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.

5.6 References

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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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Do not miss out on something that could be great just because it could also be difficult.