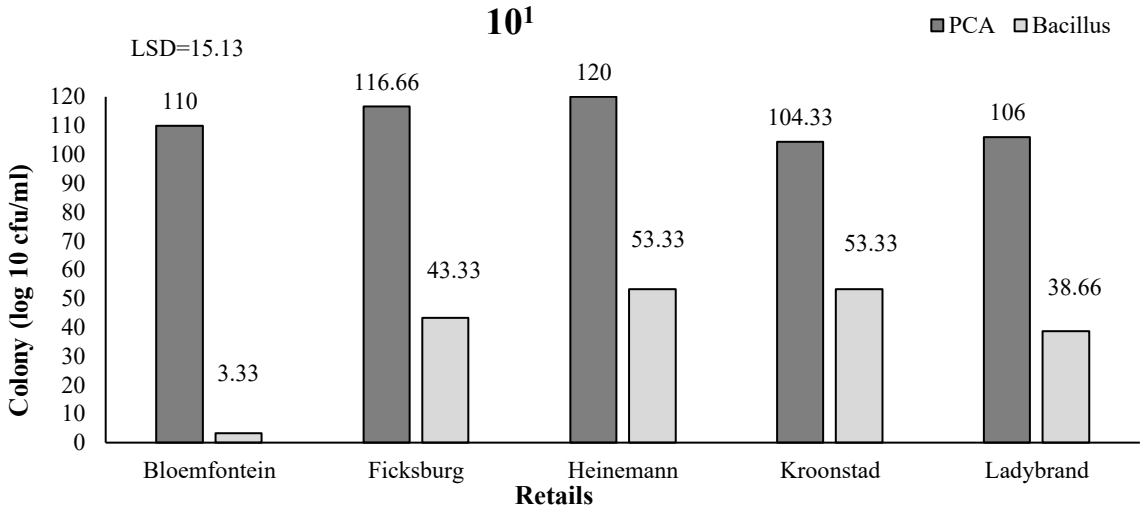

Figure 4.5: Microbial mean counts for spinach in this study are presented graphically as mean log_{10} cfu/ml (concentration 10^1) **Abbreviations**: Asterisk (*) = Interactions of interest for discussion

The highest microbial mean count for PCA 10^2 was observed in Kroonstad, Ficksburg, and Henneman followed by Bloemfontein, with the lowest count observed in Ladybrand. The Kroonstad, Ficksburg, Bloemfontein and Hennemann PCA microbial mean counts were significantly different to Ladybrand (Table 4.2). Kroonstad's microbial mean count was significantly different to Ladybrand's. The highest *Bacillus* microbial counts were observed in Hennemann and Kroonstad followed by Ficksburg, with the lowest count observed in Ladybrand, respectively. The Kroonstad microbial mean count was significantly different to Ladybrand's microbial mean count.

Bloemfontein had the highest PCA microbial mean count of 10^3 followed by Kroonstad, Ficksburg, and Hennemann, with the lowest count observed in Ladybrand retail (Table 4.2). The highest microbial mean counts for *Bacillus* were observed in Kroonstad and Ficksburg followed by Henneman, with the lowest count observed in Ladybrand, respectively. A significant difference was observed between the Ficksburg and Ladybrand *Bacillus* microbial mean counts.

Bloemfontein had the highest PCA microbial mean count of 10^4 followed by Ficksburg and Henneman, with the lowest count in Kroonstad and Ladybrand retails (Table 4.2). Kroonstad retail had the highest microbial mean count for *Bacillus* followed by Hennemann and Ficksburg, with the lowest count in Ladybrand, respectively.

Multivariate data analysis

Multivariate data analysis was applied using a PC analysis to group correlating microbial mean counts. Similar results to the ANOVA were obtained using this method (Figure 4.6). The score plot and loading matrix, based on the first and second principal components (PC1 and PC2), accounted for 88.52 % of the total variance. The biplot loading in PC 1 (67.61%) showed that the microbial mean count for PC analysis correlates with concentrations 1 and 2. Henneman, Ficksburg, Ladybrand and Kroonstad microbial mean counts correlated. Figure 4.6 below depicts the variations of spinach mean counts and their correlation in different concentrations. Different retails were compartmentalised according to their concentrations in various agars.

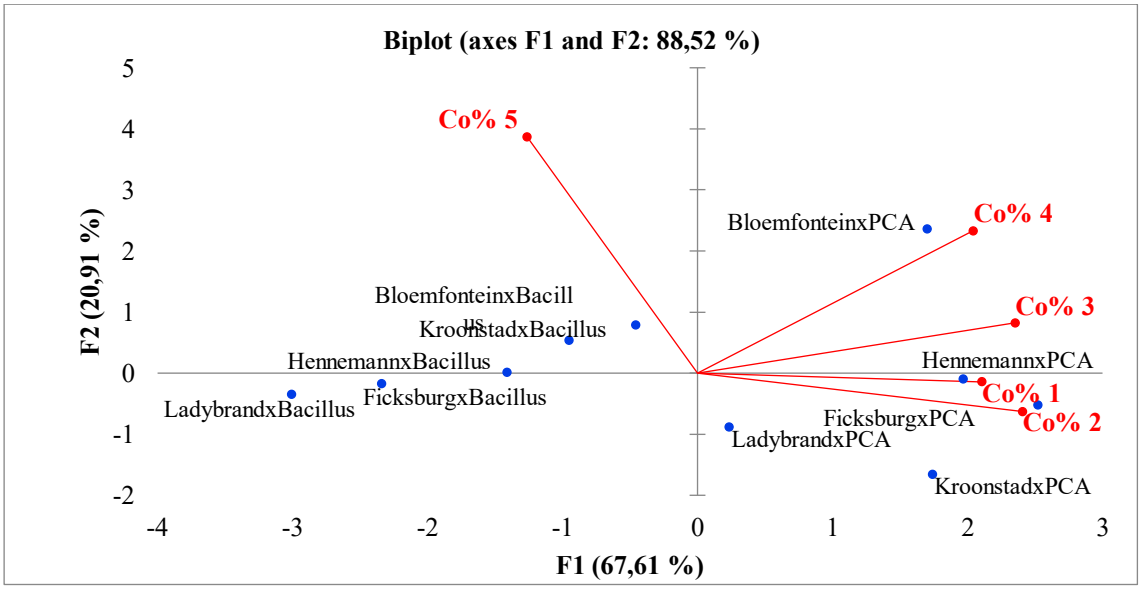

Figure 4.6: Principal component biplot illustrating the variations of spinach microbial mean count correlations in different concentrations in different farms using different growth media.

Abbreviations: PCA=Plate count agar.

4.4.3 Microorganisms identified from spinach and cabbage contamination from various retails

In this study, Henneman, Ladybrand and Kroonstad had the highest number of pathogens identified for cabbage with the least number observed in Bloemfontein and Ficksburg (Table 4.3). With regard to spinach, Henneman, Kroonstad and Ficksburg had the highest number of identified epiphytic and endophytic pathogens with the least number observed in Bloemfontein and Ladybrand. Colonies from *Listeria* and VRBA agar (total

coliforms) were considered statistically insignificant as they were less than thirty, but further test was done to identify the species.

Table 4.3: Pathogens identified from spinach and cabbage phyllosphere isolates

Isolates	Retails	Pathogens identified
Cabbage phyllosphere	Bloemfontein	*Acinetobacter haemolyticus, E.coli* (99.9%), *Pseudomona luteola*
	Kroonstad	*Pseudomona aeruginosa, Brevibacillus laterosporus, Bacillus subtilis, Yersinia enterolitica, Enterobacter aerogenes*
	Henneman	*Burkholderia cepacia* (99.7%), *Proteus mirabilis* (99.9%), *Enterobacter cloacae* (97.7 %), next taxon *Enterobacter amnigenus* with (1.5%), *Pseudomonas oryhabitants, Enterobacter aerogenes (98.8%), Acinetobacter lwoffii, E. coli* (99.9%) *Chryseomonas luteola*
	Ficksburg	*Bacillus subtilis, E. coli, Providencia alcalifaciens*
	Ladybrand	*Burkholderia cepacia, Bacillus cereus, Bacillus subtilis, Burkholderia gladioli*
Spinach phyllosphere	Bloemfontein	*Chryseobacterium indologenes* (99.7%), *E. coli, Proteus penneri, Pseudomona luteola*
	Kroonstad	*Bacillus cereus, E. coli, Bacillus lentus* (98.5%), and *Bacillus subtilis* (0.8), *Enterobacter cloacae* (99.3%) next taxon *Enterobacter asburiae* with (0.2%), *Bacillus megaterium, Listeria* spp. (*Listeria ivanovii and Listeria monocytogenes)*
	Henneman	*Pseudomonas putida, Bacillus megaterium, Enterobacter aerogenes* (98.8%), *E. coli* (99.9%), *Brevibacillus laterosporus* (97.7%), next taxon *Bacillus pumilus* (0.8%), *Bacillus cereus, Bacillus lentus* (98.5), *Proteus mirabilis* (99.8%), next taxon *Proteus Vulgaris group* (0.2%), *Chryseomonas luteola*, *Listeria ivanovii* (94.6%), *Listeria monocytogenes* (99.9%)
	Ficksburg	*Brevibacillus non-reactive, Pseudomona luteola,, Burkholderia cepacia, Pseudomanas stutzeri, Proteus mirabilis, Pseudomona aeruginosa*
	Ladybrand	*Pseudomona luteola, E. coli, Proteus mirabilis, Burkholderia cepacia*

(%) – *viable species count shown by API Web*

Cabbage phyllosphere pathogens

Acinetobacter spp. can be found in food products such as vegetables, fruits and meats which can be a source of transmission of these organisms. *Acinetobacter* spp. are aerobic gram-negative organisms and are known skin colonisers seen as normal flora of the oropharynx and skin in approximately 25% of healthy individuals. They have been identified as a cause of nosocomial infections like septicaemia, pneumonia, meningitis, urinary tract, skin and wound infections (Regalado *et al.,* 2009). In this study, it is assumed that contamination of *Acinetobacter haemolyticus* was from agronomic activities, owing to its capacity to survive long periods on dry surfaces and persist in the environment. A study by Carvalheira *et al.*

(2017) reported that *Acinetobacter* prevalence detected on lettuce (*Lactuca sativa)* from food market was 86.7% and 29.8% of the strains were classified as multidrug-resistant while 4.4% of the strains classified as extensively drug-resistant. Moreover, *Acinetobacter haemolyticus* isolates from retail spinach and cabbage also displayed 100% resistance to tetracycline, ampicillin, ciprofloxacin, and erythromycin, including penicillin (Mohapi *et al*., 2025). Another study detected *Acinetobacter baumanii* was also isolated from farm indicating of insufficient hygiene during processing and a possible cross-contamination from livestock around the farm (Mohapi *et al*., 2024). Good hygiene and handling practices associated with food processing as well as disinfection of ready-to-eat products such as vegetables and fruits, are very important to avoid and mitigate contamination (Campos *et al*., 2019).

Pseudomonas spp. emanate from soil and water environments, particularly those associated with human activity (Bloomfield *et al*., 2024). Additionally, *Pseudomonas aeruginosa* is considered an opportunistic pathogen due to its wide range of infections and is often hard to treat. Söderqvist *et al*. (2017) reported that the composition of baby spinach and mixed-ingredient salad bacterial communities changed during cold storage (8 °C), with *Pseudomonas* being the most abundant high-level taxonomic group across the samples. In this study, it is assumed that *Pseudomonas aeruginosa* and *Pseudomona luteola* presence is due to insufficient hygiene during the processing and the presence of *N*-acyl homoserine lactones (*AHL*) in *Pseudomona* which is responsible for biofilm formation and virulence.

The habitat of *Brevibacillus* overlaps with *Bacillus* and they are widespread genera of gram-positive bacteria, recorded from diverse environmental habitats, including soil (Pands *et al*., 2014). *Bacillus cereus* belongs to the same subgroup of *Bacillus* species as *Bacillus subtilis,* by both phenotypic and rRNA sequence classification. *Bacillus* species are a common cause of foodborne illnesses in humans, and one of its most distinct features and survival mode or strategy is the ability of this pathogen to produce heat-resistant spores. Several studies also reported on toxigenic diversity and cytotoxicity of the *Bacillus* spp. isolated from fresh produce and the effects of various factors on the growth of *Bacillus cereus* (Rahnama *et al*., 2022; Han *et al.,* 2023). In this study, contamination from *Bacillus* spp. which include *Bacillus cereus, Bacillus lentus, Bacillus subtilis and Bacillus megaterium* and *Brevibacillus* may be due to *Bacillus* ability to withstand certain conditions and thrive, forming part of the bacterial composition. For example, a study by Yang *et al.* (2023) mentioned that edeine is the main antibacterial peptide of *Brevibacillus* and provides a new strategy for the identification of

antibacterial products from other biocontrol bacteria hydrophobic LDPE film can act as a substratum which forms a biofilm on the low-density polyethylene (LDPE) film during biodegrading. Furthermore, Yang *et al.* (2017) showed genome sequence of *Brevibacillus laterosporus OSY-I*$_1$ strain to produce *Brevibacillin,* which combats drug-resistant gram-positive bacteria.

Enterobacter species are ubiquitous and originate from terrestrial and aquatic environments such as water, sewage, soil, and food including the intestinal tract of humans, can be present in human skin surfaces and insects. A study (Sen & Saha, 2022) found *Enterobacter* to be the dominating genus that was associated with leafy salad vegetables. In this study, Ent*erobacter cloacae* is an opportunistic pathogen due to contamination from handling and inadequate sanitation. It is identified as a contaminant on the farm with a possible succession to the retail, thus it is an indicator for cross-contamination. Another study from South Africa reported *Enterobacter, Serratia, E.coli* to be observed in irrigation water including leafy green while *Proteus* spp. was observed in irrigation water only in farming site E (Kgoale *et al*., 2023).

A study from Northwest of Spain investigated the role of fresh vegetables and their cultivation environments as reservoirs for antimicrobial-resistant *Enterobacter cloacae* complex (ECC) strains (Pintor-Cora *et al*., 2023). Furthermore, the study reported on antimicrobial-resistant *Enterobacter cloacae* complex strains isolated from fresh vegetables intended for raw consumption and their farm environments in the and reported that AmpC-producing ECC were to colistin which is deemed a last resort antibiotic. Colistin has recently been re-introduced as a last-line antibiotic to treat severe human infections due to advent of multidrug pathogens such as extended-spectrum beta-lactamase producing *Enterobacteriaceae* (Le *et al*., 2021). Besides it dominance of *E. cloacae* in leafy green vegetables, a detailed understanding of the pathogenic mechanisms and strategies of progression and succession form one niche to the other via cross- contamination is lacking.

Providencia are ubiquitous (soil, water, and sewage) gram-negative bacteria identified as part of the normal human gut flora and the genomes of some strains have been sequenced as part of the human microbiome (Rajni *et al*., 2022), including Mexican fruit flies and house flies. In this study, *Providencia alcalifaciens* may be attributed by poor personal hygiene and inadequate processing on the farm prior distribution. Dees *et al*. (2015) reported *Providencia* to be prevalent in rocket salad **(***Diplotaxis tenuifolia*) and lettuce samples due to favouring the

growth of particular taxa and microbial interactions shaping niches. Several studies have reported a higher incidence of *P. alcalifaciens* (10% - 18%) to be a cause of diarrhoea in infants and travellers in developing countries and in foodborne-associated outbreaks (Murata *et al*., 2001; Chlibek *et al*., 2002; Shah *et al*., 2015).

Burkholderia species are opportunistic pathogens found on leafy green vegetables such as spinach and cabbage. *Burkholderia* species are reported to be pathogens in many vegetables and fruits, while others have been reported as opportunistic pathogens in humans and other animals (Elshafie *et al*., 2021). These beneficial *Burkholderia* species are free-living or endophytic and form mutualistic associations with their host plants (Mannaa *et al*., 2018). In this study, the endophytic bacteria, *Burkholderia cepacia,* could be part of plant-promoting endophytic bacteria having a mutual relationship with other opportunistic bacteria available in the cabbage phyllosphere. There are no similar cases in the literature to support this hypothesis.

Spinach phyllosphere pathogens

Chryseobacterium indologenes emanates from soil, water and food products, (Alon *et al.,* 2018). In this study *C. indologenes* may be perceived as part of microbial population as a coloniser, that is resistant to disinfectant and a food spoilage organism. Other literature suggests that *C. indologenes* spp. can resist chlorination, specifically at water municipal plants, which can indicate possible water contamination from the server (Mukerji *et al.,* 2016). Future research on this subject can be useful to identify all the pathogens resistant determinants to water chlorination and develop countermeasures. Studies on this pathogen, particularly on leafy vegetables are scanty and there are no similar cases in the literature to support this hypothesis.

Proteus mirabilis is ubiquitous in the environment and is regarded as a part of the normal flora in the human gastrointestinal tract. However, reports, especially about *P. mirabilis* from fresh vegetables, are still lacking (Li *et al*., 2023). In this study, *P. mirabilis* was attributed to faecal contamination due poor sanitation and poor hygiene or by non-composted or improperly composted manure utilised as fertiliser on spinach. Among the foodborne disease incidents reported in various countries, the proportion of food poisoning caused by *P. mirabilis* remains high (Wang *et al*., 2021). For example, from 1998 to 2013, it was reported that 294 people had food poisoning caused by *P. mirabilis* in 3 provinces of China (Gong *et al.,* 2019). Another study in China mentioned that all *P. mirabilis* isolates carried *bla*$_{\text{NDM}}$, and *bla*$_{\text{NDM-1}}$ which was the most prevalent type *P. mirabilis* isolates found in vegetables may be derived

from animals because a relatively high prevalence of *P. mirabilis* isolates carrying *bla*$_{NDM-1}$ has already been found in chickens (*Gallus gallus domesticus*) manure in China (Zhu *et al*., 2021). There are no similar cases of *P. mirabilis* on leafy green vegetables in the literature to support this hypothesis.

Listeria is an important foodborne pathogen found in the intestinal tract of wild and domestic animals existing as a saprophyte and shed in faeces, it is also widely tested in food including environmental and clinical samples (Gasanov *et al*., 2005). Several reports have demonstrated that *L. monocytogenes* is commonly present in a wide variety of fresh produce samples due to contamination. A study by Tango *et al*. (2014) indicated the prevalence of *L. monocytogenes* to be highest on organic romaine lettuce and spinach and found 4 (6.4%) of 63 samples of each type of vegetable with the majority of deaths amounting to thirty-three from a single outbreak. Another study in the largest district municipality in the Eastern Cape, South Africa, reported *L. monocytogenes* in cabbages, cauliflowers (*Brassica oleracea var. botrytis*) and carrots (*Daucus carota sativus*) highlighting a significant count among the vegetables with the possibility of being contaminated on farms through irrigation water and other farm practices (Ntshanka *et al.,* 2022). In this study, *L. monocytogenes* was isolated from spinach from Kroonstad and Henneman town retails. The growth sensitivity of the organisms ranged from eleven to fifteen colonies in concentrations of 10^1 and 10^2 CFU from both retail samples, colonies were less than thirty. Owing to the *Listeria* spp. capabilities, it was assumed that it might emanate from a farm and form biofilm as a strategy to survive harsh and stressful environments. Another possibility could be the temperature fluctuation of spinach during distribution and poor sanitary practices of workers which might have proliferated the pathogen. Harter *et al.* (2017) mentioned that *L. monocytogenes* type ST121 stress survival islet (SSI-2) together with the transcriptional regulator , σ^B plays a role in the survival of the cell during detergent stresses at lethal levels in survival under oxidative and alkaline stresses response and activation of biofilms in the food processing environment. A major virulence factor, internalin gene *inlA inlABCEFJ* including hyper-virulent ST1, ST2 and ST204, and hypo-virulent ST121 and ST321 have higher prevalence in food in South Africa compared to the studied prevalence in other countries. (Mafuna *et al*., 2021). The resistance is caused by efflux pump genes located in the mobile genetic element such as *qacA Ide, qacH, mdrl, emrE* genes including *bcrABC* cassette from different ST and CC of *L. monocytogenes* (Korsak *et al*., 2019). Yoon *et al.* (2015) reported that the *L. monocytogenes* biofilm formation is the cause of increment to

quaternary ammonium compounds tolerance by increasing membrane hydrophobicity promoting further adherence to objects or surfaces.

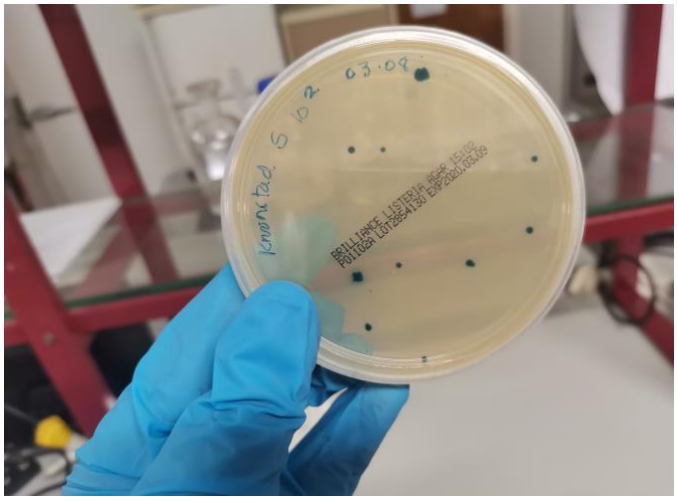

Figure 4.7: The growth of *Listeria* from spinach samples.

Previous studies reported that L. ivanovii was exclusively linked to ruminants only, but it was later highlighted that *L. ivanovii* infections occurred in humans after the ingestion of contaminated food. The same study concluded that a wide variety of food products can be a source of this pathogen and that, like *L. monocytogenes*, *L. ivanovii* can persist in the food production environment (Rossi *et al*., 2022). In another study (Nyenge *et al*., 2012), a total of 51 *L. ivanovii* strains were isolated from ready-to-eat including vegetables purchased from cafeterias in Alice, South Africa. In this study, *L. ivanovii* was isolated from spinach indicating possible contamination on farms, especially farms with livestock production, including human contamination due to inadequate sanitation and poor hygiene leading to a succession of this opportunistic pathogen. This present study is one of the few studies in South Africa to report on *L. monocytogenes* and *L. ivanovii* in food products, particularly on spinach, which was collateral study focusing on farms and retails. there's no specific information available about *L. ivanovii* resistance specifically in leafy vegetables.

Enterobacter asburiae emanates from soil, water and food products and is also known as the epiphytic bacterium (Lau *et al*., 2014). Moreover, it is speculated to be a pathogen, although more studies need to be carried out to explore the mechanisms involved in the pathogenesis of this bacteria. Furthermore, *E. asburiae* was also discovered to be involved in antagonistic communication against other microorganisms even though the system or the mechanisms involved are still unclear. Other studies reported that the organism causes

competition in the growth of human pathogens, particularly *Salmonella enterica* and *E. coli 0157:H7* and inhibits the mentioned pathogens' growth by more than 10 to 100 folds (Rezzonico *et al*., 2012; Mandal *et al*., 2013). However, further studies are required to verify the presence and function of virulence-related genes and complex proteins produced by *E. asburiae* and their regulation in interspecies microbial growth suppression (Lau *et al*., 2014). Gram-negative bacteria employ AHL as quorum sensing signals in their communication circuits to regulate a diverse array of physiological activities such as virulence antibiotic production competence including biofilm formation. Few studies shared light on *E. asburiae* isolated from leafy greens ability to produce AHL which is for the development of genetic competence, the regulation of virulence and biofilm formation including biocidal resistance of biofilms (Lau *et al*., 2013) To date, reports or studies of *E. asburiae* on fresh produce are still very limited. There are no similar cases in the literature to support this hypothesis.

Escherichia coli (*E. coli*), is a type of bacteria that normally lives in human and animals. It is often used as an indicator for poor hygiene and sanitation in food processing environments. It is highly virulent, with a low infectious dose (10 to 100 CFU). Bacteria can contaminate raw vegetables, Lettuce and spinach are prone to *E.coli* outbreaks. Intestinal illnesses will be described by the causative *E. coli* subtypes, including enterotoxigenic Escherichia coli (ETEC), enterohemorrhagic Escherichia coli (EHEC), which is also known as Shiga toxin-producing Escherichia coli (STEC) and will be referred to as EHEC/STEC, enteroinvasive Escherichia coli (EIEC), enteropathogenic Escherichia coli (EPEC), and enteroaggregative Escherichia coli (EAEC) (Priyanka *et al*., 2021). Approximately 100,000,000 organisms must be ingested to cause illness in a healthy person. ETEC is the single most important organism causing traveller's diarrhoea which is named Montezuma's revenge (Makvana and Krilov et al., 2015). In this study, *E. coli* is assumed to be prevalent due to unsanitary practises which might emanate from poor hygiene and poor post-harvest disinfectant. Another study in South Africa evaluated the prevalence of *E. coli* antibiotic-resistant genes (ARGs) in animals, humans, and the environment. The obtained results indicated that the pooled prevalence estimates (PPE) of *E. coli* ARGs was 36.3%, 34.4%, 32.9%, and 28.8% for $bla_{TEM\text{-}M\text{-}1}$, *amp*C, *tet*A, and bla_{TEM}, respectively. Eight ARGs such as $bla_{CTX\text{-}M}$, $bla_{CTX\text{-}M\text{-}1}$, bla_{TEM}, *tet*A, *tet*B, *sul*1, *sul*II, and *aad*A were detected in humans, animals and the environmental samples (Ramatla *et al.,* 2023). Ahmed *et al.* (2024) reported that the consumption of food and food-derived products contaminated with antibacterial genes

can lead to an indirect transfer from humans through food chain and in severe cases, antibiotic resistance can lead to increased mortality rates.

4.5 Conclusion

This study provides comprehensive information pathogen prevalence from farms and succession to retail highlighting poor surveillance and monitoring which appears to be increasing and spreading. The data provided is expected to support microbiological surveillance on the prevalence of pathogens from leafy greens. In effect, enumerated and characterised opportunistic pathogens indicate poor hygiene practices, while most of the pathogens found indicate succession from the farm, as most are found in the soil or environment. Understanding the complex microbiome ecosystem that is unique for each product is imperative as it provides directives on mitigation and prevention of outbreak infections including control of the pathogen during outbreak or recall. Since *Listeria* is now a notifiable communicable disease in South Africa, subsequent outbreak infections need to be reported. In 2018, South Africa experienced an outbreak of listeriosis which was identified by the WHO as the largest outbreak globally with 1060 confirmed cases and 216 deaths due to contaminated food. Surveillance systems, including inspections of farms and retails need to be active and firm to assist in the early detection and prevention of disease. Good hygiene practices must be applied until the purchase stage to ensure food safety and to maintain the farm-to-fork continuum. The study contributes to the scholarly research on the primary production and retail food safety of fresh leafy green vegetables and the understanding of contamination and potential risk parameters influenced agronomic activities leading to succession of pathogens implicating leafy green vegetables.

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CHAPTER FIVE

Antimicrobial Resistance Profiles of Bacteria Isolated from Fresh Vegetables in Free State Province, South Africa

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Abstract

Important role of antibiotics cannot be overestimated as human and animal health relies heavily on them for treatment of infectious diseases. The aim of this study was to assess the antibiotic resistance from 38 unduplicated isolates *Brassica oleracea* var. *capitata* (L.) and S*pinacia oleracea* (L.) isolates collected from farms and retails**.** As a result, *Escherichia coli* (18.4%), *Burkholderia cepacia* (15.8%), *Pseudomona luteola* (15.8%), *Staphylococcus aureus* (13.2%), *Serratia marcescens* (10.5%), and 5.3% each of *Enterobacter cloacae*, *Micrococcus luteus*, *Staphylococcus sciuri*, *Acinetobacter haemolyticus*, and *Citrobacter freundii* showed resistance to tested antibiotics. The isolates displayed high levels of antibiotic resistance (AR) against *tetracycline* (*n* = 32; 84%%), *Penicillin* (*n* = 27; 71%%), *ampicillin* (*n* = 26; 68%), *gentamycin* (*n* = 21; 58%), *erythromycin and chloramphenicol* (*n* = 20; 53%), *ciprofloxacin* (*n* = 14; 37%) *ceftazidime* (*n* = 11; 29%), *vancomycin* (*n* = 2; 5.3%) using the disk diffusion method. High multidrug resistance (MDR) rates was observed for: *Escherichia coli* with 78% (7/9), *Staphylococcus aureus* with 67% (6/9), *Pseudomona luteola* with 44% (4/9), *Serratia marcescens* with 56% (5/9) and *Burkholderia cepacia* with 33% (3/9), A total of 79% of the antibiotic-resistant isolates exhibited multidrug resistance to different classes such as *β*-lactams, chloramphenicol, tetracycline, aminoglycosides, and macrolides**.** The results of the study highlight the importance and application of monitoring the microbiological quality of green leafy vegetables, as they contain antibiotic resistant bacteria that could affect human health when consumed raw.

Keywords: Antibiotic resistance, Fresh vegetables, Bacterial prevalence, South Africa

5.1 Introduction

Antibiotic resistance is highlighted as a global health crisis that best point out and elucidate “one health approach”. The “one health approach” is outline and expressed as a conjoined discipline to provide solutions for human, animal including environment health (Djordjevic *et al*., 2023). Thus, it is imperative not only to understand antibiotic utilisation particularly in agriculture and its impact but to also have an insight of the emergence of antibiotic resistance including the complex interaction of elements in human and environmental. It is also important to gain insight into the emergence of antibiotic resistance, including the complex interaction of elements in leafy agricultural produce. A larger study regarding

the determination of the sources of pathogenic bacteria, including antibiotic resistance, is necessary in order to suggest the sources and relevant steps to mitigate the contamination (Ahmad *et al*., 2023).

Antibiotics in animals are utilised as supplements for growth efficiency, improve health status, as prophylaxis or to treat infections or diseases (Verraes *et al.,* 2013). Different studies showed that 30–90% of the antimicrobials administered to animals are excreted as the parent compound in their faeces or urine (Mohan *et al*., 2023; Swinkels *et al*., 2024). Contamination of plants by antibiotic residue can also be through different medium employed for making the soil fertile such as the use of fertilizer, biosolids, and sludge and contaminated irrigation water (Lopez-Velasco *et al*., 2011). Other studies have also reviewed the fate and transport of antibiotic residues and antibiotic resistance genes in agroecosystem following land application of manure waste (Joy *et al*., 2013; Sandberg and LaPara, 2016). The emergence of antibiotics resistance is an increasing concern world-wide and in health care facilities due to ongoing explosion of antibiotic resistance infections (Cornejo-Juarez *et al*., 2015). Antibiotic resistance is of great concern as it is associated with morbidity, mortality, and economics (Pulingam *et al*., 2022). Fresh produce is reported to be source of exposure to various antimicrobial resistant bacteria, antibiotic resistance bacteria of clinical importance (Rahman *et al*., 2021). Additionally, vegetables particularly leafy green vegetables are not treated with antibiotics but can be contaminated through various contaminants such as irrigation water, soil amendments such as biosolids including fertiliser utilised on crops. Few studies have reported and highlighted the presence of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) on fresh produce (Rahman *et al*., 2021; Kläui *et al*., 2024).

Another essential utilisation of antibiotics other than in livestock is the primary target in controlling bacterial diseases in plants (Verhaegen *et al*., 2023). However, due to inappropriate practice of misusing antibiotics on vegetables from primary sector, there has been an increase in microbial resistance (Endale *et al*., 2013). Antibiotic compounds such as tetracycline, oxytetracycline, sulfamethazine, sulfamethoxazole, tylosin, trimethoprim, ofloxacin, ciprofloxacin and amoxicillin can be absorbed by vegetables such as lettuce (*Lactuca sativa* L.), cabbage (*Brassica oleracea* L.) and spinach (*Spinacia oleracea* L.) from the growth media through their roots (Azanu *et al.,* 2016; Gudda *et al*., 2023).

It is estimated that by the year 2030 the utilisation of antibiotics will increase by 67%, with almost twice this increases in countries such as Russia, China, Brazil, India including South Africa (Van *et al.,* 2020). Each year in the United States, at least 2 million people become

infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections and many more people die from other conditions that were complicated by an antibiotic-resistant infection (CDC, 2019). A study based on 2007data estimated that 386,000 infections due to multidrug-resistant bacteria occurred in europe during that year and 25,000 patients died from those infections (Colomb-Cotinant *et al.,* 2016).

Antimicrobial resistance status specifically in sub-Saharan Africa is undefined, this is because of lack of real-time data recording, surveillance and regulation (Elton *et al.,* 2020). Phares *et al*. (2020) reported poor practices regarding the utilisation of antibiotic as well as inadequate knowledge regarding its effect on soil ecosystem amongst farmers in Ghana. A systematic review (Tadesse *et al*., 2017) reported that there is a 42.6% gap in unavailable data on antibiotic resistance, particularly in African countries. In South Africa, few studies have been conducted on fresh produce in Mpumalanga Province (Msimango *et al*., 2023), North-West Province (Njage and Buys, 2015), North-West Province (Ratshilingano *et al*., 2022). in Gauteng Province (Richter, *et al*., 2019). However, there is no information available on the antimicrobial susceptibility profile of pathogens isolated from spinach and cabbage in the Free State Province, South Africa.

5.2 Materials and methods

5.2.1 Bacterial strains

This study was conducted by procuring ninety samples of raw unpackaged spinach [*Spinacia oleracea* (L.)] and ninety samples of cabbage [*Brassica oleracea* var. *capitata* (L.)] heads from five farms, including sixty samples of raw unpackaged spinach phyllosphere and seventy-five samples of cabbage heads from five retail markets, respectively, in different local municipality districts within Free State Province, South Africa. The selected farms represent the major small-scale farms that supply most leafy greens to various retailers. Spinach and cabbage were chosen due to their minimal processing, production, demand, and purchase price. Leafy vegetables, like spinach, are available year-round, while cabbage is available during winter, so each province in South Africa is unique in terms of suitable agricultural commodities that can be produced. Additionally, the Free State Agricultural Union reports that the province has 7.515 farming units, the highest in the country. Furthermore, it accounts for 26.4% of South Africa's field crops and 15.9% of all its livestock. Moreover, Free State Province is responsible for 15% of South Africa's gross agricultural income. The sector contributes approximately 7% to the provincial gross domestic product. Consumer demand puts pressure on the fresh leafy

green vegetable industries for year-round supply. The farms selected are small-scale farms that supply retailers, small villages, street vendors, informal markets, and local supermarkets. Samples were collected in the following towns in Free State Province, South Africa: Motheo District, Mangaung Metropolitan (29.1217 S, 26.2128 E); Lejweleputwa District, Matjhabeng Local Municipality (28.9784 NS, 27.0264 E); Thabo Mofutsanyana District, Setsoto Municipality (28.9093 S, 27.5555 E); Fezile Dabi District, Moqhaka Local Municipality (27.6373 S, 27.2323 E); and Thabo Mofutsanyana District, Dihlabeng Local Municipality (28.2423 S, 28.3111 E) (Mohapi, *et al*.,2024).

5.2.2 Microbiological techniques and analysis

A total of 25 g of each collected sample was added to 90ml of sterile peptone water solution (Merck, SA) and homogenized in a stomacher (Stomacher® 400 circulation Seward, Lasec, SA) for 260 rpm for 1 min. Then, the mashed samples were filtered through a sterile folded paper filter (Lasec, SA). The sequential dilutions were prepared using filtrated samples for plate count analyses. Subsequently, ten-fold serial dilutions up to 10^5 folds of the homogenate were prepared for each sample and utilised for bacteria analysis. Each sample was serially diluted and subsequently analysed in duplicates. Plate count agar (PCA) including selective media such as MacConkey with crystal violet with salt, MacConkey without crystal violet, Baird-Parker supplemented with egg yolk, Violet-red bile and *Bacillus* (all obtained from Merck, SA) were utilised for culturing (Mohapi *et al*., 2024).

5.2.3 Identification of the isolates using API

Colonies were plated on plate counting agar plates and pure colony blood agar prior to analysis with *API 20E, 20NE, STAPH* and *50 CHB/E* for organism identification (Biomerieux, Republic of South Africa). Briefly, 1–4 colonies with identical morphology were collected from cultures (18–24 hours) and emulsified in 5 ml of sterile sodium chloride (0.85%) for *API 20E, NE, STAPH* and the turbidity was adjusted to the equivalent of turbidity of 0.5 McFarland standards. The standardized bacterial suspension was carefully distributed into the test strip tubes to avoid bubble formation. Anaerobiosis was created by overlaying with sterile mineral oil and the strips were then incubated for 18–24 hours at 37 °C in a humid atmosphere. For *Pseudomonadaceae*, an additional oxidase test was performed by adding 2-3 drops of the reagent directly to the suspected colonies on the nutrient agar plate. The colour change was observed within 10 seconds. When using Kovac's oxidase reagent, microorganisms are oxidase positive when the colour changes to dark purple within 5 to 10 seconds (Mohapi, et al., 2024).

5.2.4 Antibiotic susceptibility pattern of the isolates

The antibiotic susceptibility of the thirty-eight isolates against antimicrobials was determined by Kirby-Bauer disc diffusion method in Mueller-Hinton Agar (Merck, SA) (Ramatla, *et al*., 2024). All the isolates were analysed for antimicrobial susceptibility test against various antibiotic agent. The isolates tested were picked from identified bacteria from farms and retails. seven classes of antibiotics were tested, β-lactams [penicillin (P; 10 µg), ampicillin (AMP; 10 µg), ceftazidime (CAZ; 30 µg)], aminoglycosides [gentamicin (CN; 10 µg)], chloramphenicol [chloramphenicol (C; 30 µg)], tetracycline [tetracycline (TE; 30 µg)], glycopeptide [vancomycin (VA; 30 µg)], macrolides [erythromycin (E; 15 µg)], and fluoroquinolones [ciprofloxacin (CIP; 5 µg)] (ThermoFisher, South Africa). These antimicrobial agents were selected based on their various pharmacological categories and their availability which includes their frequency of prescription for the treatment of various bacterial infections in South Africa. The control strains of *E. coli* ATCC 25922 was used to ensure quality control during the antibiotic susceptibility test. Multidrug resistance (MDR) was taken as resistant to three or more antibiotics tested (Ramatla *et al*., 2024). Following incubation, the zones were measured to the nearest millimetre using a ruler or calliper to include the diameter of the disc in the measurement. A guidelines chart for interpretation of antibiotic susceptibility was utilised (Kirby- Bauer Guidelines, 2020).

5.3 Results

5.3.1 Identification of isolates

A total of thirty-eight non-duplicated (one isolate per sample) were confirmed by API 20E, 20NE, STAPH and 50CHB/E including *E. cloacae* (*n* = 2; 5.3%), *S. aureus* (*n* = 5; 13.2%), *M. luteus* (*n* = 2; 5.3%), *S. sciuri* (*n* = 2; 5.3%), *A. haemolyticus* (*n* = 2; 5.3%), *B. cepacia* (*n* = 6; 15.8%), *P. luteola* (*n* = 6; 15.8%), *E. coli* (*n* = 7; 18.4%), *C. freundii* (*n* = 2; 5.3%) and *S. marcescens* (n = 4; 10.5%) were recovered from farm and retail spinach and cabbage. Most isolates (60.5%) were of farm origin, with spinach and cabbage contributing 34.2% (*n* = 13) and 26.3% (*n* = 10) of the total, respectively. While fifteen isolates were obtained from the retail markets, nine (23.6%) of these isolates were from spinach and six (15.7%) were from cabbage, as shown in Table 5.1.

Table 5.1. Identification of different bacteria isolated from spinach and cabbage.

	Retail																			
	Spinach										Cabbage									
	EC1	SA	ML	SS	AH	BC	PL	EC2	CF	SM	EC1	SA	ML	SS	AH	BC	PL	EC2	CF	SM
R1		–	–	–	1	–	1	–	–	–	–	–	–	–	1	–	1	–	–	–
R2	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
R3	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	1	–	1	–	–
R4	–	–	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–
R5	–	–	–	–	–	1	1	1	–	–	–	–	–	–	–	1	–	–	–	–
	Farms																			
F 1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1
F 2	–	1	1	–	–	1	1	1	1	1	–	–	1	–	–	–	–	1	–	–
F 3	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–
F 4	–	1	–	1	–	–	–	1	1	–	–	1	–	–	–	1	–	–	–	1
F 5	–	–	–	–	–	–	–	–	–	1	–	1	–	–	–	–		1	–	–

E. cloacae=EC1, *S. aureus* (SA), *M. luteus* (ML), *S. sciuri* (SS), *A. haemolyticus* (AH), *B. cepacia* (BC), *P. luteola* (PL), *E. coli* (EC2), C. *freundii* (CF) and *S. marcescens* (SM)

Table 5.2: The antibiotic resistance profiles of the isolates from samples of spinach and cabbage.

	Spinach										Cabbage									
Ant	EC1	SA	ML	SS	AH	BC	PL	EC2	CF	SM	EC1	SA	ML	SS	AH	BC	PL	EC2	CF	SM
C	1	–	–	–		1	1	2	–	–	–	–	–	–	–	–	1	1	–	–
TE	1	–	–	–	1	1	2	2	–	–	1	–	–	–	1	1	1	1	–	–
CN		–	–	–	–	1	–	2	–	–	–	–	–	–	–	1		1	–	–
AMP	1	–	–	–	1	1	2	1	–	–	1	–	–	–	1	–	1	1	–	–
CIP	–	–	–	–	1	2	–	–	–	–	–	–	–	–	1	1	–		–	–
E	–	–	–	–	1	1	–	2	–	–	–	–	–	–	1	–	–	1	–	–
CAZ	1	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	1	–	–
VA	–	–	–	–	1	–	–		–	–	–	–	–	–	1	–	–	–	–	–
P	–	–	–	–	1	1	3	1	–	–	–	–	–	–	1	–	1	1	–	–

Farms

	Spinach										Cabbage									
C	–	–	1	–	–	1	1	2	–	2	–	–	1	–	–	1	1	2	–	2
TE	–	3	–	–	–	1	1	2	2	2	–	2	–	–	–	1	1	2	–	2
CN	–	3	1	1	–	2	–	2	2	–	–	2	1	–	–	1	–	2	–	–
AMP	–	3	1	–	–	1	3	2	2	–	–	2	1	–	–	–	1	1	–	–
CIP	–	2	–	–	–	1	–	–	2	2	–	1	–	–	–	–	–		–	2
E	–	2	–	1	–	1	–	2		2	–	2	–	–	–	–	–	2	–	2
CAZ	–	–	1	–	–	–	1	2	1	–	–	–	1	–	–	–	–	1	–	–
VA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P	–	3	1	1	–	–	1	2	–	2	–	2	1	1	–	–	1	1	–	2

Table 5.3: Resistance patterns of bacterial isolates from farm and retail leafy vegetable samples.

Species	No. of isolates	C	TE	CN	AMP	CIP	E	CAZ	VA	P
E. cloacae	2	1 (50%)	2 (100%)		2 (100%)			1 (50%)	–	–
S. aureus	5		5 (100%)	5 (100%)	5 (100%)	3 (60%)	4 (80%)		–	5 (100%)
M. luteus	2	2 (100%)	–	2 (100%)	2 (100%)	–	–	2 (100%)	–	2 (100%)
S. sciuri	2	–	–	1 (50%)	–	–	1 (50%)			2 (100%)
A. haemolyticus	2	–	2 (100%)		2 (100%)	2 (100%)	2 (100%)		2 (100%)	2 (100%)
B. cepacia	6	2 (33%)	4 (67%)	5 (83%)	2 (33%)	3 (50%)	2 (33%)		–	1 (17%)
P. luteola	6	4 (67%)	6 (100%)		5 (83%)	–		1 (17%)	–	6 (100%)
E. coli	7	7 (100%)	7 (100%)	7 (100%)	6 (86%)	–	7 (100%)	6 (86%)	–	5 (71%)
C. freundii	2	–	2 (100%)	2 (100%)	2 (100%)	2 (100%)		1 (50%)	–	–
S. marcescens	4	4 (100%)	4 (100%)	–	–	4 (100%)	4 (100%)	–	–	4 (100%)
Total	38(100%)	20(53%)	32 (84%)	22 (58%)	26 (68%)	14 (37%)	20 (53%)	11 (29%)	2 (5.3%)	27 (71%)

C=Chloramphenicol, TE=Tetracycline, CN= Gentamicin, AMP= Ampicillin, CIP=Ciprofloxacin, E= Erythromycin, CAZ= Ceftazidime, VA= Vancomycin P- Penicillin

5.3.2. Antibiotic Susceptibility for all isolates

Analysis of 38 isolates representing 10 species showed that *E. cloacae, E. coli,* and *S. marcescens* isolates exhibited resistance to chloramphenicol, while all *E. cloacae, S. aureus, A. haemolyticus, P. luteola, E. coli, C. freundii,* and *S. marcescens* isolates were resistant to tetracycline. The majority (84%) (n = 32) of the isolates showed resistance to tetracycline, followed by penicillin with 71% (n = 27). Among the tested antibiotics, vancomycin had the least number of resistant isolates, accounting for only 5.3% (n = 2) (Table 2).

Regarding multidrug resistance (MDR), 79% (*n* = 30) of the isolates from fresh vegetables were resistant to three or more classes of antibiotics (Table 3), namely, β-lactams (penicillin, ampicillin, ceftazidime), aminoglycosides (gentamicin), chloramphenicol (chloramphenicol), tetracycline (tetracycline), glycopeptide (vancomycin), macrolides (erythromycin), and fluoroquinolones (ciprofloxacin), as well as *E. cloacae* (*n* = 1; 3.2%), *S. aureus* (*n* = 5; 13.2%), *M. luteus* (*n* = 1; 3.2%), *S. sciuri* (*n* = 1; 3.2%), *A. haemolyticus* (*n* = 2; 5.3%), *B. cepacia* (*n* = 3 (9.6%), *P. luteola* (*n* = 4; 12.9%), *E. coli* (*n* = 7; 22.5%), *C. freundii* (*n* = 2; 6.5%), and *S. marcescens* (*n* = 4; 12.9%).

5.4 Discussion

Following the "One Health approach", which recognizes food as a vector for the spread of antibiotic resistance from the environment to humans, this study sought to identify fresh produce from different farms and retails based on the production and agricultural systems in the Free State Province of South Africa to assess the presence of antibiotic resistance. The nine antimicrobial drugs tested in the present study are widely used to treat bacterial infections in animals and human health.

In this study, *E. coli* (18.4%), *B. cepacia* (15.8%), *P. luteola* (15.8%), *S. aureus* (13.2%) isolates from cabbage and spinach displayed high levels of resistance to most of the antibiotics utilised while *E. cloacae*, *M. luteus*, *S. sciuri*, *A. haemolyticus*, and *C. freundii* [each 5.3%] showed the least. *Escherichia coli* isolates from retail spinach, farm spinach and cabbage displayed high levels of resistance to most of the antibiotics utilised compared to *E. coli* isolated from cabbage farm. In another study, Chinese cabbage, isolates (100%) showed high resistance levels to penicillin but varying resistant characteristics for tetracycline, ampicillin, and amoxicillin, with

resistance rates of 31.3% (30/96), 31.3% (30/96), and 31.3% (30/96), respectively (Datta *et al*., 2024). Similarly, other studies have reported higher rates of *E. coli* contamination and resistance in fresh salad vegetables in in Pakistan (32.4%) and Nigeria (24.4%) (Shah *et al*., 2015; Igbinosa *et al*., 2024). The presence of *E. coli* in food indicates possible contamination from soil or manure or water or livestock faeces or either directly or indirectly from farm personnel due to poor hygiene. The frequent isolation of *S. aureus* in vegetables has been noted in previous studies (Seo et al., 2010; Jia, et al., 2024). In the current study, all *S. aureus* (100%) isolates were susceptible to vancomycin. This is beneficial because vancomycin is the recommended antibiotic for treating MRSA infections, and the appearance of VRSA in vegetables is a concern (Jia *et al*. 2024).

In this study, *B. cepacia* isolates (15.8%) from commercially available spinach showed resistance to most tested antibiotics. Similarly, in a study conducted in USA, all *B. cepacia* isolates were resistant to ceftriaxone, and five isolates were resistant to cefepime, colistin-sulfate, and erythromycin (Karumathil, et al., 2016). *It is reported that B. cepacia* raises important ecological issues, including the evolution of pathogenicity and multi-resistant environmental bacteria through horizontal gene transfer and it is now considered an opportunist human pathogen causing respiratory and urinary tract infection including bacteraemia in humans (Sousa *et al*., 2011).

Haemolytica spp. are bacterial pathogen most frequently isolated from cattle and the prevalence of antimicrobial resistance in this pathogen has been increasing (Snyder *et al*., 2017). In this study, *A. haemolyticus* isolates from retail spinach and cabbage also displayed 100% resistance to tetracycline, ampicillin, ciprofloxacin, erythromycin including penicillin. There are no similar cases in the literature to support this hypothesis.

Serratia marcescens typically exhibits antibiotic resistance through the production of the enzymes lipase, gelatinase, and deoxyribonuclease (DNase) (Zivkovic *et al*., 2023). All *S. marcescens* isolates from farm spinach and cabbage isolates also showed 100% MDR to five antibiotics such as chloramphenicol, tetracycline, ciprofloxacin, erythromycin, and penicillin. A recent systematic review found that *S. marcescens* is resistant to a wide range of antibiotics, this included penicillin, cephalosporin, tetracycline, macrolide, nitrofurantoin, and colistin and pointed out that carbapenem should be included in the treatment of *S. marcescens* infections (Cosimato *et al*., 2024). According to the literature*, S. marcescens* is resistant to a variety of antibiotics, including tetracycline, penicillin, macrolide, nitrofurantoin, colistin and cephalosporin (Zivkovic *et al*., 2023)

In this study, *P. luteola* isolates from cabbage and spinach from both farm and retail isolates displayed high levels of resistance to most of the antibiotics utilised. According to some previous studies, *P. luteola* exhibits high resistance to trimethoprim-sulfamethoxazole, ceftriaxone, tetracycline, and ampicillin (Ahmad *et al*., 2023). *Pseudomona luteola* has been shown to be resistant to trimethoprim-sulfamethoxazole, ampicillin, tetracycline, and first and second-generation cephalosporins (Yousefi, *et al*., 2014).

The *S. sciuri* isolates from spinach and cabbage farms showed the least (5.3%) antibiotic resistance in this study. As a food-borne bacteria, *S. sciuri* spreads easily in street food markets (Yang *et al*., 2017; Makky, *et al*., 2023) and causes spoilage of dairy products, fruits and vegetables (Makky, *et al*., 2023). To date, over 100 *Staphylococcus sciuri* isolates have been characterized, and it has been found that they all carried a genetic element (*S. sciuri mecA*) that is closely related to the *mecA* gene of methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Couto *et al.,* 2000).

The majority of the multidrug resistance (MDR) isolates were reported to be resistant to 3 and 7 antibiotics phenotype. In this study, all *S. aureus* isolates showed multidrug resistant to at least six classes of antibiotics such as tetracycline (n =5; 100%), gentamycin (100%), ampicillin (100%), ciprofloxacin (n =3; 60%), erythromycin (n = 4; 80%) and penicillin (100%). Chew *et al*. (2023) reported that multidrug resistance for *S. aureus* strains was due to their ability to produce slime and biofilms which harboured SCC*mec* type IV, and belonged to different *spa* types (t022, t032, and t548), with varying profiles for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and virulence genes. The whole genome sequencing (WGS) data for the MDR SauR23 and SauR91 strains revealed that most of the antimicrobial resistance genes were present in the chromosomes, including *blaZ*, *mecA*, *norA*, *lmrS*, and *sdrM*, with only the *ermC* gene found in a small (<3 kb) plasmid. Another study highlighted certain processes including phosphorylation, glycosylation, acetylation whose inactivation or chemical transformation are reported as the major cause of the multidrug resistance in *S. aureus* (Murkherjee *et al*., 2021).

In this study, *B. cepacia* isolated showed multidrug resistance to three classes of antibiotics such as ciprofloxacin (n =3; 50%), tetracycline (n = 4; 67%) and lastly gentamycin with (n = 4; 83%). Tseng *et al*. (2014) showed that most ceftazidime-resistant (17/18, 94.4%) and chloramphenicol-resistant (16/22, 72.7%) *B. cepacia* isolates exhibited efflux pump activity and it

was identified in both trimethoprim/sulfamethoxazole-resistant concluding that the presence of efflux pump activity was significantly correlated with multidrug resistance to any of the following antimicrobial agents: ceftazidime, meropenem, and chloramphenicol (all $p<0.05$ according to the Mann-Whitney U test).

In this study, *P. luteola* isolates showed multidrug resistant to four classes of antibiotics including tetracycline and penicillin with 100% for both, while chloramphenicol, and ampicillin comprised of 67% and 83%, respectively. Von Wintersdorff *et al*. (2016) indicated that a large portion of antibiotic resistance such as tetracycline, gentamicin, chloramphenicol, and streptomycin are determined by plasmids (R plasmids) which is facilitated by the conjugative machinery which is encoded either by genes on autonomously replicating plasmids or by integrative conjugative elements in the chromosome. Mobile genetic elements are an important component of the genetic structure of *Pseudomonas*. In another study, it was demonstrated that *CbrA* and *CbrB* play an important role in various virulence-related processes of the pathogen, including multidrug resistance for *Pseudomona* but in this case *P. aeruginosa*. The study pointed out that microarray analysis revealed that under swarming conditions, *CbrA* regulated the expression of many genes, including *phoPQ*, *pmrAB*, *arnBCADTEF*, *dnaK*, and *pvdQ* consistent with the antibiotic resistance (Yeung *et al*., 2011).

In this study, *E. coli* isolates displayed multidrug resistance to all antibiotic classes including chloramphenicol, tetracycline, gentamycin, and erythromycin with 100% while ampicillin and ceftazidime displayed 86%, with penicillin displaying 71%. *Escherichia coli* bacteria can capture foreign genes through mobile elements such as mobile plasmids, integrons, mobile transposons, integrative and conjugative elements (ICE), and integrate and regulate ARGs, thereby obtaining the horizontal transfer of antimicrobial resistance making the resistance more prone to horizontal transmission (Wu *et al*., 2019). Another study reported the detection rates of *E. coli* isolates from high-to-low to be as follows: tetracycline resistance genes were *tet*(W) (98.5%), *tet*(A) (84.8%), *tet*(B) (12.1%), and *tet*(M) (6.0%); aminoglycoside resistance genes were *aph*A1 (100%) and *aad*D (36.4%), *aac*(2')-IC (7.6%); macrolide resistance gene was *vag*B (15.1%); fluoroquinolone resistance genes were *qnr*A (50.0%), *qnr*S (16.7%), and *qnr*D (3.1%); chloramphenicol resistance genes were *flo*R (78.8%) and *fex*A (9.1%); β-lactam resistance genes were bla_{TEM} with 90.9% resulting in multidrug resistance (Zhu *et al*., 2023).

In this study, *S. marcescens* isolates showed a 100% multidrug resistance to five classes of antibiotic classes such as chloramphenicol, tetracycline, ciprofloxacin, erythromycin, including penicillin. Thompson *et al*. (2007) indicated that the only characterized *S. marcescens* tetracycline resistance determinant is the Tet (B) protein expressed by the conjugative plasmid R478 through plasmid horizontal gene transfer, or as result of gene mutations. Another study reported that sequence analysis showed the presence of Ybh ABC-type transport system in the SM03 (homolog found in strains PH1a, H1q, VGH107 and W2.3 with 99% respectively); this operon involved in multidrug resistance could have been the result of gene transfer in *S. marcescens* SM03 genome sequence from *E. coli* (Srinivasan *et al.,* 2019). Each class of plasmid of various pathogens carries different types of drug resistance genes of which will determine the strategy or mechanisms that will be utilised i in multidrug resistance.

The isolates obtained from this study showed resistance to several antibiotics tested, with 79% of the isolates showing multidrug resistance (MDR). This result is higher than the results of previous studies in Nepal, South Africa and Switzerland, in which 56.9% (from chutney), 40.3% (from fresh vegetables) and 20.5% (from fresh produce) of isolates were MDR (Adhikari, *et al.,* 2023; Richter, *et al*., 2021; Kläui, *et al*., 2024) including another South Africa study with 64.7% MDR from commercial lettuce and spinach (Ratshilingano et al., 2022). Another study conducted in Bangladesh reported a high proportion (98.06%) of isolates with MDR from raw salad vegetables (Nipa *et al*., 2011). The presence of MDR in isolates from fresh vegetables must be taken seriously as they act as a reservoir and can potentially transmit resistant bacteria to humans.

The utilisation of antibiotics in animal husbandry and the simultaneous spread of antibiotic-resistant bacteria in manure mean that these bacteria can persist in agricultural soils (Sarmah et al., 2006; Karumathil et al., 2016). Soil can be considered a large reservoir of antibiotic resistance determinants since it is present in all plants, small animals, fungi, protists and soil bacteria (Monier et al., 2011; Nkhebenyane et al., 2024). In addition, cross-contamination of fruits and vegetables after harvest and horizontal gene transfer may contribute to this situation (Jiaet al., 2024). The recently published review has shown that it is difficult to disinfect contaminated vegetables, especially when the bacteria have established themselves in the plant tissue (Nkhebenyane et al., 2024). This study has several notable limitations, including a small sample size, a limited variety of vegetables, and the absence of screening for antibiotic-resistant genes.

5.5 Conclusion

This is the first study to demonstrate antimicrobial resistance in bacteria isolated from fresh vegetables in Free State Province, South Africa. The literature depicts leafy green vegetables as a reservoir for multidrug-resistant pathogens and commonly implicated in disease outbreaks worldwide. Plant uptake and the bioaccumulation of antibiotics draw attention to the need for better food safety practices in the supply chain and the identification of sources of contamination of fresh produce with antibiotic-resistant bacteria as a public health concern. The isolates from this study demonstrated high resistance characteristics to multiple antibiotic classes, including β-lactams, chloramphenicol, tetracycline, aminoglycosides, and macrolides, mostly from farm origin. To ensure safe fresh vegetable production and distribution, minimising antibiotic-resistant bacteria risk is crucial. Additionally, regulated parties must oversee and promote safe handling practices throughout the production chain. The AR profile comparisons across vegetables can guide future mitigation strategies.

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CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The overall goal of this study was to identify and characterise bacterial species from both farms and retails spheres to gain knowledge and identify the type of microorganisms found on leafy green vegetables in addition to determining what consumers purchase and consume from these small-scale farms. Identification of the proliferation and succession of opportunistic pathogens and their aetiology on leafy green vegetables at retails and how these sources of contamination can be mitigated was an important aspect of this study. Moreover, the study also revealed the occurrence of resistance and susceptibility from antibiotics utilised on leafy green samples obtained from farms and retails and the need for appropriate control measures to mitigate antibiotic consumption. Furthermore, the results demonstrate that some opportunistic pathogen communities in fresh leafy vegetables are diverse and can be a health risk to consumers leading to food poisoning and foodborne illnesses which could potentially lead to death.. The pathogens found on different fresh leafy greens vegetables sold at the small-scale farms and retails in different regions in the Free State were not significantly different. Thus, small-scale farmers and retailers need strict regulations, codes and regular training regarding contamination including education and awareness. The study found that small-scale farmers face several challenges, including the government sector's inconsistent assistance, which forces farmers to compromise and adopt different farming practices. For instance, using broilers and livestock farm-made manure instead of fortified and processed fertiliser for vegetables. There is a shortage of personnel who strictly work with vegetables while others work strictly with livestock. Different packaging stations, like bigger areas for storing vegetables, are required. It is crucial to identify the origins of pathogenic bacteria, their features, and the characteristics of antibiotic residues to suggest appropriate control measures to prevent contamination and antibiotic residue consumption in humans.

6.2 Synthesis

The major empirical findings of this study were summarized within the specific chapters. This current section will serve to synthesize the findings to explain the three main objectives of this study.

6.2.1 Enumeration and identification of microbiota species isolated from spinach and cabbage at small-scale farm level by analysing spinach, cabbage and storing crates

During minimal processing, safety may be compromised due to stakeholders such as retails and markets demands for large purchase compromising the process safety which in turn will presents a challenge to consumer health. The goal of food hygiene and food safety is to prevent any hazardous event that may cause illness to human health through the consumption. The foodborne risk is directly related to the prevalence and concentration of pathogenic bacteria. The agri-food supply chain including distributors must comply with food safety management systems, risk management and hygiene guides. Compliance with food safety standards is a prerequisite for safe food.

Firstly, the results point towards the presence of abundant opportunistic pathogens from both personnel and processing facilities which indicates a lack of hygiene or poor hygiene practices. The United States Food and Drug Administration recommends all growers conduct appropriate risk assessments and, where necessary, implement risk mitigation strategies. Growers should also be aware of and take into consideration adjacent land use practices, particularly as they relate to the presence of livestock and the interface between farmland, rangeland, and other agricultural areas (USFDA, 2021). While this study sheds light on small-scale and retail microbial characterisation and microbial hazards throughout the farm-to-fork continuum, further research could address the gap or deficit of knowledge regarding consumer perceptions and practices at home, including knowledge of safe perishable fresh produce handling and specifically on microorganisms identified by this study. This study showed that both cabbage and spinach microbiomes are highly diverse and fluctuate vigorously due to extrinsic and intrinsic parameters which include both storage and refrigeration temperatures. Further research is also necessary to directly correlate the composition and changes in strategies including diversity behaviour with regard to how opportunistic pathogens function, to fully comprehend the succession and survival until purchased and to determine which parameters stress the microorganisms and when and how to interfere with survival strategies during such times. It is recommended that processors ensure that intervention methods are utilised by their growers and distributors and that these methods are adopted from hygiene practice regulations and codes for fresh leafy green vegetables for farms and retails.

6.2.2 Enumeration and identification of microbiota species isolated from packaged spinach and cabbage at retail

Secondly, the hygiene of leafy greens at a point of sale can be challenging as consumers pick and pay for what they prefer, this refers to both farm and retail establishments. The method of picking the best can introduce or add bacteria to a product and this is where regular hygiene practices should always be implemented in frequent intervals to reduce contamination load. Both farmers and retailers require quantifiable knowledge regarding all these best practices of hygiene and safety regarding contamination and succession of pathogens, including risk factors along the food and supply chain.

Millions of tons of vegetables are produced by farmers in South Africa and are exported to various destinations, including retails. Additionally, farm-to-farm exchange is another crucial aspect that requires attention as it contributes to cross-contamination of produce which might lead to foodborne outbreaks and have a potential economic impact. Another essential required feature is a precise guideline, specifically for guiding small-scale farms with regard to supply chain, microbial hazards, infrastructure, and the type of commodity produced, as many small growers share farms with livestock. Farmers who sell fresh produce to retails, particularly leafy green vegetables, need regular and effective agricultural practice audits and effective sanitation. The findings also support the following conclusions:

- Small-scale farmers and personnel require environmental health education, including food safety, to ensure safer production.
- The study also found that many small-scale farms lack information regarding GAPs which could be a barrier to good food safety practices.
- Overproduction to reach the end goal or status for the day leads to compromising food safety, especially if personnel must work overtime to complete the work.
- Lack of supervision also adds to compromising food safety.
- Negligence of food safety during minimal processing.

6.2.3 Antimicrobial susceptibility profile on pathogens isolated from spinach and cabbage against antibiotics

Important role of antibiotics cannot be overestimated as human and animal health relies heavily on them for treatment of infectious diseases. The study illuminates gram-negative and gram-positive bacteria and their involvement in the transfer of bacteria and genes between humans

and crops. As a result, the misuse of contaminants such as manure and others can lead to antibiotic residues on leafy green vegetables and the environment as well as the development of antibiotic resistance. Necessary control measures must be taken to mitigate contamination from small-scale farms to avoid the progression of antibiotic resistance to the next sphere and to avoid antibiotic consumption.

6.3 Recommendations

Risks and hazards are mostly caused by negligence in food production facilities. The minimization of food hazards in a processing facility is always essential. The following can be adopted for the minimization of microbial hazards and the promotion of food safety:

- Guidance for Industry; Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables
- Good agricultural practices (GAPs) and Good manufacturing practices (GMPs)
- Hazard analysis and critical control points (HACCP)

An essential requirement is that the implementation of an effective food system must be accompanied by continuous routine monitoring. Regardless of the size of the farm, if it produces enough produce that can be sold to marketplaces, food safety education and risk awareness are required to reduce unforeseeable circumstances in practical settings. The guides specify the parameters for raw commodities, strict monitoring and risk assessment based on a qualitative assessment which includes all the critical points. This is essential to minimise microbial hazardsand also to avoid potential consequences. Without accountability to ensure a smooth process, there will be no progress, and everything will be subjected to failure.

Farms should be regularly monitored with frequent surveillance by either food safety representative at the farm, once in a while by either Environmental health practitioner or food scientist to enhance good safety safety outcomes. Good agricultural practise certificate should also be provided to farms with good performance.

In-store customer surveys are helpful regarding services and food safety, specifically how the consumer should handle food at home, although conducting these surveys may be challenging. Staff training is essential and data safety sheets should always be in visible places for consumers and staff. Managers should elect at least one personnel member who will be responsible for in-depth monitoring of food safety. Self-audit meetings to maintain high standards of food safety are

also required. In-depth inspection should also include an inspection of the delivery vehicle when possible. This is to verify that the vehicle and incoming products are free of signs of cross-contamination. The temperature where leafy green vegetables are stored should be monitored regularly, including calibration verification of the refrigerators or any other utilised tools. The above mentioned methods are useful to enhance food safety. Other useful guidelines regarding microbial hazards and microbial food safety guidance for the industry include the following:

- Quality assurance schemes and code of practice for fresh leafy greens vegetables
- Guidance for industry: guide to minimize microbial food safety hazards for fresh fruits and vegetables
- Microbiological risk assessment series: Microbiological hazards in fresh fruits and vegetables, meeting report
- Standards regarding food safety and food hygiene of regulated agricultural food products of plant origin destined for export
- Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale
- A guide to the food safety standards and microbiological guidelines for food

Codex Alimentarius guides are helpful in risk assessment of growers and retail establishments. They are implemented for assurance and good practice and are required for risk assessment.

It is reported that foodborne diseases are under-reported and poorly investigated in South Africa due to poor surveillance systems and poor integrated management (Shonhiwa *et al.,* 2019). Shonhiwa *et al*. (2019) reviewed the foodborne disease outbreaks and reported to the outbreak response unit at the NICD from 2013 to 2017, They concluded that there has not been a noticeable improvement in the notification and investigation of foodborne disease outbreaks since then and that the notification and investigation process remains the same. Lack of under-reporting from healthcare services, delayed response to such outbreaks, lack of appropriate clinical and environmental sample collection and testing, and the variable scope of testing performed at different laboratories contribute to the under-reporting of foodborne diseases (Ramalwa *et al.,* 2020). Local farmers rely heavily on food safety awareness and education since these are critical components that help reduce food contamination and increase knowledge of microbial infections.

Several studies in South Africa show that investigations of foodborne outbreaks, including preventative measures by industries that supply food and the government's efforts, are inadequate. Foodborne disease (FBD) outbreaks are a common occurrence that is either not investigated or poorly investigated and according to anecdote evidence gathered, it was found that this is due to the non-uniformity of environmental health practices in South Africa (Mbonane *et al*., 2020). The study further elaborated that the results indicated that there are gaps and challenges in available knowledge, while the practices were not consistent amongst environmental health practitioners. However, the attitude of Environmental Health Practitioners was positive concerning their role in foodborne disease outbreak investigations. Surveillance data from the notification system is suboptimal and limited and does not provide adequate information to guide public health action and inform policy (Ntshoe *et al*., 2021). The study concluded that efforts should be made to set up systems and develop applications that can improve data collection and quality of foodborne disease outbreak investigations.

6.4 Future Research

i. The recent chitosan studies related to the treatment and maintenance of the quality of post-harvest produce by regulating the elicitation processes and limiting bacterial growth have not been given much attention, particularly concerning perishable leafy produce compared to fruits in South Africa. This presents an interesting prospective research study.

ii. Chitosan is described as a naturally occurring compound often referred to as a polymer and is commercially produced from seafood shells (Sharif *et al*., 2018). Betchem, Johnson and Wang (2019) reviewed the application of chitosan in the control of post-harvest diseases and concluded that it provides an alternative control for pathogenic microorganisms.

iii. Another study investigated the effects of this chitosan compound residue on the growth and post-harvest quality of lettuce and concluded that the treatment may be the best all-year-round supplement to maximise yields and post-harvest quality (Muymas *et al.*, 2015).

In conclusion, strict measures, monitoring, and good agricultural practices have to be in place to avoid outbreaks. Knowledge of tracing investigation procedures is essential regarding foodborne outbreaks. The aim is to minimise as much microbial contamination and microbial proliferation as possible to prevent the proliferation and progression of pathogens. Quality is an important aspect of leafy green vegetables or any final product that is consumed.

6.5 References

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