



UNIVERSITY of the
WESTERN CAPE

Identification and characterisation of potential virulence genes of *Salmonella* from rooibos tea

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Magister Scientiae

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DECLARATION

I, the undersigned, declare that **'Identification and characterization of potential virulence genes of *Salmonella* from rooibos tea'** is my own work and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Miss Nashleen Johannes

Signature: N. Johannes

Date: 2016-03-17



This thesis represents a compilation of articles where each chapter is an individual entity and repetition between chapters has been unavoidable.

The format of this thesis follows the *Journal of Applied Microbiology*.

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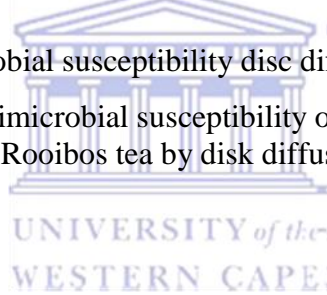
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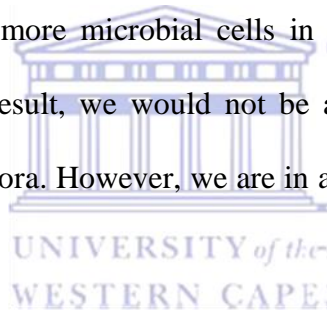
ABR	Antibiotic resistance
ARB	Antibiotic resistant bacteria
API 20E	Analytical profile index
BPW	Buffered peptone water
CECs	Contaminants of emerging concern
EHIA	European Herbal Infusion Association
HACCP	Hazard Analysis and Critical Control Point
h.p.i	Hours post infection
ISO	International organisation for standardization
MDR	Multidrug resistance
MH	Muller-Hinton agar
NCCLS	Formerly known as the “National Committee on Clinical Laboratory Standards,” NCCLS is an educational organisation that develops updated consensus standards and guidelines for the healthcare community on an annual basis.
ORFs	Open reading frames
PAI	Pathogenicity islands
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RV	Rappaport vassiliadis broth
SCV	Salmonella-containing vacuole
SIF	Salmonella-induced filaments
SKIP	SifA kinesin interacting protein
SPIs	Salmonella pathogenicity islands
T3SS	Type III secretion system
TSA	Tryptone-based soy agar
TSB	Tryptone-based soy broth
WHO	World Health Organization
XLD	Xylose lysine desoxycholate agar

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

In our daily regime, moments subsequently to birth, we continuously respire, eat, and come into physical contact with masses of microbes, until our bodies are eventually consumed by it at death (Jones 1996). Most microbes are harmless evanescence ('that which appears momentarily and fades away'). Others turn out to be part of the normal flora that we retain throughout our life. Several of the billions of bacteria, fungi, and protozoa that are inhabitants of our normal flora have never been cultured before and, mathematically, we possess more microbial cells in comparison to our own cells (Kessel *et al.* 2001). As a result, we would not be able to live long without our perpetual normal microbial flora. However, we are in a subtle equilibrium with these microorganisms.



Undeniably, our immune system is largely a mirror image of our requirements to endure the ambush of a cosmos of microbes that surrounds us. Therefore, if the inherent immunity is being compromised, the harmless microbes can rapidly become detrimental to our survival. Additionally, amongst the microbes that we come across daily, there are the ones whose survival hinges on its capability to cause cellular destruction to their host (Mead *et al.* 1999). It is thus this group of microbes, called the pathogens, which are predominantly fatal to our existence.

A pathogen must come into contact with a host, discover an exclusive niche, by-pass competitive microbes, host defence barricades and proliferate adequately to launch itself, or to be transferred to a new susceptible host (Jones 1996). These bacterial

pathogens impair their host by intoxication or impeding on the integrity of cells. In several cases, the impairment is not that severe, but susceptible hosts are likely to suffer from blatant disease, or it could even result in death (Jones 1996).

Salmonella is a foodborne pathogen of great significance in human illnesses globally (Nascimento *et al.* 2015; Saikia *et al.* 2015). Infections by *Salmonella enterica* are a significant public health concern around the world. *Salmonella enterica* is a leading cause of infections in many low- and middle-income countries (Saikia *et al.* 2015). The growing centralism and industrial development of our food chain supply have heightened the distribution of these robust microorganisms. It causes gastroenteritis, bacteraemia, and succeeding focal infection (Saikia *et al.* 2015). *Salmonellae* have a widespread range of hosts and they are also relatively linked with agricultural products (Mead *et al.* 1999).

Typhoid fever was estimated to cause about 21.7 million illnesses in 2000, and 216 000 deaths; paratyphoid fever caused about 5.4 million illnesses (Crump *et al.* 2004). Typhoid and paratyphoid fevers were estimated to account for 12.2 million disability-adjusted life years (Murray *et al.* 2012) and 190 200 deaths (Lozano *et al.* 2012); these illnesses were included in the Global Burden of Disease 2010 (GBD, 2010) project. The International Vaccine Institute approximated that there were 11.9 million typhoid fever illnesses and 129 000 deaths in low- and middle-income countries in 2010 (Mogasale *et al.* 2014). Typhoid fever appears to have become more common in sub-Saharan African countries as well (Breiman *et al.* 2012). *Salmonella* related illnesses are spread mainly through water, or food contaminated with faeces. The risk for infection is high in low- and middle-income countries where typhoidal *Salmonella* is

endemic and that have inadequate sanitation and lack of access to safe food and water (Crump *et al.* 2004).

The contamination of food from animal origin with *Salmonella* is well documented and has been widely studied (Alemayehu 2014; Batista *et al.* 2014; Erganis *et al.* 2014; Uzzau *et al.* 2005). The way in which these foods are contaminated is clearly understood and difficult to avoid because of the wide distribution of *Salmonella* among animals of all kinds, as well as contamination from the environment. However, fresh fruit and vegetables are now increasingly recognised as sources of *Salmonella* outbreaks (Jacobsen and Bech 2012; Lynch *et al.* 2009). During the last three decades, the number of documented infections associated with the consumption of fresh produce has increased (Behraves *et al.* 2011; Nascimento *et al.* 2010; Saikia *et al.* 2015).

In 1987, Swanepoel revealed one of the first studies of *Salmonella* in Rooibos tea. Rooibos (*Aspalathus linearis*) is known for its richness of flavonoids and its lack of physiologically active chemicals, such as caffeine (Gouws *et al.* 2014). It also does not have the harshness of common tea because it is low in tannins (Beltran-Debyn *et al.* 2011; Joubert *et al.* 2008). These properties make it a preferred substitute for common tea. Regardless of the prevalence of *Salmonella* in nature, low-moisture foods, such as dried herbs and spices, are not usually considered as high risk with respect to salmonellosis because the low water activity is a barrier for bacterial growth (Keller *et al.* 2015). However, several worldwide *Salmonella* outbreaks have been associated to low-moisture foods, such as nuts, peanut butter, paprika-flavored potato chips, black and red pepper (Behraves *et al.* 2011; Joubert and de Beer 2011). And there have been at least two documented outbreaks of salmonellosis associated with herbal teas

(Koch *et al.* 2005; Llic *et al.* 2010), not including Rooibos tea. Symptoms usually appear 12-36 hours after the ingestion of raw contaminated food. Symptoms include acute enterocolitis with sudden onset of headache, abdominal pain, diarrhoea, nausea, vomiting and fever (Adak *et al.* 2002; Crump *et al.* 2015). Dehydration may occur predominantly amongst susceptible populations, i.e. infants, the immuno-compromised and the elderly. The illness usually lasts 2-5 days and is normally self limiting (Lhocine *et al.* 2015). Complications can lead to systemic infections and the infective dose can differ depending on the strain, the immuno-competence of the individual and the nature of the food (Crump *et al.* 2015; Miller and Pegues 2000).

Salmonella has been recognised as a potential threat for the agricultural industry (ICMSF 2011); however, there are not many South African survey reports, and little in terms of data published internationally, on contamination of *Salmonella* or other *Enterobacteriaceae* in end products of herbal teas. The popularity of rooibos has now for many years been associated with its health related benefits only based on nutrition, whereas the microbial status of this product was often rendered unimportant to the consumer. Perhaps the focus has been so intense that the microbial factor has not been given chance to rise. This however seems unreal as life at present is consumed with the effects of microorganisms in our food products, but due to the lack of research in rooibos, not much could be concluded. It is therefore time that research surrounding this world wide consumed product is resurrected.

The aim of this study was to investigate the presence of *Salmonella* in the tea processing environment, to identify and detect potential virulence genes isolated from *Salmonella* in the tea, and to determine the antibiotic resistance levels of *Salmonella* isolated from fermented Rooibos.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Salmonella is a facultative intracellular pathogen which, depending on the serotype and host, can cause diseases ranging from gastroenteritis to typhoid fever (Newell *et al.* 2010; Tsai *et al.* 2015). They can invade macrophages, dendritic and epithelial cells (Que *et al.* 2013; Siriken 2013; Wang *et al.* 2015). Despite the preventative control measures that have already been put into place, *Salmonella* infection that arises from contaminated food continues to be an immense problem, with millions of cases occurring annually throughout the world (Crump *et al.* 2015). Detection of *Salmonella* before contaminated food is consumed is therefore an essential feature of safeguarding public health (Jacobsen and Bech 2012). Surveillance of *Salmonella* in all the different stages of feed-food chain is an important element in the exploration of epidemiology of foodborne salmonellosis, and in the development and implementation of efficient *Salmonella* control strategies (Gouws *et al.* 2014).

Salmonella has two species, namely *Salmonella bongori* and *Salmonella enterica*. The latter includes six subspecies: *S. enterica* subsp. *houtenae*, *arizonae*, *diarizonae*, *enterica*, *salamae* and *indica* (Grimont and Weill 2007; Prakash *et al.* 2015). *S. enterica* subsp. *enterica* includes the human pathogenic *Salmonella*, consisting of more than 2 000 serovars, including Typhimurium (of which more than 500 phage types are recognised), Typhi, Dublin, Enteritides, Montevideo, Newport etc. (Crump *et al.* 2015; Prakash *et al.* 2015). *Salmonella* spp. are resilient bacteria and can adapt to extreme environmental conditions (Prakash *et al.* 2015). Overall, *Salmonella* spp.

requires several genes for full virulence, as it reflects a complex set of interactions within its host (Lhocine *et al.* 2015). The majority of these genes are found in ‘*Salmonella* pathogenicity islands’ (SPIs) clustered on the chromosome in distinct regions (Karunasagar *et al.* 2012; Que *et al.* 2013).

Most knowledge about SPIs and other *Salmonella* virulence genes is based on observations with *Salmonella enterica* ser. Typhimurium, and thus far only a fraction of these genes have been identified in other serovars, including serovar Enteritidis (Hu *et al.* 2008; Lhocine *et al.* 2015). *S. enterica* ser. Typhimurium possess at least five such SPIs, which confer specific virulence traits and are thought to have been acquired by horizontal gene transfer from other organisms (Siriken 2013). Moreover, the virulence genes responsible for invasion, survival, and extra intestinal spread are located in *Salmonella* SPIs (Lhocine *et al.* 2015; Que *et al.* 2013). Some of these SPIs are conserved throughout the *Salmonella* genus, and some of them are specific for certain serovars (Siriken 2013). There are differences between *Salmonella* serotypes in terms of adaptation to host cell, virulence factors and the resulting infection (Karunasagar *et al.* 2012). Virulence genes that are involved in the intestinal phase of infection are located in SPI-1 and SPI-2; the remaining SPIs are required for intracellular survival, fimbrial expression, magnesium and iron uptake, multiple antibiotic resistances and the development of systemic infections (Almeida *et al.* 2013; Campioni *et al.* 2012; Siriken 2013).

Important virulence factors of *Salmonella* contributing to adherence, invasion of and replication within the vertebrate host cells are encoded within SPIs. Thus far, 17 SPIs have been identified (Deekshit *et al.* 2013; Karunasagar *et al.* 2012). Five SPIs (SPI1-5) have been shown to be conserved amongst *Salmonella* spp., (Karunasagar *et al.*

2012); the distribution and structural organisation of the other SPIs reflect functional features of specific serovars and might be responsible for host-specificity (Siriken 2013). More than 30 SPI-1 proteins have been identified, comprising structural components of a type III secretion system (T3SS) (Lhocine *et al.* 2015). T3SS, (Injectisome or Injectosome) is a protein appendage found in several Gram-negative bacteria such as *Salmonella* (Wee and Hughes 2015). In pathogenic bacteria, the needle-like arrangement is utilised as a sensory probe to identify the presence of eukaryotic organisms, and it secretes proteins that assist the bacteria to infect them (Lhocine *et al.* 2015). The effector proteins are secreted directly from the bacterial cell into the eukaryotic host cell (Wee and Hughes 2015), where they apply several effects that aids the pathogen to survive and to escape an immune response (Troxell *et al.* 2015). T3SS is one of the most intricate secretion systems and its configuration shows many similarities with bacterial flagella, which are elongated, rigid, extracellular structures used for motility (Lhocine *et al.* 2015; Wee and Hughes 2015). Some of these bacteria possess a T3SS, flagella and are motile, such as *Salmonella* (Troxell *et al.* 2015).

Virulent chromosomal gene, *invA* is recognised as an international standard for detection of *Salmonella* genus (Mezal *et al.* 2013). It is a structural component critical for T3SS function encoded within SPI-1 (Troxell *et al.* 2015). This gene is essential for full virulence in *Salmonella* and is thought to trigger the internalisation required for invasion of deeper tissue (Wang *et al.* 2015). *Salmonella* virulence depends on an ability to invade host cells, which is in turn dependent on a T3SS encoded in SPI-1 (Lhocine *et al.* 2015; Troxell *et al.* 2015). Several protein targets of the SPI-1-encoded secretion system are translocated into host cells, where they subvert cellular processes

that contribute to bacterial invasion, actin rearrangement, membrane ruffling and other aspects of virulence (Lhocine *et al.* 2015; Troxell *et al.* 2015).

There are several methods for detecting *Salmonella*; however, due to the low infective dose of *Salmonella*, methods for its detection are needed to prove the presence of one cell in a defined food sample (Gouws *et al.* 2014). Cultural methods for *Salmonella* isolation involve a number of selective pre-enrichments, followed by selective enrichment and plating on selective and diagnostic agars (Prakash *et al.* 2015). The standard method for the isolation of *Salmonella* has been developed and evaluated in relation to the analysis of food and feed. As the matrix has a substantial influence on the performance of the method due to levels of competitive flora, methods developed for analysis of food cannot be assumed to be appropriate for analysis of materials from primary animal production, e.g. faeces. In recent years, efforts have been made to develop and evaluate a standard bacteriological method for the isolation of *Salmonella* from samples from primary animal production (EFSA 2006). Presumptive colonies are confirmed biochemically and serologically; the complete test involves three to four days to attain a negative result and up to seven days to get a confirmed positive result (Anon 1995).

A number of rapid methods for the detection of *Salmonella* in foods have been developed, including electrical techniques, immunoassays and nucleic acid probe analyses (Prakash *et al.* 2015). The serological tests developed for *Salmonella* spp. are based on the detection of antibodies against the somatic O-antigens (EFSA 2006). Most tests are indirect enzyme-linked immunosorbent assays, based on Lipo-Poly-Saccharide antigens, which is a part of the cell wall of many bacteria but it is very

specific for each kind of bacteria. However, there are still problems with their sensitivity and specificity (EFSA 2006).

Polymerase chain reaction (PCR) has been developed and used within research and industry. This relates specifically to environmental, clinical and food products. The amplification and detection of specific DNA sequences unique to microorganisms allow for PCR to rapidly increase the speed, sensitivity and accuracy for bacterial detection (Troxell *et al.* 2015). Species-specific PCR has shown to be a rapid and reliable molecular method to detect a bacterial strain without culturing the colonies (Mezal *et al.* 2013). Samples from various types of environments such as food and environmental samples do however allow for more inhibitors than compared to already isolated colonies and extracted DNA.

Several PCR assays have been developed by targeting various *Salmonella* genes (Mezal *et al.* 2013). This method has been used to detect *Salmonella* genus or serovars by using a variety of genes including: SPI-1 encoded gene such as virulent chromosomal gene, *invA* (Troxell *et al.* 2015). The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application (Arnold *et al.* 2004; Barthel *et al.* 2003; Bulte and Jakob 1995; Chiu and Ou 1996; Cocolin *et al.* 1998; Cocolin and Comi 1998; Malorny *et al.* 2003; Ochman and Groisman 1996; Pathmanathan *et al.* 2003; Perelle *et al.* 2004; Raffatellu *et al.* 2005; Rahn *et al.* 1992; Scholz *et al.* 2001; Troxell *et al.* 2015). This gene is recognised as an international standard for detection of the *Salmonella* genus (Lhocine *et al.* 2015; Troxell *et al.* 2015). The secreted effectors critical for the efficient invasion of intestinal epithelial cells within SPI-1 (Hur and Lee 2011; Raffatellu *et al.* 2005), and has also been explored as a suitable PCR target as well as

genes located in SPI-2 (Shah *et al.* 2011). Genetically, SPI-2 encodes 31 virulence genes. These 31 virulent SPI-2 associated genes are organised in at least four operons (Almeida *et al.* 2013; Campioni *et al.* 2012; Hur and Lee 2011; Miao and Miller 2000; van der Heijden *et al.* 2015).

Real-time Polymerase Chain Reaction (real-time PCR) could also be used to detect *Salmonella* and it has the ability to monitor the progress of the PCR as it occurs (i.e. in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR (Rodriguez-Lazaro *et al.* 2003).. This completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. The main advantages of real-time PCR are high sensitivity, high specificity, excellent efficiency reduced amplicon size, no post-PCR step that reduce risks of cross-contamination (Rodriguez-Lazaro *et al.* 2003).

Rooibos is a herb harvested from the plant *Aspalathus linearis*, this herb has shown to be rich in nutritional components often sought after by the consumer (Gouws *et al.* 2014) Some of these benefits include slowing the ageing process, the influence the progression of various diseases and other positive attributes. No negative effects of Rooibos have been shown from the nutritional aspect; this however may be untrue when looking from the microbial aspect of the plant and the final tea product.

According to Omogbai and Ikenebomeh (2013) the bacterial isolates that are associated with herbal teas include: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella typhimurium*, and *Escherichia coli*. Fungal isolates were *Aspergillus niger*, *Aspergillus flavus*,

Penicillium expansum, *Rhizopus stolonifer* and *Fusarium solanii*. (Omogbai and Ikenebomeh 2013). Of all the bacterial isolates, *Bacillus subtilis* showed the highest occurrence (100%) and the least was *Salmonella typhimurium* (3%). Among the fungi, *Aspergillus niger* had the highest occurrence (100%) and the least was *Rhizopus stolonifer* (10%) (Omogbai and Ikenebomeh 2013).

A few reports that demonstrates microbial contamination of herbs from several countries exist in the literature. Rizzo *et al.* (2004) showed that medicinal plants in Argentina harbored toxigenic fungi such as *A. flavus*, *A. parasiticus* and various members of the Genus *Fusarium*. Efuntoye (1999) indicated that dried medicinal plants from Nigeria herb markets contained *A. flavus*, *A. parasiticus* and *A. ochraceus*. These fungal isolates were capable of producing mycotoxins when grown on semi-synthetic media. Martins *et al.* (2001), after evaluating several medicinal herbs obtained from Portuguese markets, reported that the supplies were contaminated with moulds such as *Aspergillus* and *Fusarium spp.*

The occurrence and numbers of bacteria could be elucidated by the fact that some of these organisms like *Bacillus* produce spores which are resilient to harsh processing. Therefore they can survive for a long period of time in the product in a dormant state. Martins (2001), also isolated *Bacillus cereus* and *Clostridium perfringens* from Chamomile and other herbs. Part of the bacterial bio-burden may have originated from the personnel handling the tea materials after processing, especially if stringent good manufacturing practices (GMPs) and hygienic conditions were not followed (Omogbai and Ikenebomeh 2013). *Staphylococcus aureus* and *Staphylococcus epidermidis* are organisms which can be transmitted from humans to teas during processing as well.

Some of these microorganisms are capable of causing human infections and/or producing endotoxins which makes them health risks (Dohmae *et al.* 2008).

2.2 *SALMONELLA* TAXONOMY AND SEROLOGICAL CLASSIFICATION

Salmonella, the genus, forms part of the *Enterobacteriaceae* family; they are bacilli-shaped facultative anaerobes, catalase-positive, oxidase-negative and gram-negative (Figure 2.1), which are usually motile (Andino and Hanning 2015). This pathogen is non-spore forming with a size of $0.7\text{-}1.5\mu\text{m}\times 2.0\text{-}5.0\mu\text{m}$ and it generally produces colonies approximately 2-4mm in diameter (Crump *et al.* 2015; Le Minor 1984).

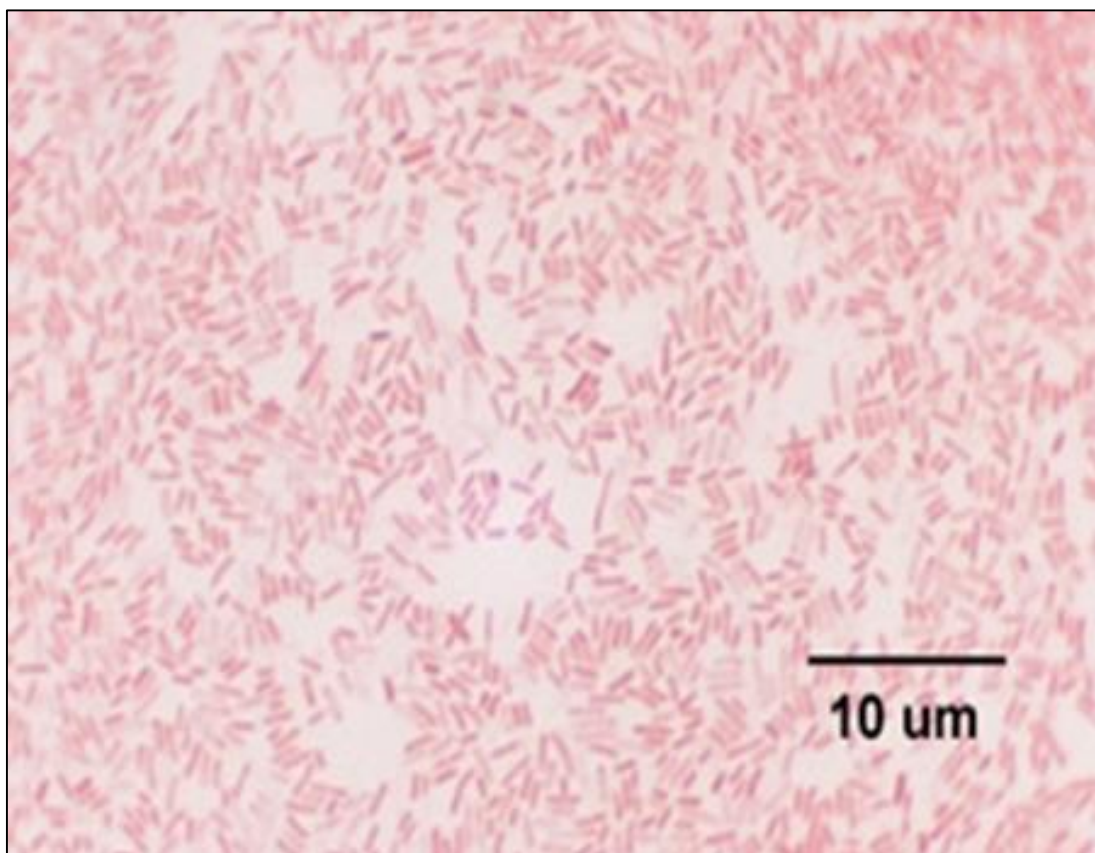


Figure 2.1. Gram stain reaction of *Salmonella*

Source: <http://faculty.ccbcmd.edu/courses/bio141/lecguid/unit3/bacpath/diseases/salmonella/gnrod.html>

Salmonella was named after the American bacteriologist D.E. Salmon, who identified *Salmonella choleraesuis* in 1884 (Salmon 1884). There are two species, namely *S.*

enterica and *S. bongori* (formerly subspecies V) that are currently recognised (Prakash *et al.* 2015). *S. enterica* has been further divided into six subspecies (subsp.), *S. enterica* subsp. *enterica* (designated subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV) and *S. enterica* subsp. *indica* (subspecies VI) (Crump *et al.* 2015; Grimont and Weill 2007; Porwollik *et al.* 2004). Subspecies I strains are generally isolated from human tissue and warm-blooded mammals (Porwollik *et al.* 2004) and the latter subspecies are commonly from cold-blooded animals and from environmental samples (Bertrand *et al.* 2008; Grimont and Weill 2007; Prakash *et al.* 2015).

Salmonella, can trace its lineage to a common ancestor with *Escherichia coli*, approximately 120-160 million years ago, roundabout the same time of the origin of mammals (Jones 1996). The first salmonellae parasitised reptiles and birds, as well as mammals (Cox 1999; Wray and Sojka 1977). Many *Salmonella* strains reveal a significant degree of host-species specificity (Wee and Hughes 2015).

Conferring to the White-Kauffmann-Le Minor scheme (Grimont and Weill 2007), subspecies are further divided into serovars/serotypes by means of serological tests with monovalent antisera (Crump *et al.* 2015; Prakash *et al.* 2015). Serotyping is extensively utilised as an epidemiological and typing methodology for *Salmonella*. Roughly 99 percent of the 2 579 now known serovars comprise this genus, falling under *S. enterica* (Andino and Hanning 2015; Prakash *et al.* 2015).

The DNA sequence alignment parallel between serovars is 96-99 percent (Edwards *et al.* 2002). Serotyping is established on antigenic changeability at lipopolysaccharide moieties (O-antigen), the phase-1 and phase-2 flagellin (H1 and H2 antigen), and some

serovars on the capsular polysaccharide (Vi-antigen). Overall, 67 diverse serological groups are demarcated by O-antigens, categorised by the White-Kauffmann-Le Minor scheme (Le Minor 1984). Serovars are titled by their antigenic formulae (O-antigen:H1-antigen:H2-antigen) succeeding the subspecies name. Serovars that belong to *Salmonella enterica* subspecies I are entitled with a name that is correlated to the geographical habitation where the serovar was initially isolated (Grimont and Weill 2007).

2.2.1 Salmonellosis

2.2.2.1 Foodborne Salmonellosis

Salmonella is a zoonotic pathogen found ubiquitously in the natural environment, causing salmonellosis (Jacobsen and Bech 2012). *Salmonella* is one of the leading causes of foodborne illnesses worldwide (Mezal *et al.* 2014) (Figure 2.2).

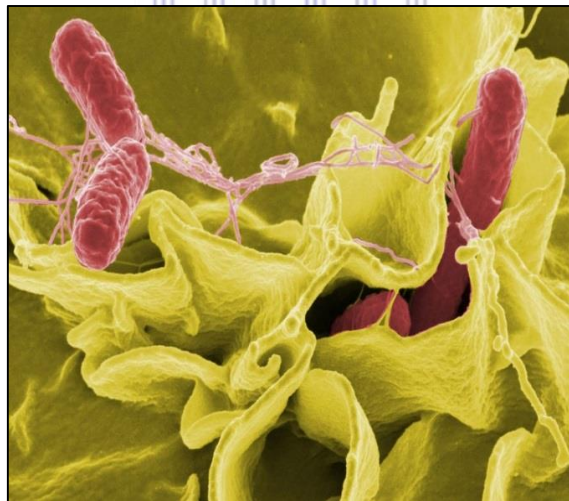


Figure 2.2. *Salmonella*, a common cause of food poisoning, invading an immune

cell Source: <http://indianapublicmedia.org/eartheats/consumers-prevent-food-borne-illnesses/>

Salmonella can be transmitted via numerous possibilities, such as the consumption of contaminated foodstuffs i.e. fresh produce, avian species, livestock, physical contact with wildlife and their environment (Mezal *et al.* 2014; Prakash *et al.* 2015), as well as cross-contamination through direct interface of foods with tainted surfaces (Denny *et al.* 2007; Jacobsen and Bech 2012). Contaminated animal feed and animals have also been recognised as imperative entrance sites in the food chain in farm livestock (Skov *et al.* 2008) and, as a result *Salmonella*, can be difficult to eradicate from food-processing environments and products (Mezal *et al.* 2014; Prakash *et al.* 2015). Worldwide, it is suggested that *Salmonella* causes 93.8 million human infections and 155 000 deaths annually (Azriel *et al.* 2015). With the rising alarm over food safety and prevention of foodborne illnesses, further studies to preclude, detect, and control foodborne pathogens is very much needed (Wong and Chen 2013).

2.2.2.2 *Salmonella in Animals*

The poultry industry is afflicted with *Salmonella* due to the migration and feeding behaviours of the carriers and the entrance of wild carriers (such as rodents) into poultry houses and processing facilities (Feng *et al.* 2012). Serovar Enteritidis, specifically, can flourish within the shell and it contaminates the egg itself by trans-ovarian transmission. This transpires prior to the formation of the shells (Batista *et al.* 2014). This is of utmost apprehension to the poultry industry, due to the overall healthy appearance of the infected avians (Feng *et al.* 2012).

Salmonella spp. are also prevalent amongst the bovine and swine meat industries (Asai *et al.* 2010; Kuo *et al.* 2014; Langridge *et al.* 2015). These meats are often contaminated due to environmental sources, animal feed contamination and infection

from parental transmission (Xie *et al.* 2015). Infected livestock can emit up to 10^8 CFU/g of faeces and further contaminate the environment and uninfected animals (Asai *et al.* 2010; Kuo *et al.* 2014; Langridge *et al.* 2015). Certain livestock are proficient in being active carriers of *Salmonella* i.e. animals that do not display clinical symptoms; however, it excretes the organism and, as a result, it becomes an issue to food production (Kuo *et al.* 2014; Langridge *et al.* 2015). Swine have also been revealed to excrete serovar Typhimurium through their faeces, for up to 28 weeks subsequent to initial infection (Xie *et al.* 2015). Farming animals are largely associated as carriers of *Salmonella* (Langridge *et al.* 2015). As *Salmonella* locates its way to farming areas, the risk of contamination will be mainly influenced by its survival proficiencies in compost, soil and plants (Jacobsen and Bech 2012). A number of studies have isolated *Salmonella* spp. both in agricultural animals and environmental samples (Figure 2.3) (Alemayehu 2014; Batista *et al.* 2014; Erganis *et al.* 2014; Jacobsen and Bech 2012; Langridge *et al.* 2015; Omogbai and Eze 2011; Omogbai and Ikenebomeh 2013; Xie *et al.* 2015). Both Baloda *et al.* (2001) and Sandvang *et al.* (2000) found that the serovar Typhimurium clone persevered and subsisted in a Danish farm (or in swine's with asymptomatic infection), elucidating recurrence of infection in the masses up to about 300 days.

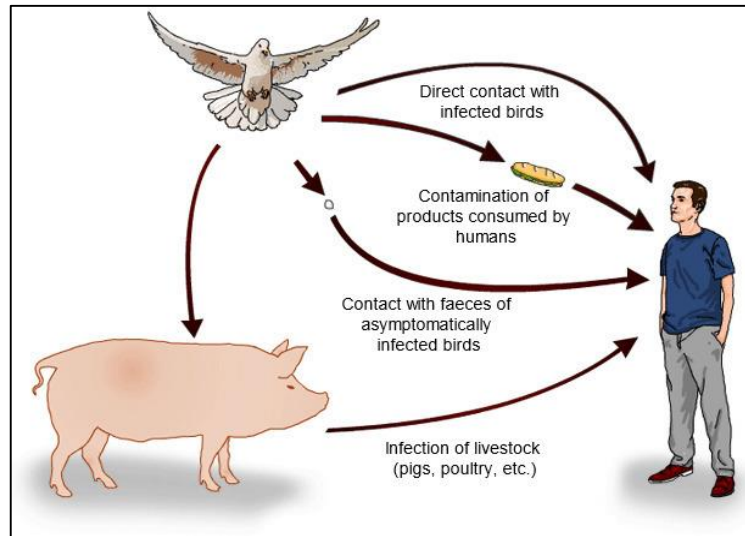


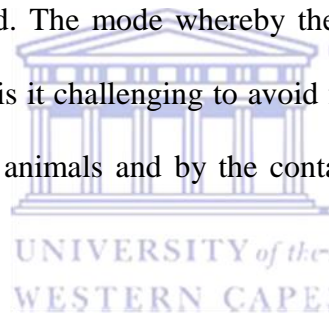
Figure 2.3. Possible causes of salmonellosis

Source: [http://www.pig333.com/what_the_experts_say/salmonella-in-wild-birds-](http://www.pig333.com/what_the_experts_say/salmonella-in-wild-birds-a-risk-for-swine_7185)

[a-risk-for swine_7185](http://www.pig333.com/what_the_experts_say/salmonella-in-wild-birds-a-risk-for-swine_7185)

The nature of *Salmonella* infections in the agricultural environment will fluctuate depending on various influences, including; animal species, health and size of the herds, age and farmhouse environment (Kuo *et al.* 2014; Langridge *et al.* 2015; Ojha and Kostrzynska 2007; Payne *et al.* 2007; Rajic *et al.* 2007). *Salmonella* is significantly dispersed in the environment; however, most *Salmonella* serovars are related to animal species, for example sheep (*S. enterica* ser. Abortusovis) (Alemayehu 2014; Uzzau *et al.* 2005), pigs (*S. enterica* ser. Choleraesuis) (Asai *et al.* 2010; Xie *et al.* 2015), poultry (*S. enterica* ser. Gallinarum) (Batista *et al.* 2014; Langridge *et al.* 2015), horses (*S. enterica* ser. Abortus-equi) (Erganis *et al.* 2014) and cattle (*S. enterica* ser Dublin) (Langridge *et al.* 2015) *Salmonella* can also be dispersed into the environment by animal carriers (Gouws *et al.* 2014).

Salmonella has previously been isolated from various animal species such as birds (Butterfield *et al.* 1983), muscoid flies (Mian *et al.* 2002), reptiles (Mitchell and Shane 2001) and mice (Davies and Wray 1995). These carriers play a vital role in the epidemiology of *Salmonella* contamination, particularly in food production (Gouws *et al.* 2014). These animals may pass spontaneously through the agricultural estates and excrete *Salmonella* into its environs. In a study of 7 680 animal and environmental samples from a U.S. dairy, 13-72 percent of the livestock samples, and more than 50 percent of the soil, water, insect, and bird faeces samples yielded *S. enterica* (da Cruz *et al.* 2006; Jacobsen and Bech 2012; Kruse *et al.* 2004; Pangloli *et al.* 2008). The contamination of food animals with *Salmonella* is quite well understood and it has been extensively documented. The mode whereby these foods are contaminated is evidently implicit; however, is it challenging to avoid for the reason that *Salmonella* is widely distributed among animals and by the contamination of the environment (Gouws *et al.* 2014).



2.2.2.3 *Salmonella* in Plants

Fresh fruit and vegetables are now increasingly recognised as sources of *Salmonella* outbreaks. During the last three decades, the number of documented infections associated with the consumption of fresh produce has increased (Keller *et al.* 2015; Koch *et al.* 2005; Llic *et al.* 2010; Nascimento *et al.* 2010); this has been linked with human salmonellosis. Fresh produce can be contaminated in several means: contamination can surface through the roots, stems, and shoots of different fruits and vegetables (Behravesh *et al.* 2011; Joubert and de Beer 2011; Keller *et al.* 2015; Nascimento *et al.* 2015).

However, it is deemed that the main source of contamination of fresh produce is via untreated irrigation waters (Gemmell and Schmidt 2012; Jacobsen and Bech 2012) and, as a result of all of these original sources of contamination, pre- and post-processing facilities may possibly become contaminated with *Salmonella* (Nascimento *et al.* 2015). Fruit and vegetables contaminated by *Salmonella* spp. in the farming environment could likely be the prime route for infection of consumers (Gouws *et al.*, 2014). The ever-increasing emphasis on uncooked vegetables as a healthy food source is accenting the necessity to guarantee that the fresh produce is *salmonella*-free (Behravesh *et al.* 2011; Joubert and de Beer 2011; Keller *et al.* 2015; Nascimento *et al.* 2015).

Subsequently, since 2008, studies from Behravesh *et al.* (2011), Franz and van Bruggen (2008), Holden *et al.* (2009), Teplitski *et al.* (2009), Berger *et al.* (2010) and Critzer and Doyle (2010) have been published, focussing on bacterial pathogens in farming production. It is commonly supposed that the risk of contaminating of fresh produce in the farm is subjective to two factors: firstly, the concentration of the microbe in the soil (this is however influenced by soil and fertiliser type), and secondly, the distance from the soil to the edible portion of the vegetation (Gonzalez-Escalona *et al.* 2012; Jacobsen and Bech 2012; Oliveira *et al.* 2010). One of the primary routes of *Salmonella* spp. transfer to plants are germinating seeds in compost-amended soil (Jacobsen and Bech 2012).

Oat seeds were revealed to be colonised by *S. enterica* ser. Typhimurium in aboveground parts and roots. This occurred subsequent to the seeds being germinated in an experimental container with mixed soil and slurry, spiked with *Salmonella* (Semenov *et al.*, 2010). The soil density influenced the comparative distribution of

serovar Typhimurium on root and shoots. Whereas similar densities were existent on roots and shoots, on seeds cultivated in soil/manure mixtures with amplified quantities of serovar Typhimurium the numbers on the roots were lesser than on the shoots when the seeds were cultivated in soil/manure mixtures, with low numbers of serovar Typhimurium (Semenov *et al.* 2010).

S. enterica was shown to infect tomato seeds, several weeks subsequent to *S. enterica* being introduced to the soil (Barak and Liang 2008). *S. enterica* was also shown to be capable of the colonisation of tomato plants, from both seeds germinating in *S. enterica* infected soil, and via irrigation with *S. enterica* infected water. However, the highest number of the *Salmonella* was observed when exposed to irrigation water (Barak *et al.* 2009). Semenov *et al.* (2010) publicised the notion that feeding the infected plants to grape snails caused colonisation of *Salmonella* in the snails. This direction is fascinating as the snails can travel and, as a result, it can spread *Salmonella* spp. from seeds on the infected ground to trees (Semenov *et al.* 2010). Vegetal leaves are inhabited by a mass of various bacteria and protozoa; however, the overall understanding is that the microbial community conformation of vegetation is not widespread (Raymond *et al.* 2010). Even though systematic cleaning and sanitation measures within a food processing unit is imperative, contamination must initially be addressed at the basic level i.e. prevention (Gouws *et al.* 2014). As *Salmonella* is capable of infecting a large number of animal species, detection of the source of environmental contamination might not always be feasible. Despite that, survival of *Salmonella* in the soil and in water supplies the bacterium with a greater possibility of infecting a new host (Jacobsen and Bech 2012). *Salmonella* does not proliferate expressively outside its natural environment, but the bacterium can subsist in soil if

the temperature, humidity and pH conditions are favourable (Gouws *et al.* 2014; Jacobsen and Bech 2012). The ubiquitous distribution of *Salmonella* in its natural environment, and its pervasiveness in the global food chain, the physiological adaptableness, virulence of the bacterial pathogen and its profound economic influence on the food industry, highlights the need for sustained awareness and rigorous controls at all levels of food production (Gouws *et al.* 2014).

Enterobacteria is normally limited to water supplies contaminated by faeces in the environment (Jacobsen and Bech 2012). And, as a result, untreated foods of plant origin would undoubtedly be contaminated with natural soil microflora (Gouws *et al.* 2014; Jacobsen and Bech 2012). The use of wastewater for irrigation is also commonly practiced in developing countries, for instance South Africa, due to local fresh water scarceness; however, little is understood in these developing countries about the possible risks related with its usage (Maimon *et al.* 2010).

2.2.2.4 *Salmonella* in *Rooibos* tea

Aspalathus linearis is a prevalent South African species of fynbos, which is cultivated to make the renowned herbal tea, Rooibos (Morton 1983). Currently, it is retailed in more than 37 countries, including Germany, the Netherlands, the United Kingdom, Japan and the USA, representative of 86 percent of the trade market in 2010 (Anon 2011). Rooibos tea is caffeine-free and has a relatively low tannin grade; collective with its probable healthy properties, particularly its antioxidant activity, contributes to its popularity (Beltran-Debyn *et al.* 2011). The use of Rooibos has furthermore encouraged products beyond herbal tea, to transitional value-added products, for

instance extracts for the beverage, nutrition, nutra-ceutical and cosmetic markets (Gouws *et al.* 2014; Joubert *et al.* 2008).

The recent South African regulations concerning the quality standards for Rooibos deals with moisture content, pesticide deposits, microbial contamination and the fraction of white stems permissible (Gouws *et al.* 2014)). Rooibos ought to ‘have the fresh, specific taste and aroma and clear distinct colour of Rooibos’ (Beltran-Debyn *et al.* 2011). Alternative description usage by the industry is ‘typical’. This means that companies have the liberty to customise their particular quality standards in terms of colour, savour and mouth sensation of Rooibos brews (Joubert and de Beer 2011).

Rooibos (Figure 2.4) is a subtle tea which could be unpleasantly affected by the harsh processing and refining techniques (Beltran-Debyn *et al.* 2011). Hence, procedures must be taken to guarantee that from the plant, to tea and lastly to the consumer, that the product remains free from pathogens and the microbiological content of the tea is adequately conferring to the law to ensure, not only good quality tea but is also safe for consumption (Gouws *et al.* 2014). These concerns have led to regulations being acquired for Rooibos, as shown in Table 2.1 (Anon 2008) (Foodstuff, Cosmetics and Disinfectants Act 1972) and on the European market [European Herbal Infusion Association (EHIA)] (Anon 2005). Using these regulations, Rooibos will be regarded as safe for the consumer as well as for exportation purposes.



Figure 2.4. A mature Rooibos plant *Aspalathus linearis*

Source: <http://www.imvusabs.8k.com/lenquarooibos.html>

Table 2.1: Microbiological regulations for Rooibos tea in Europe and South Africa

Microbial group	Specification	
	South Africa*	Europe[†]
Total viable count (Aerobic plate count)	Shall not exceed 75 000 CFU/g (Bulk) Shall not exceed 150 000 CFU/g (Retail)	Shall not exceed 1 000 000 000 CFU/g
<i>Escherichia coli</i>	Shall not exceed 20 CFU/g	100 000 CFU/g
<i>Salmonella</i>	Absent in 25g	Absent in 5 X 25g

*Guidelines extracted from European Herbal Infusion Association Microbiological Guidelines

(Kolb 1999) [†]Regulations governing microbiological standards for foodstuffs and related matters, Foodstuffs, Cosmetics and Disinfectants Act of South Africa

Source: Anon 2008

As observed in Table 2.1, the groups of immediate distress are the total viable counts, *E.coli* and *Salmonella*. Of these, *Salmonella* is of foremost significance as this microbe is a risk to the consumer.

2.3 THE PROCESSING OF ROOIBOS TEA

In the late summer months of February and March, seeds are cultivated in well-prepared seedbeds, where after systematic irrigation is applied to guarantee maximum germination (Gouws *et al.* 2014). About four to six days later, two cotyledons surface above the ground, later followed by needle-like leaves. For the duration of the cooler winter months, from June to August (after the first rainfall), the seedlings are uprooted in rows at plantations (Joubert and de Beer 2011). During these earlier stages, effective insect and weed control is applied to ensure the maximum enterprise percentage (Gouws *et al.* 2014). No fungicides and antibiotics are utilised during cultivation and pesticides are used generously and only if required (Gouws *et al.* 2014). Subsequently, 18 months after the Rooibos has been cultivated, the plants are pruned for the first time and, thereafter, it is harvested yearly by cutting the branches 50cm above the ground (Joubert and de Beer 2011). These shoots are meticulously bound into bundles and transported to the processing yards (Gouws *et al.* 2014). The sheaves are then cut by commercial cutting machinery into a uniform length for the final product, which is then bruised between rollers to trigger the significant chemical process of fermentation (Joubert and de Beer 2011).

Thereafter, proper aeration and watering is needed; the tea is then left to ferment in low heaps at ambient temperature for roughly 12-14 hours (Gouws *et al.* 2014). A process of enzymatic oxidation occurs whereby the product changes from green to the characteristic brick-red colour and develops the distinct flavour and sweet aroma of Rooibos (Gouws *et al.* 2014; Joubert and de Beer 2011). Subsequent to fermentation, the Rooibos is sprayed out in large drying tea courts using tractors, where it is allowed to dry under the hot African sun (Gouws *et al.* 2014). Commercial machinery then

collects the dried Rooibos, after which it is transported to the factory for further processing (Joubert and de Beer 2011). Significantly, the tractors and boots used by farm workers are merely used on the tea courts to avoid cross-contamination (Omogbai and Ikenebomeh 2013). This is followed by screening, blending and steam pasteurisation, utilising state-of-the-art equipment (Gouws *et al.* 2014). During this process, bacteriological control is vigorously examined by laboratory testing to guarantee that the final product can be certified as a hygienic, safe, superior quality Rooibos (Gouws *et al.* 2014; Joubert and de Beer 2011). Throughout the steam pasteurisation the tea leaves reaches a temperature of 85-92°C for 2.5 minutes to diminish any microbial contaminants present, which is then conducted off to a set of sifts to eliminate excess moisture (Gouws *et al.* 2014). The desiccation of the tea is important, as the low water activity will inhibit the growth of moulds (Gouws *et al.* 2014). Finally, the tea is then sent to a large container for bulk packaging ready for dispatch to consumers worldwide (Joubert and de Beer 2011).

Rooibos is typically regarded a herb, and as of late, herbs have been increasingly recognised as potential sources of *Salmonella* outbreaks (Omogbai and Ikenebomeh 2013). Several environmental sources could be involved in the contamination of Rooibos tea, such as pastures via contaminated irrigation water or fertilisers, which could be the reason for contamination (Gouws *et al.* 2014; Jacobsen and Bech 2012; Maimon *et al.* 2010). Similarly, some of the water in the processing cycle could have been contaminated with *Salmonella* (Ahmed *et al.* 2009; Gemmell and Schmidt 2012; Gemmell and Schmidt 2013). Almost all animals could act as reservoirs for salmonellae, and these animal species can transmit the microorganisms to the tea throughout the different processing stages (Alemayehu 2014; Batista *et al.* 2014;

Erganis *et al.* 2014; Jacobsen and Bech 2012; Langridge *et al.* 2015; Omogbai and Eze 2011; Omogbai and Ikenebomeh 2013; Xie *et al.* 2015). Yet, distinctive allusion ought to be made to the wide diversity of salmonellae that can be harboured by reptiles, specifically lizards (Swanepoel 1987; Tournas and Katsoudas 2008), as salmonellae seem to be part of their normal gut flora. The main reservoirs for serovar Arizonae subspecies are reptiles and poultry, though they have been infrequently isolated from humans (Swanepoel 1987).



2.4 *SALMONELLA* PATHOGENESIS AND CLINICAL FEATURES

Subsequent to ingestion of an infectious dose of *Salmonella*, the bacterial cells surpass through the stomach to the lower small intestine (the ileum), where invasion begins in the intestinal epithelium (Crump *et al.* 2015; Miller and Pegues 2000). The early targets of invasion are the M-cells (specialised epithelial cells), which conveys the bacteria to the fundamental lymphoid tissue (Figure 2.5) (Lhocine *et al.* 2015). At this point they attack intestinal lymphoid follicles and the draining mesenteric lymph nodes, allowing some bacteria to spread to the liver and spleen (Ferreira *et al.* 2015) where it subsists and proliferates inside mononuclear phagocytic cells (Azriel *et al.* 2015). This gestation period lasts 7-14 days, once the bacteria is released into the bloodstream (Lhocine *et al.* 2015). It is generally at this point that patients experience the commencement of fever and other symptoms (Adak *et al.* 2002). If the infection remains untreated, the bacteria can become extensively circulated throughout the bacteraemic phase, spreading to the liver, spleen, bone marrow and gall bladder (Ferreira *et al.* 2015; Lhocine *et al.* 2015).

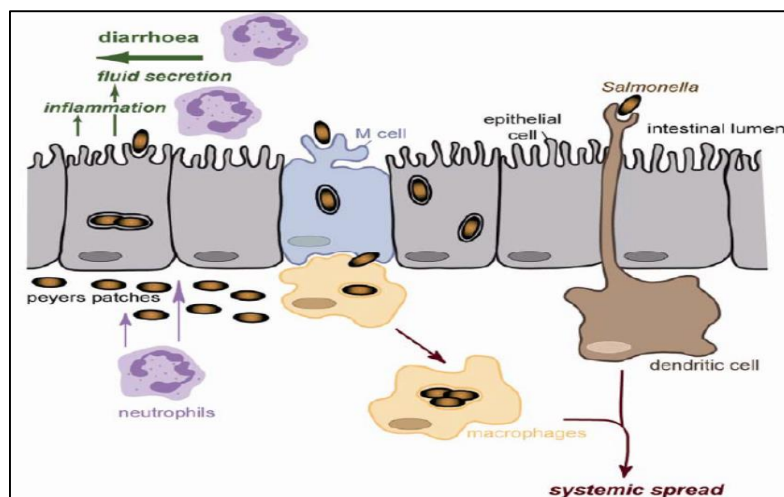
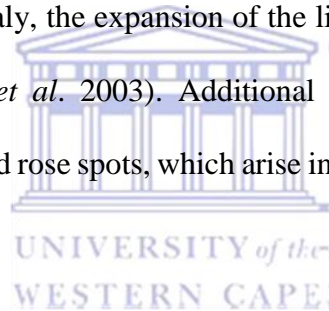


Figure 2.5. Pathogenesis of *Salmonella* infection

Source: Cossart and Sansonetti (2004)

The main medical conditions related to *Salmonella* infections are enteric (typhoid) fever and gastroenteritis (Anon 2010; Lhocine *et al.* 2015). Enteric fever is a prolonged systemic illness which is caused by infection with the human pathogen, serovar Typhi and serovar Paratyphi (Anon 2010; Crump *et al.* 2015). Clinical symptoms include fever, abdominal pain, ephemeral diarrhoea and/or constipation, and sometimes a maculopapular rash (Cunha *et al.* 2013). Throughout the bacteraemic phase of infection, patients usually have persistent fever of up to 40°C, though other symptoms differ broadly amongst patients (Bhutta *et al.* 2014; Grassl and Finlay 2008). Some patients can have influenza like symptoms, a dismal frontal headache, malaise, anorexia, a dry cough, sore throat, and occasionally epistaxis (Cunha *et al.* 2013). Hepatomegaly or splenomegaly, the expansion of the liver or spleen respectively, are occasionally found (Zhang *et al.* 2003). Additional somatic signs include coated tongue, sensitive abdomen and rose spots, which arise in 1-30 percent of cases reported (Crump *et al.* 2015).



The trademark of enteric fever is generally mononuclear cell infiltration and hypertrophy of the reticuloendothelial system (Medzhitov and Janeway 2000). In patients suffering from acute typhoid fever, the average concentration of bacteria is one colony-forming unit per ml of blood (two-thirds of which is present in phagocytes and approximately ten times this in bone marrow) (Wiles *et al.* 2005). Most patients with acute enteric fever continue to excrete *Salmonella* serovar Typhi or Paratyphi A in their stool or urine, days after starting antimicrobial treatment, and up to 10 percent may do so for up to three months (Crump *et al.* 2015). Several non-typhoidal *Salmonella* strains, such as serovar Enteritidis and Typhimurium, infect a widespread range of animal hosts, including poultry and cattle (Feasey *et al.* 2012). The case

fatality ratio among cohorts of children with invasive non-typhoidal *Salmonella* disease across Africa has been reported to be 20 to 28 percent, and highest among children under 2 years of age (Crump *et al.* 2015).



2.5. SALMONELLA PATHOGENICITY ISLANDS

Genomic regions which explicitly arise in pathogens and are inattentive in apathogenic bacteria of similar or interrelated species have been formerly defined by Hacker and Goebel in the 1980s (Hacker *et al.* 1990). These large DNA loci (>20kb) were designated 'pathogenicity islands' (PAI). They are furthermore categorised by a G+C content which contrasts from the residual genome and it encodes at least one virulence gene (Karunasagar *et al.* 2012; Que *et al.* 2013). The islands are frequently initiated next to the 3' end of tRNAs fringed by direct repeats or insertion sequence elements. In bacteria like uropathogenic *E. coli*, *Yersinia* spp., *Helicobacter pylori*, PAI incorporates instable DNA regions which are spontaneously deleted from the chromosome. On the basis of all these features, PAI's are thought to have established from mobile genetic elements attained by horizontal gene transfer through evolution (Hacker *et al.* 1997; Lhocine *et al.* 2015).

Significant virulence factors of *Salmonella*, which contributes to adherence, invasion of and replication within the host cells (Figure 2.5) are encoded within SPI (Lhocine *et al.* 2015). Currently, 17 SPIs have been identified (Deekshit *et al.* 2013; Karunasagar *et al.* 2012). Five SPI (SPI1-5) are conserved amongst *Salmonella* spp.; the dissemination and structural organisation of the other SPI reveal efficient features of specific serovars and it could be accountable for host-specificity (Deekshit *et al.* 2013; Ohlson *et al.* 2008; Que *et al.* 2013).

2.5.1 SPI-1

SPI-1 is the most characterised, and most significant, virulence attribute of *Salmonella*. (Mezal *et al.* 2013; Troxell *et al.* 2015). A 40kb DNA region is contained in this locus, located at 63 centisomes on the serovar Typhimurium chromosome, flanked by the

fhlA and mutS genes (Mills *et al.* 1995). SPI-1 exists in species *S. bongori*, along with *S. enterica*, where it facilitates active invasion into non-phagocytic cells, initiates apoptosis of phagocytic cells and encourages intestinal inflammation (Almeida *et al.* 2013; Campioni *et al.* 2012; Hapfelmeier *et al.* 2004).

Bacteria that are present in the gut express the virulence genes in response to specific stimuli in the intestinal lumen; however, absent within the host (Hu *et al.* 2008). Succeeding linkage to the intestinal epithelium, the T3SS then assembles and assists as a molecular syringe, introducing a cocktail of virulence effectors right into the cytoplasm of the host system (Lhocine *et al.* 2015). A group of seven translocated proteins triggers the mechanism of bacterial uptake, involving a reverse of actin cytoskeleton redistribution and membrane disruption, which follows bacterial internalisation (Cossart and Sansonetti 2004; Lhocine *et al.* 2015; Troxell *et al.* 2015).

Virulent chromosomal gene, *invA*, is essential for full virulence in *Salmonella* and is thought to trigger the internalisation required for the invasion of deeper tissue (Que *et al.* 2013). In particular, the actin binding proteins such as *sipA* and *sipC* combine forces in order to promote effective actin nucleation (which is the creation of actin trimers from monomers), and aggregation (Hur and Lee 2011; McGhie *et al.* 2001; Raffatellu *et al.* 2005). Genes located in SPI-1 such as *sipA*, *sopB*, *sopD* and *sopE2* activates *sopA*, ultimately leads to diarrhoea in the bovine host (Zhang *et al.* 2002). The *Salmonella* invasion protein A, *sipA*, is a secreted effector critical for the efficient invasion of intestinal epithelial cells within SPI-1 (Lhocine *et al.* 2015; Zhang *et al.* 2006). It is an actin-binding protein that contributes to host cytoskeletal rearrangements by stimulating actin polymerisation and counteracting F-actin destabilising proteins (Lhocine *et al.* 2015). T3SS-1 secretes proteins across the inner

and outer membranes of the bacterial cell (Lhocine *et al.* 2015); some of the secreted effectors include *sipA* and *sspA*. It suggests that *sipA* mediates a separate process to accelerate bacterial invasion (Hur and Lee 2011). The utility of *sopB* was primarily associated to neutrophil as a fluid inflow into the intestinal lumen (Galyov *et al.* 1997; Norris *et al.* 1998). Moreover, *sipA*, *sopB*, *sopE/E2* have been revealed to disrupt constricted junctions of diverged epithelia, a process suggested to be required for the development of diarrhoeal disease (Boyle *et al.* 2006).

Throughout enteric infections, *Salmonella* exploits the proinflammatory host response for its own advantage in order to overawe the commensal gut flora (Lupp *et al.* 2007; Stecher *et al.* 2007; Ackermann *et al.* 2008). *AvrA* plays a role in provoking a self-destructive, critical immune response in support of an effective, local inflammation. This theory is reinforced by the high expression of *avrA*, along with *sopD* and *sopB*, mainly in prevalent enteritis strains of serovar Typhimurium; however, this is absent in strains triggering a systemic progression of infections (Hu *et al.* 2008; Streckel *et al.* 2004).

SopA, *sopB*, *sopE/E2*, *slrP*, *sopD* of the SPI-1 T3SS-translocated effectors, are encoded outside of the SPI-1 locus, dispersed in the chromosome (Forest *et al.* 2010). However, *sopE2* is highly conserved and encoded by a residue bacteriophage; *sopE* has been discovered in only a small number of Typhimurium serovars and is produced by the bacteriophage *sopE* (Bakshi *et al.* 2000; Miroid *et al.* 1999). Virulence genes that are expressed, in accordance with the SPI-1 region, is synchronised in an intricate mode (Hur *et al.* 2011; Zou *et al.* 2012) and instigated by several environmental factors like pH, low oxygen settings, short-chained fatty acids, cationic antimicrobial peptides and salt (Altier 2005).

2.5.2 SPI-2

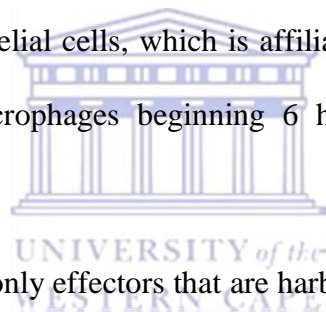
A specific hallmark of *Salmonella* is the expression of a functionally diverse second T3SS, encoded by the *Salmonella* pathogenicity island 2 (SPI-2) (van der Heijden *et al.* 2015) and it is required for the intracellular existence of the pathogen SPI-2 is genetically comprised of a 40kb region composed of 42 open reading frames (ORF) which is positioned at the 30.7 centisome (Shea *et al.* 1996). From the 40kb region only 25kb, encoding 31 virulence genes, are required for the virulence characteristic of *Salmonella* (Almeida *et al.* 2013; Campioni *et al.* 2012). The additional 15kb fragment is expendable for the phenotype throughout systemic infection which encrypts the tetrathionate reductase complex in conjunction with its regulatory elements, along with numerous ORF's with undefined functions (Hensel *et al.* 1999a; Hensel *et al.* 1999b). The SPI-2, with the 31 virulence genes, are prearranged in at least four operons and they are designated as follows:

- i) Two operons encode the structural constituents of the T3SS apparatus [*ssa*];
- ii) Additional transcriptional unit expressing translocated effector proteins [*sse*] and their chaperons [*ssc*];
- iii) The fourth operon encrypts the two-component regulatory system [*ssrAB*] (Almeida *et al.* 2013; Campioni *et al.* 2012; Cirillo *et al.* 1998).

Active invasion of epithelial cells is facilitated by SPI-1 or passive uptake by phagocytes; *Salmonella* is present inside the macropinosome or phagosome, correspondingly (Almeida *et al.* 2013; Campioni *et al.* 2012). Initially, subsequent to entering, the regulatory elements of SPI-2 were expressed while genes encoding

structural and effector proteins of the T3SS were triggered with an interval delay of approximately two hours in macrophages (Cirillo *et al.* 1998).

SPI-2 activity promotes functional sequestration of the bacterium enclosing the membrane-bound vesicle from the regular endocytic pathway, with the purpose of establishing an intracellular niche for bacteriological survival, killing resistance and duplication: the supposed SCV (van der Heijden *et al.* 2015). SPI-2 effectors encourage the development of lysosomal glycoproteins enriched tubular aggregates, known as *Salmonella*-induced filaments (sif), that is prolonged from the SCV to create an intracellular network (Henry *et al.* 2006; Knodler and Steele-Mortimer 2005; van der Heijden *et al.* 2015). Sif are highly active structures that emerge three to four hours post infection (h.p.i) in epithelial cells, which is affiliated with bacterial replication. This delays process in macrophages beginning 6 h.p.i. (Drecktrah *et al.* 2008; Rajashekar *et al.* 2008).



SpiC, *sseF* and *sseG* are the only effectors that are harboured by the SPI-2 locus (van der Heijden *et al.* 2015). The first protein described to be translocated through the SPI-2 encoded T3SS, and mandatory to block synthesis of the SCV with lysosomes and endosomes, was *spiC* (Uchiya *et al.* 1999). *SseF* and *sseG* demonstrate little likeness to one other; however, it reveals no sequence resemblance to any former identified protein (Cirillo *et al.* 1998). These proteins are mutually secreted in vitro and it is translocated into the cytoplasm of the host cell where it is allied with the SCV, playing a role in sif formation (Guy *et al.* 2000; Hansen-Wester *et al.* 2002; Kuhle and Hensel 2002; Kuhle *et al.* 2004). Although *sseF* is involved in microcolony formation of replicating bacteria, *sseG* contributes in SCV targeting to the Golgi complex (Abrahams *et al.* 2006; Salcedo and Holden 2003).

PipB2, a linker for kinesin-1, *SopD2* and *sifA* are effectors translocated outside of the SPI-2 locus, which is restricted to the SCV and involved in the induction of Sif (Henry *et al.* 2006; Knodler and Steele-Mortimer 2005). *SifA* is essential to maintain the integrity of the SCV. The binding of kinesin via SKIP (*sifA* kinesin interacting protein) and *sseJ* reveals a necessity for *sifA* to instigate sif formation (Jackson *et al.* 2008; Ohlson *et al.* 2008). *SifA* regulates the assembly and tubulation of the *Salmonella* phagosome (Henry *et al.* 2006; Knodler and Steele-Mortimer 2005; van der Heijden *et al.* 2015). Several of the SPI-1 secreted proteins have been ascribed effector functions. As of yet, no virulence function has been associated to *sifB*, a protein similar to *sifA* (van der Heijden *et al.* 2015), though it has been noted that *sifB* targets with *sseJ* to the SCV and its tube-shaped postponements (Freeman *et al.* 2003). *SlrP* and *sspH1*, already described as SPI-1 effectors, are similarly translocated by means of the SPI-2 T3SS (Miao and Miller 2000). The T3SS complex is comprised of basal component proteins in the inner membrane (including *ssaR*) (Brumell *et al.* 2001; Hoiseh and Stocker 1981; Hur *et al.* 2011; Zou *et al.* 2012), proto-channel proteins, outer membrane proteins, proteins forming a hollow tube generating the needle and outer ring proteins (including *sseB*); this forms the translocon that passes through the host vacuolar membrane (Forest *et al.* 2010).

2.5.3 SPI-3

SPI-3 is a 17kb DNA-fragment which is integrated genetically, amid the selC tRNA locus at 82 centisome. This region infers ten ORFs that is organised into six operons, not distributed equally within salmonellae. A designated gene that is encoded within SPI-3 is *misL*, an autotransporter protein. This gene performs as a fibronectin-binding

adhesion molecule needed for intestinal colonisation, as well as *marT*, regulating *misL* expression (Dorsey *et al.* 2005; Tukul *et al.* 2007).

2.5.4 SPI-4

SPI-4 was classified by comparative genomic hybridisation of *E. coli* K-12 genome, together with that of *S. enterica* serovar Typhimurium LT2. It has a region of 25kb, is composed of six ORF's and links the single stranded DNA binding protein and superoxide response regulatory gene at 92 centisome (McClelland *et al.*, 2001). Preliminary screening proposed a sequence segment positioned inside SPI-4, designated for survival of intramacrophages (Baumler *et al.* 1994). Additional analyses demarcated SPI-4 as a virulence locus that is necessary for optimum colonisation of calves, as well as facilitating intestinal inflammation in the murine colitis model (Morgan *et al.* 2004; Gerlach *et al.* 2007b). SPI-4 was also revealed to be a factor for adhesion and invasion of opposed epithelial cells, co-regulated along with combination of SPI-1 invasion genes (Gerlach *et al.* 2007a; Gerlach *et al.* 2007b; Main-Hester *et al.* 2008).

Conferring to its virulence utility as explained by Morgan *et al.* (2004), the SPI-4 genes are denoted as *Salmonella* intestinal infection genes. *SiiC*, *siiD* and *siiF* are three genes, homologous to the structural constituents of a type I secretion system and convene *siiE* secretion. *SiiE* is the biggest recognised gene present in *S. enterica* ser. Typhimurium proteome. It is an exceedingly repetitive structure as it functions as an adhesion. *SiiA* and *siiB* could, however, be essential for the constricted retention of *siiE* throughout host cell interaction, although their molecular action still remains unclear (Gerlach *et al.* 2007b).

2.5.5 SPI-5

The classification of the DNA-region of the invasion gene, *sopB*, steers the characterisation of the *Salmonella* pathogenicity island 5 (SPI-5) positioned at 25 centisome. This insertion is lined by the *serT* tRNA and the *copS/copR* gene and it secretes *pipA*, *pipB*, *pipC*, *pipD* and *orfX*, as well as *sopB* (Wood *et al.* 1998). SPI-5 genes are suggested to be involved in enteropathogenicity; strains lacking *pipA*, *pipB*, *pipD* or *sopB* are without an intestinal secretory along with inflammatory response and it shows insignificant attenuation in systemic mice (Galyov *et al.* 1997; Wood *et al.* 1998).



2.6. ANTIBIOTIC RESISTANCE OF SALMONELLA

Foodborne salmonellosis is of great public health concern worldwide. Human *Salmonella* epidemics are related to the consumption of contaminated food products (WHO 2006). The rise in antibiotic-resistant *Salmonella* has been problematic globally (Chiu *et al.* 2002; White *et al.* 2002). Antibiotics are generally accessible ‘over the counter’ and the regulation of antibiotic practise is poor (Aarestrup *et al.* 2008).

Antibiotics are utilised extensively in animal husbandry, for numerous reasons including therapeutics, prophylaxis and growth promotion (Chiu *et al.* 2002). The usage of antibiotics in animals not only picks out antibiotic-resistant bacteria but it could also escalate the antibiotic-resistant bacteria in humans, via the food chain (Pappaioanou 2004; White *et al.* 2004). Antibiotic resistance is demarcated as the ability of bacteria to halt the inhibitory (bacteriostatic), or killing (bacteriocidal), effects of antimicrobials to which it was previously sensitive and effective (Hedberg 2011). There is ample evidence of the development of antibiotic-resistant pathogens that was spread from an animal reservoir to humans (Aarestrup *et al.* 2008; White *et al.* 2002).

Even though antibiotics are not needed for the treatment of several cases of salmonellosis, it could however save lives in invasive infections that generally take place in children and the elderly. The selections of treatment for invasive *Salmonella* infections are ceftriaxone and ciprofloxacin in humans (Glynn *et al.* 1998; Hohmann 2001). The resistance of quinolone and fluoroquinolone are frequently linked with point mutations at the Quinolone Resistance Determining Region of genes, *gyrA* and *parC* (Hooper 2001). *Salmonella* infections, resistant to numerous antimicrobials, are related to increased morbidity and mortality (Hedberg 2011; Spapen *et al.* 2011), and

the occurrence of these entities is leaving clinicians with limited, or no, treatment options (Hohmann 2001).

Salmonella species are becoming progressively resistant, making it more problematic when treating patients with severe infections (Rusul *et al.* 2012). This makes multidrug-resistant *Salmonella* an imperative subject area of exploration and a foremost concern for food safety (Abakpa *et al.* 2015; Levantesi *et al.* 2012; Rusul *et al.* 2012). *Salmonella* has the capability to adapt to diverse external conditions, including low pH or high temperature; this allows it to survive outside the host organism (Semenov *et al.* 2007). Undeniably, *Salmonella* is able to attach and adhere to plant surfaces prior to dynamically infecting the interior of various plants, steering to the colonisation of plant organs (Gu *et al.* 2011; Klerks *et al.* 2007), and destruction of the plant immune system (Schikora *et al.* 2012). In addition, *Salmonella* originating from plants retains virulence toward animals (Schikora *et al.* 2012). Thus, plants are an alternative host for *Salmonella* pathogens, and have a role in its transmission back to animals.

Recently, several studies have indicated the emergence, and spread, of multidrug-resistant *Salmonella* clones in Africa (Gordon *et al.* 2008, Kingsley *et al.* 2009; Vandenberg *et al.* 2010). These clones often have a dissimilar epidemiology than in developed countries, obscuring control and prevention stratagems (Okoro *et al.* 2012). It is of the utmost importance that novel multidrug-resistant clones are detected as quickly as probable, to obstruct further distribution (Hedberg 2011). The microbiological testing of food products for the incidence of these pathogens is obligatory (Helms *et al.* 2005). *Salmonella* that is multi-drug resistant has also gained concern globally (Deekshit *et al.* 2012, 2013).

Antimicrobial resistance is a matter of profound importance for public health globally (Bagudo *et al.* 2014; Zakar *et al.* 2012). Both climatic change, and urbanisation, has impeded Earth's freshwater resources (Zakar *et al.* 2012). These factors cause freshwater to become progressively scarcer worldwide; many countries resort to utilising treated wastewater directly for domestic, manufacturing, and agricultural uses (Hamilton *et al.* 2006). Nevertheless, the detection of residue level antimicrobials and pathogens have frequently been reported in wastewater emissions and in recycled wastewater (Fatta-Kassinos *et al.* 2011). Due to extended exposure to antimicrobial remaining in wastewater, some bacteria, such as *Salmonella*, acquire resistance to these antimicrobial compounds (Abakpa *et al.* 2015). In the occurrence of low levels of antimicrobials, bacteria have revealed the capability to attain antibiotic resistance genes (Gal-Mor 2010). Genetically modified crops with antibiotic resistant marker genes (microbes added intentionally or unintentionally to the food chain that could potentially transfer antimicrobial resistance genes), and food processing technologies that used slightly lethal dosages are also matters for concern (Gay and Gillespie 2005; Gouws 2015).

Antibiotic-resistant *Salmonella* has become a problem due to several reasons. There is an urgent need for alternative classes of antimicrobial compounds that can be used in the management of *Salmonella* infections (Khan *et al.* 2007). Some authors have described that the antimicrobial resistance of *Salmonella* strains could be related with the expression of certain virulence genes, including *invA* (Dione *et al.* 2011).

To minimise the possibility of salmonellosis from the ingestion of low-moisture foods, it is essential for companies to apply paramount efforts to control several risk factors that could lead to cross-contamination. Available literature on *Salmonella*

contamination in low-moisture foods has been outlined, due to inadequate sanitation practices, poor equipment design, inappropriate maintenance, poor operational practices, inadequate ingredient control, and other factors (Isaacs *et al.* 2005; Uesugi *et al.* 2007).

Biofilm formation is an alternative way by which *Salmonella* endures the hostile settings of the environment. But, on the basis of existing literature, it is unclear whether *Salmonella* cells form biofilms in low-moisture conditions. In high-moisture conditions, cellulose production and biofilm formation could be a significant factor for the survival of serovar Enteritidis on surface environments (Solano *et al.* 2002).



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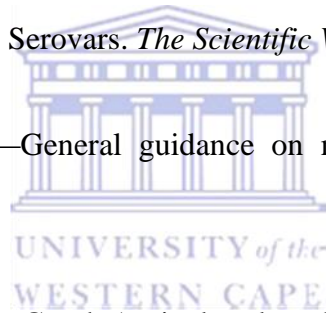
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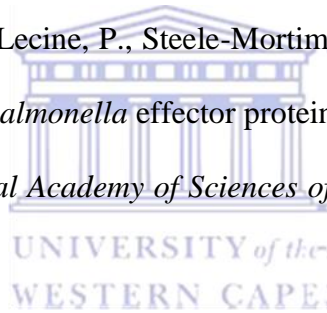
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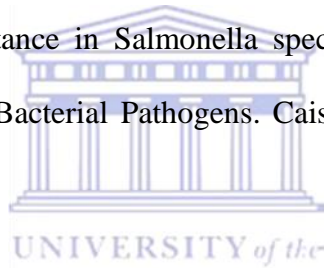
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CHAPTER 3

VIRUTYPING OF *SALMONELLA* STRAINS ISOLATED FROM FERMENTED ROOIBOS

3.1 ABSTRACT

Aim: The objective of this study was to determine the prevalence of *Salmonella* isolated from Rooibos tea. This experiment was designed to explore the characterisation and genotyping of potential virulence genes associated with *Salmonella* after natural fermentation of Rooibos tea.

Methods and Results: Rooibos samples from various farms were collected after fermentation and post-pasteurisation from the processing plant. *Salmonella* detection was carried out using traditional microbial methods based on the ISO (International Organisation for Standardization 1993) method. To reduce the possibility of attaining false negative results, a non-selective pre-enrichment, a selective enrichment and two different selective media's were used. Media for isolation included a pre-enrichment in non-selective medium Buffered Peptone Water (BPW), selective enrichment in Rappaport Vassiliadis broth (RV), sub-cultivation on Xylose Lysine Desoxycholate (XLD) and Tryptic Soy agar (TSA). Virutyping of *Salmonella* genes was explored by cultural dependent techniques. It was concluded that 26/60 isolates were confirmed to be that of the *Salmonella* genus, totalled to 43 percent. This revealed that throughout the processing of Rooibos, *Salmonella* contamination occurred. The overall prevalence of virulence genes *invA*, *sipA*, *sifA* and *ssaR* from the *Salmonella* isolates was 19/60 (32 percent), 8/60 (13 percent), 7/60 (12 percent) and 6/60 (10 percent) respectively.

Conclusion: The results revealed that unpasteurised herbal teas are potential mechanisms of transmission of pathogenic *Salmonella* isolates; this could be linked to the prevalence of salmonellosis and other *Salmonella* related infections. However the samples from post-pasteurisation revealed no that no *Salmonella* was present, this gives significant insight to the efficacy of the sterilization process. This study reports the first data that validates the potential transmission of *Salmonella* strains harbouring virulence determinants from herbal infusions to cause infections in humans, specifically genes from SPI-1 and 2.

Significance and impact of study: Determining the prevalence of virulence genes of *Salmonella* could be significant in predicting the risk of contracting salmonellosis from food products. This study will lead to a better understanding of the fate of *Salmonella* in the processing environment and it is the first to identify virulence genes in herbal tea. Several studies of virulence determinants in *Salmonella* have focused on clinical and veterinary strains only, but this study focused on virulence genes in *Salmonella* isolated from plant material. To fully understand the scale of virulence in *Salmonella*, and to combat this emergent problem, it is also vital to consider consumer exposure to potential virulence genes present in food products. Furthermore, this study provides evidence about the possible public health risks associated with unpasteurised herbal teas. It is crucial to identify any trends in *Salmonella*-related infections by conducting routine virutyping of genes. Currently, no reports have been published about virulence genes in *Salmonella*, isolated from herbal teas in South Africa. Studies on these virulence factors may provide new insights into the prevention and treatment of salmonellosis. These virulence genes are involved in the intestinal phase of infection and are located in SPI-1 and SPI-2. SPI's generally take part in intracellular

replication, as it interferes with host responses and favours *Salmonella* survival. Regardless of the controls that have previously been set into place, *Salmonella* infection that arises from contaminated food remains a great issue, with numerous worldwide outbreaks occurring annually. Detection of *Salmonella* prior to contaminated foods being consumed is therefore an indispensable attribute of safeguarding public health and parenthetically preserving the statuses and affluences of food manufacturers and processors. Scrutiny of *Salmonella* in all the various phases of feed-food chain creates a vital component in the investigation of epidemiology of foodborne salmonellosis, and in the progress and execution of proficient *Salmonella* control stratagems.



3.2 INTRODUCTION

Tea is a product of botanical origin, which is used for preparing a beverage or to be prepared from such product. *Aspalathus linearis* is a well-known herbal tea, known as Rooibos (Joubert and de Beer 2011). Aspalathin seems to have in vitro antioxidative and antimutagenic effects (Hamilton-Miller 1995; Ivanova *et al.*, 2005; Joubert and de Beer 2011). In 2007, the Swiss Business Hub South Africa (Anon 2007) stated that, ‘Rooibos is seemingly headed to becoming the second most frequently consumed tea beverage worldwide, after regular tea (*Camellia sinensis*)’ (Gouws *et al.* 2014). Rooibos tea is well established in South Africa and it is enjoyed over an estimated 10.9 million households (Gouws *et al.* 2014). No deleterious effects of Rooibos have been publicised from the nutritive aspect (Joubert and de Beer 2011). However, this may well be fallacious when considering it from the microbial aspect of the plant and the final product (Gouws *et al.* 2014). The outcomes of most studies express potential health benefits of tea, when consumed, including prevention of cancer and heart disease (Joubert and de Beer 2011; Omogbai and Ikenebomeh 2013).

However, there are risks involved with the microbial contamination and succeeding adversative events. The reason of these adverse effects are common microbial pathogens such as yeasts, moulds, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Pseudomonas flourecens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Serratia marcenscens*, and *Salmonella typhimurium*; therefore, it is crucial to detect them in herbal teas (Omogbai and Eze 2011; Omogbai and Ikenebomeh 2013). It is imperative to diminish such contamination of food and the consequences of microbial growth (Joubert and de Beer 2011). Hence, microbiological standards have been set in South Africa (Foodstuff,

Cosmetics and Disinfectants Act) (Anon 2008) and on the European market (European Herbal Infusion Association (EHIA)) (Anon 2005). These standards provide parameters for the allowed level of a microbe that can be present per gram of Rooibos tea. South African standards enforce a sterner acceptance level, in comparison to Europe (Gouws *et al.* 2014). Nonetheless, although the parameters vary between Europe and South Africa, both have a zero tolerance for the presence of *Salmonella* in Rooibos tea (Gouws *et al.* 2014).

Rooibos seeds are disseminated during the period of February to March and the seeds are transplanted a few months after this. The plants requires at least 18 months to grow and develop before it can be first harvested (Gouws *et al.* 2014; Joubert and de Beer 2011). During spring the plant produces a yellow flower containing legumes which have a seed inside. These seeds are then released once ripened. No fungicides and antibiotics are utilised during cultivation, pesticides are however used generously and only, if required (Gouws *et al.* 2014; Joubert and de Beer 2011). The sheaves are then cut for the final product, which is then bruised to trigger fermentation (Joubert and de Beer 2011).

Rooibos is typically regarded as a herb, and as of late, herbs have been increasingly recognised as potential sources of *Salmonella* outbreaks (Omogbai and Ikenebomeh 2013). Documented outbreaks by Franz and van Bruggen (2008) and Jacobsen and Bech (2012) may well be associated with a change in consumer decree towards a greater consumption of fresh fruits and vegetables (Lynch *et al.* 2009). Jacobsen and Bech (2012) and Lynch *et al.* (2009) have detailed three stages in the management of fresh produce where contamination is almost certainly to occur: Firstly in the field, then for the duration of the initial processing and, thirdly, throughout the final

preparation in the kitchen (Lynch *et al.* 2009). It is presumed that compost and irrigation water are of the main sources of contamination of vegetation in the field (Jacobsen and Bech 2012; Lynch *et al.* 2009).

Food animals are also commonly documented as carriers of *Salmonella* spp. (Jacobsen and Bech 2012; Lynch *et al.* 2009). As soon as *Salmonella* discovers its mode to farm areas, the risk of contamination will likely be contingent on its survival competencies in compost, loam and in/on plants (Jacobsen and Bech 2012). Outbreaks that are related to the survival of *Salmonella* in soil and the transference to water and to fresh produce (Jacobsen and Bech 2012; Lynch *et al.* 2009) is necessary to control salmonellosis. The contamination of fresh produce is can also be through water splashes during the period of rain events (Jacobsen and Bech 2012). Fresh produce can either be contaminated by cells attached to the surface, or by internal colonisation of the plant cells (Jacobsen and Bech 2012; Noel *et al.* 2010).

As a foodborne pathogen, *Salmonella* causes gastroenteritis, bacteraemia, and succeeding focal infections (Gouws *et al.* 2014; Jacobsen and Bech 2012). Pathogenesis and immune responses related to *Salmonella* infections is influenced by the infecting *Salmonella* serotype, virulence of the infected serovar and infected hosts, genetics and immune responses (Deekshit *et al.* 2013; Que *et al.* 2013). Variations in virulence among *Salmonella* serovar and in the course of *Salmonella* infections in various host species, have been attributed to the variable acquisition and evolvement of virulence genes (Deekshit *et al.* 2013).

In serovar Typhimurium, at least 80 different virulence genes have been identified. A large part of these genes are clustered on the chromosome in SPIs (Karunasagar *et al.*

2012; Que *et al.* 2013). Currently, 17 SPIs have been identified (Deekshit *et al.* 2013; Karunasagar, *et al.* 2012). From the total range of virulence genes, the attainment of distinctive SPIs is predominantly reflected to have extended the adaptive mechanisms of the diverse *Salmonella* serovars (Karunasagar *et al.* 2012), facilitating the crossing of host barriers and the occupation of niches in new hosts (Deekshit *et al.* 2013; Que *et al.* 2013).

SPI-1 has been related with the ability of *Salmonella* to penetrate the epithelium, encodes genes essential for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory responses (Que *et al.* 2013). In contrast, SPI-2 encodes genes crucial for intracellular replication, but in the mouse enteric fever model, it is needed for formation of systemic infection beyond the intestinal epithelium (Forest *et al.* 2010).

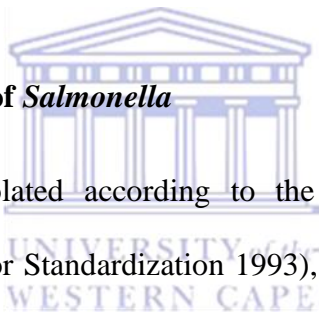
These resilient pathogens are particularly challenging in an extensive variability of immune-compromised persons, including (but not limited to) patients with malignancy, HIV patients, or diabetes, and others receiving immunotherapy agents (Abakpaa *et al.* 2015). The escalation in outbreaks is most likely allied with improved surveillance, greater consumer demand and adjustment in production and distribution (Jacobsen and Bech 2012). Therefore, it is important to meet the legislative requirements for the production, import and export of tea, tea extracts and preparations thereof, for handling it and introducing it on the market as is demarcated by the legislation (Gouws *et al.* 2014).

3.3 MATERIALS AND METHODS

3.3.1 Sample Collection of Rooibos tea

Sixty samples of Rooibos tea was collected from several different processing plants in the Cederberg area (within the Western Cape region), from August to December during 2013-2014. The samples were collected just after fermentation and drying; several samples were assembled before, and others after, pasteurisation. The dried tea leaves were then packaged into sterile whirl-pak bags and transported to the laboratory. Samples were labelled according to its specific origin, with date of collection, prior to and after pasteurisation. The samples were subjected to a controlled environment until it arrived at the laboratory, where it was stored in a cool arid place for further analysis.

3.3.2 Bacterial Isolation of *Salmonella*



Salmonella strains were isolated according to the Standard ISO-6579 method (International Organization for Standardization 1993), with some modifications. For the pre-enrichment of *Salmonella*, 25g of each sample was homogenised in sterile bags with 225ml of BPW (Merck Biolab) and incubated at 37°C for 18-24 hours. Following the incubation, 0.1ml of PBW was added to 10ml of RVS broth (Merck Biolab) followed by further incubation at 41.5°C for 24 hours. A loopful of culture was then taken from the selective enrichment RVS broth, streaked onto XLD (Merck Biolab), and incubated at 37°C for 24 hours. Subsequent to incubation, the black colonies, indication of Hydrogen sulfide production, were assumed to be presumptive positives of *Salmonella*. The colonies were then re-streaked onto TSA (Merck Biolab) which was incubated for 24 hours at 37°C.

Several single colonies were inoculated into Tryptic Soy broth (TSB) (Merck Biolab) to characterise each *Salmonella* isolate culture for further identification. The isolates were then subjected to Gram stain analysis to confirm Gram reaction and morphology. The biochemical analysis of each isolate was done, as a confirmatory test using the API 20E (analytical profile index) (Biomerieux) system for accurate identification and confirmation.

3.3.3 Characterisation and Virotyping of *Salmonella* Genes

3.3.3.1 Biochemical Confirmation of *Salmonella*

Biochemical identification of *Salmonella* isolates was performed by utilising the API 20E system (Biomerieux) for characterisation and confirmation. The API 20E system (Biomerieux) is a plastic strip that consists of 20 microtubes, containing parched substrates (Murray, 1979). The API 20E (Biomerieux) was ultimately developed for the identification of Enterobacteriaceae and it includes various tests such as: o-nitrophenile- β -D-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilisation, hydrogen sulphide production, urease, tryptophan deaminase, indole production, acetoin production by the Voges-Proskauer test, gelatinase and fermentation of glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdaline and arabinose. One colony representing a presumptive *Salmonella* isolate was re-suspended in deionised water and this was inoculated into the API 20E (Biomerieux) test strips. This was done according to the manufacturer's instructions to identify the Gram negative organism.

3.3.3.2 *Salmonella* DNA Extraction

For DNA extraction, the cells were streaked onto TSA; then, a couple of single isolates was suspended in 200µl phosphate buffered saline solution (PBS). The ZR Fungal/Bacterial DNA MiniPrep Kit™ (Zymo Research Corporation) was utilised according to the manufacturer's recommendations, then further subjected to PCR amplification.

3.3.3.3 PCR Detection of *Salmonella* (ST11 and ST15)

PCR was performed to amplify a 429bp portion of a *Salmonella* specific gene by using the primers ST11 and ST15 (Table 3.1). These primers were specific to the *Salmonella* genus (Table 3.1) (Aabo *et al.* 1995). Reference strain *S. enterica* ser. Typhi (ATCC 14028) was used as a positive control, whereas water and *Staphylococcus aureus* (ATCC 25923) was used as negative controls in this study. Negative and positive controls were utilized for amplification of every sample, and also for all genes. The positive control was (ATCC 14028) previously cultured *Salmonella*.

Table 3.1: Primer set for the amplification of *Salmonella* spp

Primer Description	Primer Sequence	Amplicon Size (bp)
<i>Salmonella</i> spp. specific primer set	ST11: 5'-AGCCAACCATTGCTAAATTGGCGGA-3'	429
	ST15: 5'-GGTAGAAATCCAGCGGGTACT-3'	

Source: Aabo *et al.* (1995)

For a 25µl reaction, the mixture was composed of: 5X MyTaq reaction buffer (Bioline), 1U MyTaq DNA polymerase (Bioline), 1µl DNA and 0.3µM of each primer (ST11 and ST15) (Whitehead Scientific) with the addition of Milli-Q (Merck). Amplification was carried out in the T100™ Thermal Cycler (Bio-Rad) with the following program: initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for ten minutes.

3.3.3.4 PCR Detection of *Salmonella* Virulence Genes (in SPI-1 and 2)

To determine the temperature which was optimal for primer annealing and extension, gradient PCR was employed using the T100™ Thermal Cycler (Bio-Rad). The amplification of genes: SPI-1, *invA*: 2.168kb, *sipA*: 3.24kb and SPI-2, *sifA*: 1kb, *ssaR*: 1.628kb (Hu *et al.* 2008) was carried out in the T100™ Thermal Cycler (Bio-Rad) (Table 3.2). Optimal annealing temperatures was observed between 47 and 60°C. *S. enterica* ser. Typhi (ATCC 14028) was used as a positive control and *Staph. aureus* (ATCC 25923) was used as a negative control. Negative and positive controls were utilized for amplication of every sample, and also for all genes. The positive control was (ATCC 14028) previously cultured *Salmonella*.

Table 3.2: SPI-1 and 2 Primers

Gene	Forward Primer	Reverse Primer	Conditions
<i>invA</i>	5'-CAGCGATATCCAAATGTTGC-3'	5'-AAATGGCAGAACAGCGTCGTA-3'	95°C/10 min; 35 cycles of 94°C/30 s, 60°C/1 min, and 72°C/2.5 min; and 72°C/10 min
<i>sipA</i>	5' ATGGGTACCAGGCGGCTACTAAA ATCC-3'	5'-ATGGAGCTCCAAGCGAGAGAAAA ATCTACAC-3'	94°C/2 min; 35 cycles of 94°C/30 s, 50°C/1 min, and 68°C/4 min; and 68°C/10 min
<i>sifA</i>	5'-ATGGTCGACATGCCGATTACTATA GGGAATGG-3'	5'-ATGGGATCCTTATAAAAAACAACA TAAACAGCCG-3'	95°C/10 min; 35 cycles of 94°C/30 s, 55°C/1 min, and 72°C/1 min; and 72°C/10 min
<i>ssaR</i>	5'-GTTCCGATTCAATTGCTTCGG-3'	5'-TCTCCAGTGACTAACCCCTAACCAA-3'	95°C/10 min; 35 cycles of 94°C/30 s, 47°C/1 min, and 72°C/2 min; and 72°C/10 min

Source: Hu *et al.* (2008)

For a 20µl reaction, the mixture was composed of: 5X MyTaq reaction buffer (Bioline), 1U MyTaq DNA polymerase (Bioline), 1µl DNA and 0.2µM of each primer (Whitehead Scientific) with the addition of Milli-Q (Merck). Amplification was carried out in a T100™ Thermal Cycler (Bio-Rad) with the program depicted in Table 3.2 (Hu *et al.* 2008) with slight modifications.

3.3.4 Agarose Gel Electrophoresis

Subsequently, the DNA that was amplified from the PCR was electrophoresed on agarose gels. 2.5µl of the final product with the addition of 1µl loading dye, was resolved into amplified fragments by electrophoresis on a 1 percent agarose gel at 80V for 1.5 hours. To estimate the molecular weights of fragments, a 1kb and 100bp molecular weight ladders was run on each of the respective gels. The agarose gels were then immersed in a 3X gel red solution as a post staining procedure for about 30

minutes. The amplicons were then observed using UV illumination (AlphaInnotech Corporation) and the photographs were acquired utilising AlphaEase FC™ software (version 4.0.0).

3.4 RESULTS AND DISCUSSION

Table 3.3: *Salmonella* biochemical confirmation using the API 20E system

Isolate Number	Biochemical Confirmation
2	<i>Salmonella</i> spp.
2A	<i>Salmonella</i> spp.
3	<i>Salmonella</i> spp.
3B	<i>Salmonella</i> spp.
4	<i>Salmonella</i> spp.
4A	<i>Salmonella</i> spp.
7	<i>Salmonella</i> spp.
7A	<i>Salmonella</i> spp.
8	<i>Salmonella</i> spp.
9	<i>Salmonella</i> spp.
9A	<i>Salmonella</i> spp.
11	<i>Salmonella</i> spp.
11B	<i>Salmonella</i> spp.
12B	<i>Salmonella</i> spp.
13	<i>Salmonella</i> spp.
13A	<i>Salmonella</i> spp.
14	<i>Salmonella</i> spp.
14A	<i>Salmonella</i> spp.
15A	<i>Salmonella</i> spp.
16A	<i>Salmonella</i> spp.
17	<i>Salmonella</i> spp.
18	<i>Salmonella</i> spp.
20	<i>Salmonella</i> spp.
54 (1)	<i>Salmonella</i> spp.
54 (6)	<i>Salmonella</i> spp.
54 (7)	<i>Salmonella</i> spp.

Table 3.3 indicates that the isolates were confirmed to be that of the *Salmonella* genus. API tests base its confirmation solely on biochemical characteristic and as a result it would merely define the bacterium on a genus level; additional testing can be utilised for the identification of species. *Salmonella* was shown to be present in Rooibos throughout processing and fermentation (Table 3.3). This is, however, not considered to be that imperative as the final product is pasteurised and no *Salmonella* was detected in the post-pasteurised Rooibos samples in this study. Most of the microorganisms should have therefore been eradicated, thereby reducing the risk for pathogen detection. This proves that the sterilization process is quite effective. However these results show implications for possible public health risks associated with unpasteurised herbal teas. For the purpose of this study only *Salmonella* strains were isolated, however according to API 20E system there were quite a few *Klebsiella pneumoniae* isolates detected in the Rooibos samples. Although *K. pneumoniae* is found in the normal flora of the mouth, skin and intestines, it could cause destructive changes to human and animal lungs resulting in bloody sputum. *K. pneumoniae* occurs naturally in the soil and it has been shown to increase crop yields in agricultural conditions (Ryan and Ray 2004).

Up to now, over 2 610 serovars of *S. enterica* have been recognised worldwide, and virtually all are capable of causing illness in humans and animals (Guibourdenche *et al.* 2010). *Salmonella* has the ability to spread between hosts; for instance, bacteria can channel from septic farm animals to fresh produce as a result of field fertilisation with raw, contaminated manure and *Salmonella* can penetrate, inhabit and persist on plants (Davies and Wray 1995; Schikora *et al.* 2011).

Despite the frequency of *Salmonella* in the environment, low-moisture foods, such as dried herbs and spices, are not conventionally considered as high risk with respect to salmonellosis because the low water activity is a barrier for bacterial growth (Keller *et al.* 2015). There have been at least two documented outbreaks of salmonellosis related to herbal teas. In 2003, a cluster of *S. enterica* ser. Agona infections, mainly amongst infants, occurred in Germany (Koch *et al.* 2005). These infections were linked with the consumption of herbal tea containing aniseed. A second outbreak caused by *S. enterica* ser. Senftenberg was associated with the consumption of fennel seed tea; this occurred in Serbia from March 2007 to September 2008 (Llic *et al.* 2010).

According to a survey done by Zweifel and Stephan (2012), of 374 samples of herbal tea, 1.3 percent was contaminated with *Salmonella*. The incidence of Rooibos tea has been reported to be high; 20-100 percent of South African Rooibos samples tested was contaminated with several serovars of *Salmonella* (Mossel and Struijk 1991).

In this study, 60 samples of Rooibos tea were tested; 43 percent was contaminated with *Salmonella* spp., following isolation and identification (Table 3.3). Omogbai and Ikenebomeh (2013) isolated *S. enterica* ser. Typhimurium (3 percent), from 26 samples of herbal tea. A study performed by Keller *et al.* (2015), showed that all brewed teas (chamomile, peppermint, and green tea) tested supported the growth of *Salmonella*. It was suggested that, if *Salmonella* can survive after storage, it could also survive and propagate after a home brewing process (Keller *et al.* 2015).

South Africa produces approximately 15 000 - 20 000 tons of Rooibos tea annually, with 6 000 tons being consumed locally and the remainder being exported. In South Africa, Rooibos tea fills roughly 17-20 percent of the available shelf space (Anon

2014). During the processing of Rooibos, the presence of microbes is likely; nonetheless, when pathogens are identified, awareness needs to be implemented, although we would like the product to be without any foodborne pathogens, even before pasteurisation.

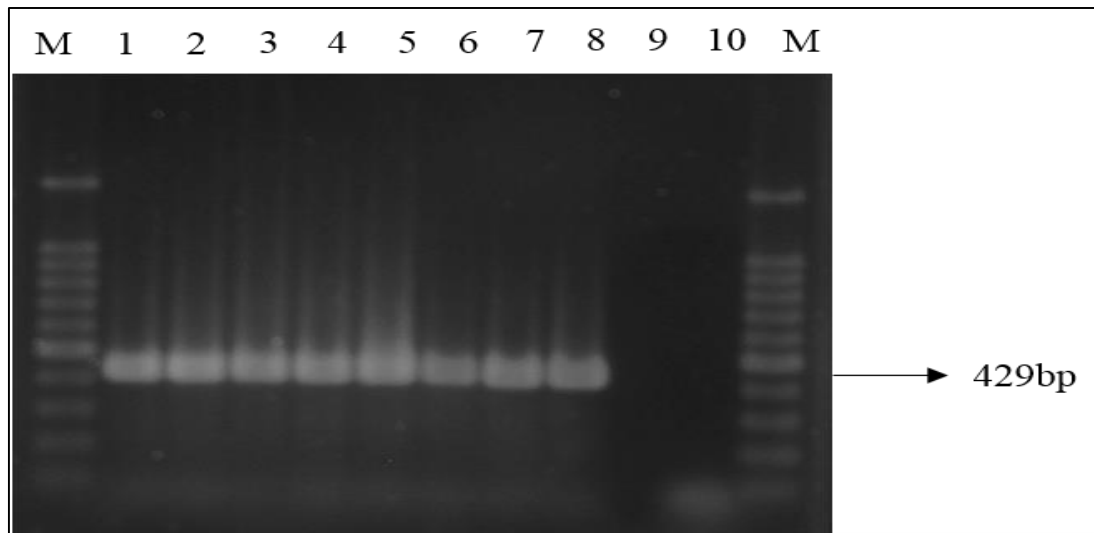


Figure 3.1: Gel electrophoresis pattern - 429bp

Figure 3.1 Representative gel showing amplification of PCR product, with primer set ST11 and ST15, 429bp, as analysed on a 1.5 percent agarose gel. Lane M: DNA ladder (Thermo Scientific GeneRuler 1kb); lane one: *S. enterica* ser. Typhi (ATCC 14028); lanes two-eight: *Salmonella* isolates with ST11 and ST15 primer set taken from Rooibos tea; lane nine: water (negative control); lane ten: *Staph. aureus* (ATCC 25923) (negative control) and lane M: DNA ladder (Thermo Scientific GeneRuler 1kb).

The golden standard for *Salmonella* PCR detection is the 429bp amplification band (Aabo *et al.* 1995) (Figure 3.1). This fragment was identified in all 26 *Salmonella* isolates from Rooibos, which was already confirmed by biochemical testing. The ST11

and ST15 primer set was very discriminating, as only the *Salmonella* genes were amplified. The negative control was therefore not amplified, and the positive control demonstrated that the PCR for 429bp gene was specific and herein confirms that the bacteria isolated was that of the genus *Salmonella* (Figure 3.1).

Inadequate procedures of cultivation and storage, improper treatment, unsatisfactory transportation, long-term desiccation, pitiable hygiene at production level and natural climatic conditions could all cause the Rooibos plants to be susceptible to microbial contaminants (Stevic *et al.* 2012),

A variety of environmental sources could have been implicated in the contamination of the Rooibos tea in this study. Rooibos tea, like any other herbal product, naturally contains a high microbial load. However, downstream processing stages of this product should generally help in diminishing any contaminants present. A study conducted by Du Plessis and Roos (1986), this demonstrates the influence of fermentation on microbial growth. Their data showed that the colony count of Rooibos tea artificially spiked with *E. coli* and *S. enterica* ser. Enteritidis, augmented from 10^4 CFUg⁻¹ to 10^8 CFUg⁻¹ subsequent to fermentation for both microorganisms tested (Du Plessis and Roos 1986).

The presence of *Salmonella* in Rooibos is not well understood. The literature currently accessible is also limited and out-of-date. Studies have identified the presence *S. enterica* ser. Enteritidis and *S. enterica* ser. Lindrick from fermentation heaps (after 23 hours) throughout processing. *S. enterica* ser. Greiz was also isolated from the product after a two minute steam pasteurisation procedure (Du Plessis and Roos 1986).

The first outbreak of salmonellosis associated with consumption of plant products was documented in Vojvodina, Serbia. This occurred from March 2007-September 2008. Fourteen cases of *S. enterica* ser. Senftenberg infection was reported. Outbreaks related to leafy greens from the period 1973-2006, revealed 502 outbreaks, more than 18 000 illnesses, and 15 deaths, with 35 of the outbreaks caused by *Salmonella*; this was reported by the CDC (Herman *et al.* 2008). At least 12 outbreaks have been associated with tomatoes contaminated with *Salmonella* (more than 1 600 cases) since 2000, and three outbreaks related to *Salmonella* contaminated cantaloupes (72 cases) (CDC 2003). However, there are currently no reported outbreaks of salmonellosis that was traced to Rooibos.

The percentages of *Salmonella* positives from 774 samples on leafy vegetables in eight distinct studies were 0 (0/151), 0 (0/63), 0.6 (1/159), 0.9 (1/116), 3.5 (2/57), 6.3 (5/80), 7.1 (2/28), and 68 percent (82/120) (Castillo *et al.* 2004; Froder *et al.* 2007; Harris *et al.* 2003), in comparison to this study 26/60 (43 percent) which is relatively high considering the amount of samples tested.

A study conducted by Gemmell and Schmidt (2012) recognised potential relationships between river water quality and the microbiological quality of vegetation that is irrigated with this water (De Bon *et al.* 2010; Drechsel *et al.* 2006; Obi *et al.* 2002). Consequently, the farming sector is under increased pressure to generate adequate magnitudes of food to sustain the rising demand (Addo 2010; Costello *et al.* 2009; De Bon *et al.* 2010; Schaefer 2008).

In South Africa, the majority of mortalities among 1-14 year olds in 2009 was caused by intestinal infectious diseases (Statistics South Africa 2013), emphasising the

possible risks of consuming products contaminated by wastewater. However, farmers generally use fresh water for irrigation purposes. In this perspective, it is of significance to note that South Africa has been recognised as the country with the highest prevalence of typhoid fever in Africa (Crump *et al.* 2004). To facilitate microbiologically safe production of fresh produce in a short duration, providing small-scale farmers with microbiologically safer drip irrigation systems could be a footstep in the right direction. River water needs improved water treatment facilities and adequate sanitation systems if it is used for irrigation (Gemmell and Schmidt 2013) to reduce the manifestation of microbial pathogens in wastewater or contaminated water sources (Mrayyan 2005). The presence of faecal contamination is detected by selected indicator microbes that are frequently used in risk assessments (Maimon *et al.* 2010). The presence of faecal coliforms is generally supposed to identify the possible presence of other faecal pathogens such as *Salmonella* spp., *Shigella* spp. or pathogenic strains of *E. coli* which is often found on plant material including Rooibos (DWAF 1996; Maimon *et al.* 2010; Mara *et al.* 2007; WHO 1989, 2006).

Workers' hygiene is also influenced by the accessibility and user-friendliness of wash and lavatories on the farms, and proper training of staff. Another concern is the incidence of sick workers in the fields which could also be one of the reasons for the occurrence of *Salmonella* in this study (CDC 2003; FAO/WHO 2008; Herwaldt *et al.* 1997). This underlines the necessity for compulsory health screening. Rooibos, with its comprehensive appeal and usage by worldwide population groups, including high-risk individuals, initiates public safety concerns calls for further microbiological evaluation of the product.

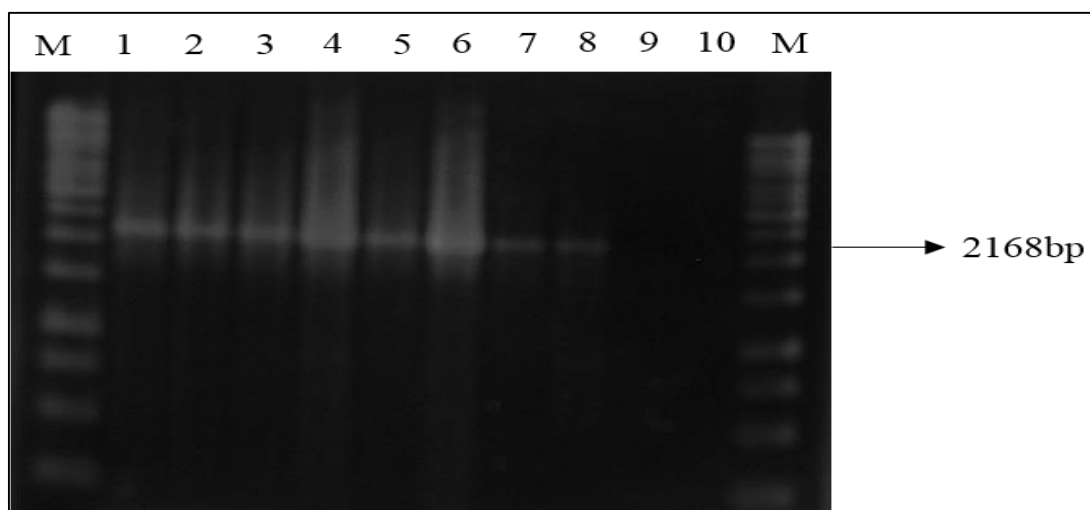


Figure 3.2: Gel electrophoresis pattern - 2168bp

Figure 3.2. Representative gel showing amplification of PCR product, with primer *invA*, 2168bp, from SPI-1 of the *Salmonella* isolates, as analysed on a 1 percent agarose gel. Lane M: DNA ladder (Thermo Scientific GeneRuler 1kb); lane one: *S. enterica* ser. Typhi (ATCC 14028); lanes two-eight: *Salmonella* isolates with *invA* gene extracted from Rooibos tea after fermentation; lane nine: water (negative control); lane ten: *Staph. aureus* (ATCC 25923) (negative control) and lane M: DNA ladder (Thermo Scientific GeneRuler 1kb).

This virulent chromosomal gene, *invA* was depicted on a representative gel (Figure 3.2). *InvA* is recognised as a universal standard for detection of *Salmonella* genus (Lhocine *et al.* 2015; Mezal *et al.* 2013; Troxell *et al.* 2015; Wang *et al.* 2015). The *invA* gene of *Salmonella* comprises sequences exclusive to this genus and has been proved to be a suitable PCR target with potential diagnostic application (Anderson *et al.* 2011; Hu *et al.* 2008; Lhocine *et al.* 2015; Mezal *et al.* 2013).

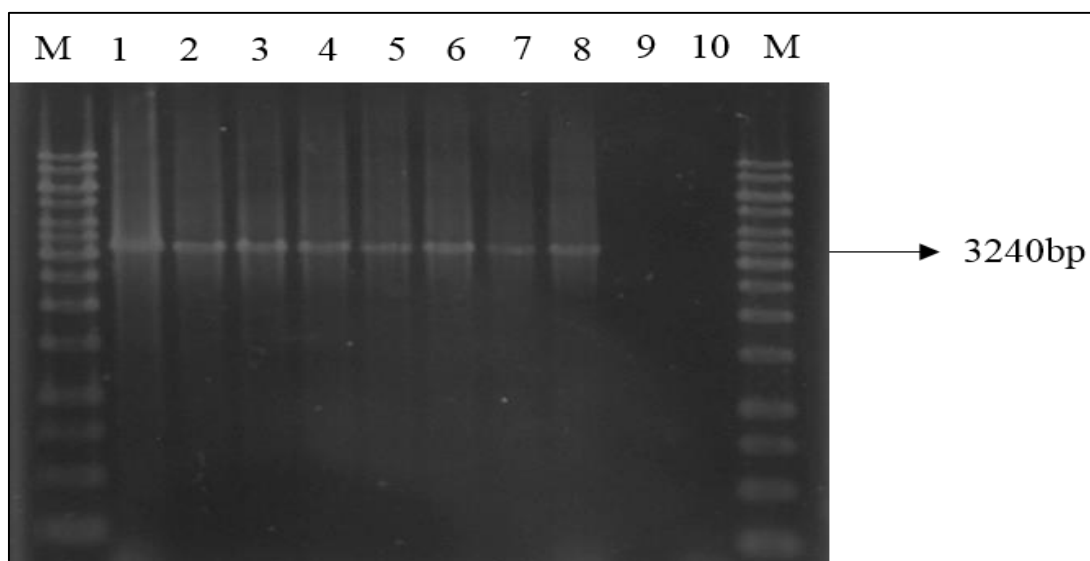


Figure 3.3: Gel electrophoresis pattern - 3240bp

Figure 3.3. Representative gel showing amplification of PCR product, with primer *sipA*, 3240bp, from SPI-1 of the *Salmonella* isolates, as analysed on a 1 percent agarose gel. Lane M: DNA ladder (Thermo Scientific GeneRuler 1kb); lane one: *S. enterica* ser. Typhi (ATCC 14028); lanes two-eight: *Salmonella* isolates with *sipA* gene extracted from Rooibos tea after fermentation; lane nine: water (negative control); lane ten: *Staph. aureus* (ATCC 25923) (negative control) and lane M: DNA ladder (Thermo Scientific GeneRuler 1kb).

Salmonella invasion protein A (*sipA*) as depicted on a representative gel (Figure 3.3), is a secreted effector crucial for the proficient invasion of intestinal epithelial cells within SPI-1 (Hur and Lee 2011; Lhocine *et al.* 2015; Raffatellu *et al.* 2005; Wang *et al.* 2015).

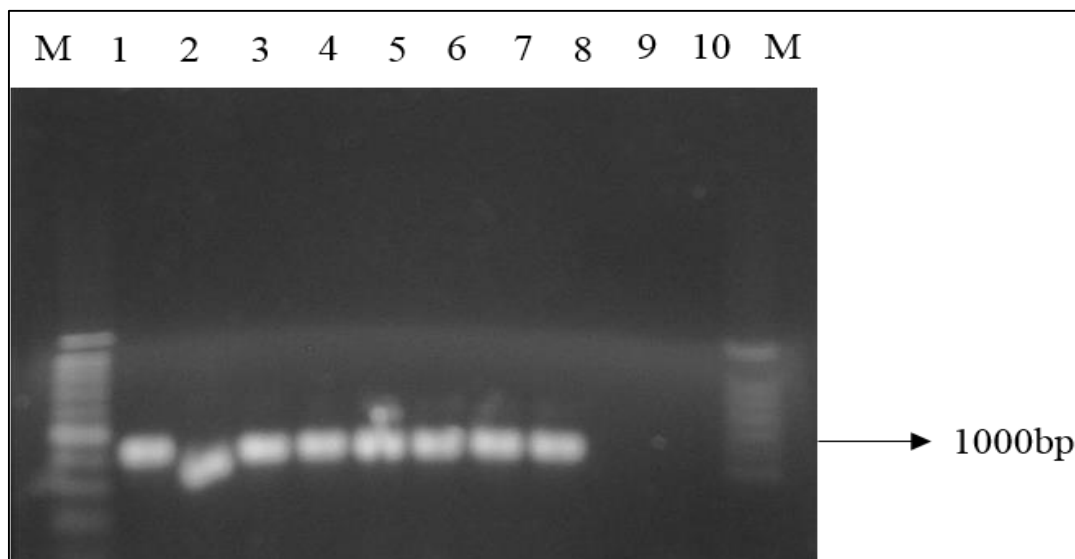


Figure 3.4: Gel electrophoresis pattern - 1000bp

Figure 3.4. Representative gel showing amplification of PCR product, with primer *sifA*, (1000bp), from SPI-2 of the *Salmonella* isolates, as analysed on a 1 percent agarose gel. Lane M: DNA ladder (Thermo Scientific GeneRuler 1kb); lane one: *S. enterica* ser. Typhi (ATCC 14028); lanes two-eight: *Salmonella* isolates with *sifA* gene extracted from Rooibos tea after fermentation; lane nine: water (negative control); lane ten: *Staph. aureus* (ATCC 25923) (negative control) and lane M: DNA ladder (Thermo Scientific GeneRuler 1kb).

Figure 3.4 shows the detection of the secreted effector protein, *sifA*, isolated from *Salmonella* on a representative gel. This serovar Typhimurium effector protein regulates the assembly and tubulation of the *Salmonella* phagosome Drecktrah *et al.* 2008 Hur and Lee, 2011; Rajashekar *et al.* 2008; van der Heijden *et al.* 2015).

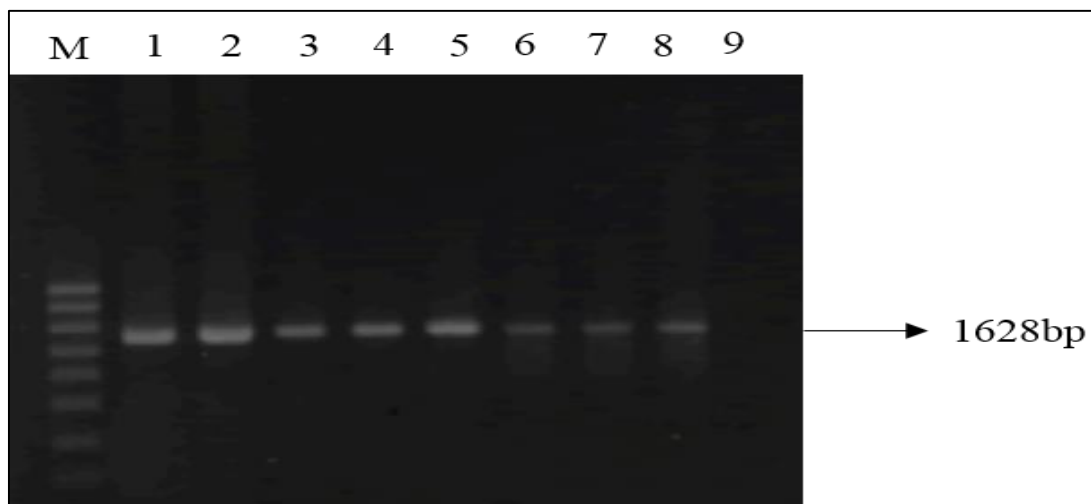


Figure 3.5: Gel electrophoresis pattern - 1628bp

Figure 3.5. Representative gel showing amplification of PCR product, with primer *ssaR*, 1628bp, from SPI-2 of the *Salmonella* isolates, as analysed on a 1 percent agarose gel. Lane M: DNA ladder (Thermo Scientific GeneRuler 1kb); lane one: *S. enterica* ser. Typhi (ATCC 14028); lanes two: *S. enterica* ser. Enteritidis (ATCC 13076); lane three-eight: *Salmonella* isolates with *ssaR* gene extracted from Rooibos tea after fermentation and lane nine: *Staph. aureus* (ATCC 25923) (negative control).

SsaR is a secretion system apparatus protein depicted on a representative gel (Figure 3.5), it is a component critical for the SPI-2 T3SS apparatus (Hur *et al.* 2011). It is usually associated with survival and replication within host cells (Fass *et al.* 2009; Giacomodonato *et al.* 2007; Hu *et al.* 2008; McGhie *et al.* 2009; Zou *et al.* 2012).

Table 3.4: *Salmonella* virulence-related genes isolated from fermented Rooibos

Isolate Number	Virulence Genes			
	<i>invA</i>	<i>sipA</i>	<i>sifA</i>	<i>ssaR</i>
2	+	-	-	-
2A	+	-	+	+
3	-	-	-	-
3B	+	+	-	-
4	-	-	-	-
4A	+	-	-	+
7	-	-	-	-
7A	+	+	+	+
8	+	-	-	-
9	+	-	+	-
9A	+	-	+	-
11	-	-	-	-
11B	+	+	-	-
12B	+	-	-	-
13	+	+	-	+
13A	+	+	-	+
14	-	-	-	-
14A	+	+	-	-
15A	+	+	+	+
16A	+	-	-	-
17	+	-	+	-
18	+	-	-	-
20	+	+	-	-
54 (1)	-	-	-	-
54 (6)	+	-	+	-
54 (7)	-	-	-	-

+/- = tested positive/ negative for virulence gene.

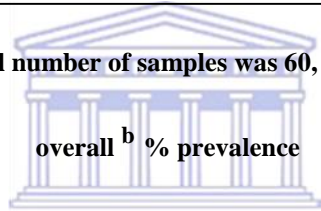
Salmonella spp. involves several genes for complete virulence as it exhibits a multifaceted set of interactions in its host. Most of these genes are located in ‘pathogenicity islands’ gathered on the chromosome in specific regions (Callewaert *et*

al. 2008). Table 3.4 indicates the presence or absence of virulence genes from *Salmonella* in Rooibos.

Table 3.5: Frequency of virulence-related genes in *Salmonella* isolated from fermented Rooibos

Virulence Genes	Number of Positive Strains (%)	Overall ^b % Prevalence
<i>invA</i>	19/26 (73.1)	31.7
<i>sipA</i>	8/26 (30.8)	13.3
<i>sifA</i>	7/26 (26.9)	11.7
<i>ssaR</i>	6/26 (23.1)	10

^b = against all samples. The total number of samples was 60, and this was used to calculate the overall ^b % prevalence



The virulence gene, *invA*, frequency amongst the 26 isolates totalled to 73.1 percent (Table 3.5). Virulence genes that participate in the intestinal phase of infection are found in SPI-1 and SPI-2 and the remaining SPIs are required for intracellular survival, fimbrial expression, magnesium and iron uptake, multiple antibiotic resistances and the development of systemic infections (Almeida *et al.* 2013; Campioni *et al.* 2012; Hapfelmeier *et al.* 2004).

Additionally to invasion, SPI-1 also takes part in intracellular replication, interferes with host responses and favours *Salmonella* survival (Hur *et al.* 2011; Zou *et al.* 2012). This occurrence is, however, not restricted to animals. *Salmonella* isolated from *Arabidopsis thaliana* leaves maintain similar virulence towards animals. This indicates

that illness or diseases may possibly be developed from contaminated plants; however, it is unclear how encoded effectors correlate with plants (Schikora *et al.* 2011).

Eight of the 26 *Salmonella* positive isolates harboured the *Salmonella* invasion protein A gene, *sipA*, (30.8 percent) and the overall prevalence of *sipA* was 13.3 percent (Table 3.5). It was suggested that, by modulating actin dynamics, *sipA* might focus F-actin at the point of bacterial entry and contribute to membrane ruffle formation by promoting their outward extension (Lhocine *et al.* 2015). Moreover, *sipA* has been revealed to disrupt constricted junctions of diverged epithelia (Hur and Lee 2011), a process suggested to be required for the development of diarrhoeal disease (Boyle *et al.* 2006).

Formerly, SPI-1 was thought to be vital for intestinal *Salmonella* pathogenicity. However in 2008, Hu *et al.* showed that SPI-1-deficient *S. enterica* serovars caused human enteropathogenic infection. Furthermore, intestinal inflammation in animal prototypes of *S. enterica* infection has been shown to occur in the absence of SPI-1, in a manner dependent on SPI-2 (Coombes *et al.* 2005; Hapfelmeier *et al.* 2005; Hu *et al.* 2008). Even though there are variances concerning the bovine and murine infections described, the development of SPI-1-independent *Salmonella* enterocolitis possibly will characterise a comparable process. Detecting which virulence factors are involved in SPI-1-independent human intestinal salmonellosis will now be a fascinating and significant opportunity of investigation.

InvA has previously been used as the molecular marker of *Salmonella* diagnosis (Arnold *et al.* 2004; Barthel *et al.* 2003; Bulte and Jakob 1995; Chiu and Ou 1996; Cocolin *et al.* 1998; Cocolin and Comi 1998; Malorny *et al.* 2003; Ochman and Groisman 1996; Pathmanathan *et al.* 2003; Perelle *et al.* 2004; Raffatellu *et al.* 2005;

Rahn *et al.* 1992; Scholz *et al.* 2001; Troxell *et al.* 2015); however, one should re-evaluate molecular detection techniques for *Salmonella* that are based on the absolute presence of SPI-1 in human disease-causing strains of *S. enterica*.

Biochemical tests and PCR confirmed that all the isolates were *Salmonella* serovars. However, some isolates lacked genes critical for SPI-1 function (Table 3.5) *Salmonella* strains that lack the invasion-associated locus SPI-1 have been previously isolated from foodborne disease outbreaks; this indicates that SPI-1 is not necessarily needed for human gastroenteritis (Hu *et al.* 2008).

Table 3.5 shows the prevalence of the secreted effector protein, *sifA*, isolated from SPI-2, which totals to 26.9 percent. SPI-2 also encodes a T3SS, regulatory proteins and effector proteins essential for systematic infection (van der Heijden *et al.* 2015). Genetically, SPI-2 encodes 31 virulence genes. These 31 virulent SPI-2 associated genes are organised in at least four operons (Almeida *et al.* 2013; Campioni *et al.* 2012). *SifA* is encoded by a gene located in the potABCD operon (Hur and Lee 2011). A specific hallmark of *Salmonella* is the expression of a functionally diverse second T3SS encoded by SPI-2 (Shah *et al.* 2011). This SPI-2 locus has been recognised by using the signature tagged mutagenesis method to the systemic mouse model (Hu *et al.* 2008). Comprehensive studies also exposed a diminished colonisation rate of the spleen and liver of a SPI-2 mutant, in comparison to exponentially growing wild-types; henceforward, the island was primarily associated with systemic infections (van der Heijden *et al.* 2015).

Currently, a function of SPI-2 has been exposed in the stimulation of enterocolitis (Coburn *et al.* 2005; Hapfelmeier *et al.* 2005). This locus, *in vitro*, is merely of minor

significance for intracellular phenotypes. A 10-fold lowered rate of intracellular replication has been monitored in cell culture models (Hensel *et al.* 1998). SPI-2 activity promotes functional sequestration of the bacterium enclosing membrane-bound vesicle from the regular endocytic pathway with the purpose of establishing an intracellular niche for bacteriological survival, killing resistance and duplication, the supposed SCV (van der Heijden *et al.* 2015).

The sif are prolonged from the SCV to create an intracellular network (van der Heijden *et al.* 2015). *SifA* are effectors translocated outside of the SPI-2 locus which are restricted to the SCV and involved in the induction of sif (Henry *et al.* 2006; Knodler and Steele-Mortimer 2005; van der Heijden *et al.* 2015). The binding of kinesin via SKIP and *sseJ* reveals a necessity for *sifA* to instigate sif formation (Ohlson *et al.* 2008).

Only six of the 26 *Salmonella* positive isolates harboured the *ssaR* gene (10 percent) (Table 3.5). SPI-2 mutants were detected in primary macrophages and numerous macrophage-like cell lines, as well as in epithelial cell lines previously (Cirillo *et al.* 1998; Hensel *et al.* 1998; Ochman and Groisman *et al.* 1996). Also, *ssaR* mutants of serovar Typhimurium are flawed in intracellular replication in murine macrophages, are weakened in BALB/c mice and are incapable of secreting SPI-2 effectors (Coombes *et al.* 2005; Shea *et al.* 1996). Wild-type serovar Typhimurium or isogenic mutants deficient in *ssaR* (SPI-2 secretion system defective) have previously been described (Brumell *et al.* 2001; Hoiseth and Stocker 1981; Hur *et al.* 2011; Zou *et al.* 2012).

Forest *et al.* (2010) revealed that none of the particular deletion mutations in SPI-2-T3SS-encoding genes (including *ssaR*) altered bacterial uptake or survival, as the amount of bacteria detected at various intervals (subsequent to infection) was comparable to that of the wild-type strain. Astonishingly, mutations in *ssaR* (basal component) which revoke SPI-2 T3SS function or expression, did not influence serovar Typhi uptake and survival in human cells. Akiyama *et al.* (2011) and Mezal *et al.* (2013), also recently showed that the isolates from the environment carried virulence genes similar to clinical isolates, which is responsible for causing human infections.

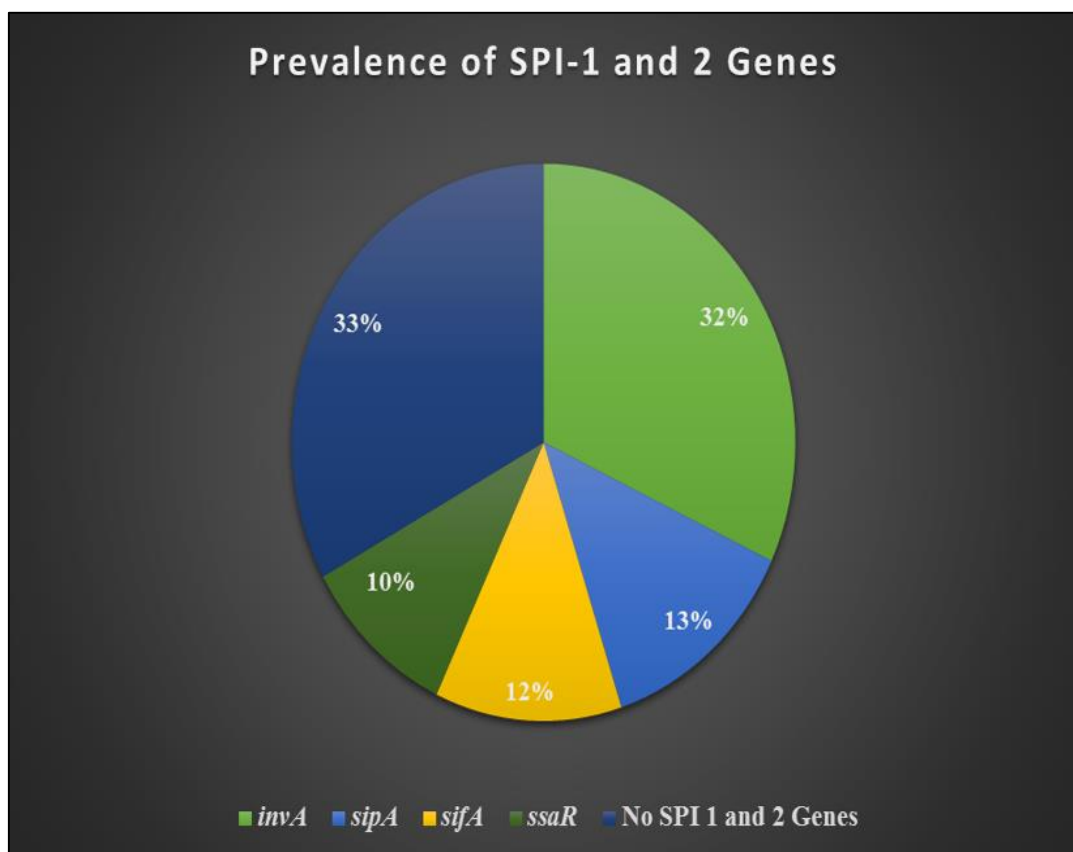


Figure 3.6. The overall prevalence of SPI-1 and 2 genes in *Salmonella* isolated from fermented Rooibos

The overall prevalence of *invA*, *sipA*, *sifA* and *ssaR* was 32 percent, 13 percent, 12 percent and 10 percent respectively (Figure 3.6) and 3.3 percent of the isolates contained all the virulent genes tested. 33 percent of the *Salmonella* isolates lacked the specified SPI-1 and 2 genes. Smith *et al.* (2015) showed the prevalence of the *invA* gene to be 38.6 percent, in comparison to 32 percent in this study (Figure 3.6). However, in a recent study from Korea, the prevalence of the *invA* gene was about 96 percent (Mezal *et al.* 2013). The use of *invA* gene for *Salmonella* detection in food samples is suggested; however, according to a study by Smith *et al.* (2015), the majority of the isolates lacked the virulence-related genes described. One possibility could be that not all isolates comprise virulent plasmids, as lower proportions were described in some studies for the plasmid virulence genes (Gorman 2000; Mezal *et al.* 2013; Todd *et al.* 2008).

Among 25 strains isolated in 2000-2001, 100 percent carried the *sipA* gene, and amongst 17 strains isolated in 2005-2006 approximately 90 percent carried *sipA* (Hur and Lee 2011). However, in this study, only 13 percent of the isolates carried the *sipA* gene (Figure 3.6). The presence of SPI-1-deficient strains of *S. enterica* serovars in various environmental reservoirs, including livestock feed and aquatic environments, has been previously reported in literature (Hu *et al.* 2008; Rahn *et al.*, 1992). These strains, however, are revealed as having reduced invasiveness in vitro, and were considered to be improbable that they would cause human infection due to the weakening of invasion and the conjectured requisite for SPI-1 for in vivo intestinal pathogenesis (Hu *et al.* 2008).

PCR analysis revealed that among 25 *Salmonella* positive isolates in 2000-2001, 96 percent carried the *sifA* genes (Hur and Lee 2011); however, in this present study, *sifA*

was only detected in 12 percent of the isolates (Figure 3.6). *SifA* is translocated across the membrane of the SCV by the SPI 2-encoded T3SS; SPI-1 and 2 genes are regulated differentially, and respond to numerous environmental signals. Mutant strains lacking these SPI-2 genes encoded within this region are highly weakened, subsequent to intraperitoneal, circulatory or oral infection of mice, whereas SPI-1-lacking mutant strains are only weakened after it is orally administrated (Hu *et al.* 2008; Shea *et al.* 1996). This data was confirmed by directed mutagenesis. SPI-2 is relatively conserved amongst the *S. enterica* subspecies; however, it is absent in *S. bongori* (Hu *et al.* 2008).

Among 25 strains isolated in 2000-2001, 100 percent carried the *ssaR* gene, and amongst 17 strains isolated in 2005-2006 approximately 90 percent carried *ssaR* gene (Hur and Lee 2011). In another study of 42 *Salmonella* positive isolates, only 14 percent contained the *ssaR* gene (Hu *et al.* 2008), which is somewhat comparable to this study as only 10 percent contained *ssaR* genes (Figure 3.6). Forest *et al.* proposed in 2010 that the SPI-2 T3SS of serovar Typhi is not essential for survival in human macrophages. However, serovar Typhimurium strains lacking SPI-2 are highly attenuated in the murine systemic infection model (Hensel *et al.* 1998; Shea *et al.* 1996).

Salmonella, lacking SPI-2 genes, was observed throughout survival in whole human blood, invasion and survival of human epithelial cells. It is probable that SPI-2 effectors may be translocated by the SPI-1 T3SS when the SPI-2 T3SS is mutated, but a double SPI-1 (*invA*)/SPI-2 (*ssaR*) mutant in serovar Typhi survives and replicates to a level similar to that of the isogenic wild-type strain (Forest *et al.* 2010), and for this reason the lack of these virulence related genes in *Salmonella* isolates should not be considered as non-pathogenic strains.

3.5 SUMMARY

Recently, promotion of healthier regimes for public wellbeing and consumer demands has led to increased consumption of herbal teas. This trend could also be related to the increase in the amount of outbreaks recorded of foodborne diseases and illnesses. Based on these results, it cannot be assumed that the Rooibos teas available on the market are safe for consumption. Even though the tea has not been pasteurised yet, the alarming presence of *Salmonella* throughout fermentation is intolerable. As some strains such as serovar Senftenberg has a much higher heat resistance than most other *Salmonella* serotypes, it might not be killed during pasteurisation. Current outbreaks of serovar Senftenberg infection resulted from consumption of fresh produce and partially processed foods. Therefore, products processed from plant material should endure more rigorous testing for pathogens, or improved approaches of infection control must be expended. The European Food Safety Authority has stated that all herbs or herbal preparations might become perilous as a result of flaws in the production procedure; for that reason, companies should follow the Hazard Analysis and Critical Control Point (HACCP) systematic approach. To diminish potential risks, economical and effectual methods to decrease microbial loads in contaminated water used for irrigation must be applied. Maintaining good manufacturing and hygiene practices with the use of HACCP at farming, harvesting and processing of material is indispensable. Cold atmospheric gas plasma treatment can be used as an alternative method for the decontamination of fresh and nominally processed food. The presence of the virulence genes that were investigated in this study highlights the pathogenic potential of the *Salmonella* strains, which causes disease in humans. Determining the prevalence of virulence genes of *Salmonella* is significant in predicting the risk of

contracting salmonellosis from food products. Several studies of virulence determinants in *Salmonella* have focused on clinical and veterinary strains only, but this study focused on virulence genes in *Salmonella* isolated from plant material. To fully understand the scale of virulence in *Salmonella* and to combat this emergent problem, it is also vital to consider consumer exposure to potential virulence genes, present in food products. Furthermore, this study provides evidence about the possible public health risks associated with unpasteurised herbal teas. However, the relatively low frequency of these virulence genes should not disregard the fact that *Salmonella* is present in these Rooibos samples, and according to legislation *Salmonella* should be absent in 25g. This product should be free from pathogens and the microbiological status of the tea should be acceptable (according to the regulations), to not only ensure high-quality tea but also that which is safe to consume. It is important to identify any trends in *Salmonella* related infections by conducting routine virutyping of genes. Regardless of the controls that have previously been set into place, *Salmonella* infection that arises from contaminated food remains a great issue, with numerous worldwide outbreaks occurring annually. Detection of *Salmonella* prior to contaminated foods being consumed is therefore an indispensable attribute of safeguarding public health and, parenthetically, preserving the statuses and affluences of food manufacturers and processors. Scrutiny of *Salmonella* in all the various phases of feed-food chain creates a vital component in the investigation of epidemiology of foodborne salmonellosis, and in the progress and execution of proficient *Salmonella* control stratagem.

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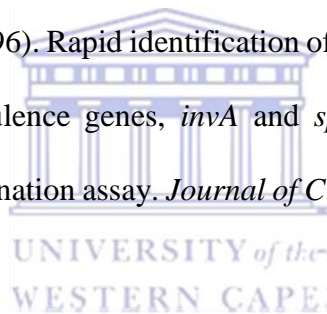
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CHAPTER 4

ANTIBIOTIC RESISTANCE OF *SALMONELLA* STRAINS ISOLATED FROM FERMENTED ROOIBOS

4.1 ABSTRACT

Aim: The objective of this study was to determine the level of antibiotic resistance in *Salmonella* isolated from Rooibos tea.

Methods and Results: Rooibos samples from various farms were collected, after fermentation from the processing plant. *Salmonella* detection was carried out using traditional microbial methods based on the ISO method. Eight different antibiotics i.e. ampicillin, tetracycline, kanamycin, streptomycin, nalidixic acid, sulphafurazole, chloramphenicol and ceftriaxone, all which are conventionally used against *Salmonella*, were evaluated for antimicrobial resistance. The bactericidal resistance of the selected antibiotics were determined by disk diffusion assays. Antimicrobial resistances exhibited among the isolates were to ampicillin (30 percent), tetracycline (11 percent), kanamycin (92 percent) streptomycin (100 percent), ceftriaxone (4 percent), sulphafurazole (92 percent), chloramphenicol (16 percent) and nalidixic acid (16 percent).

Conclusion: The results showed that unpasteurised herbal infusions are possible modes of transmission of pathogenic *Salmonella* isolates that could contribute to the occurrence of salmonellosis and other *Salmonella* related infections. This study presents the first data that validates the potential transmission strains of *Salmonella* harbouring virulence traits, and resistance dynamics from herbal infusions, to cause infections in humans. And it is of significance to note that from the results no

recommendation can be made as a first line drug of treatment for salmonellosis, as no *Salmonella* strain revealed complete susceptibility to any antimicrobial compound.

Significance and impact of study: Determining antimicrobial resistance levels for microbes is important in predicting the risk of proliferation of the microbes in the environment. This study will lead to a better understanding of the fate of *Salmonella* in the processing environment, and is the first to identify antimicrobial resistance in herbal tea. Several studies of resistance in *Salmonella* have focused on clinical and veterinary strains only, but this study focused on *Salmonella* isolated from plant material. To completely appreciate the scale of antimicrobial resistance and to oppose this emergent problem, it is also essential to consider consumer exposure to resistant strains present in food products. Additionally, this work provides information about the possible public health risks associated with unpasteurised herbal teas. It is also imperative to recognise any trends in antimicrobial resistance by conducting routine screening. Currently, no reports have been published about antibiotic resistance in *Salmonella*, isolated from herbal teas, in South Africa.

4.2 INTRODUCTION

Salmonellae are considered as potential human pathogens, as it generally causes human infections and diseases (Abakpa *et al.* 2015). Traditionally, *Salmonella* is divided into a small number of human-restricted invasive typhoidal serotypes (*S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A) and thousands of non-typhoidal *Salmonella* serotypes, these serotypes characteristically have a broad vertebrate host range and cause various diseases (Gordon *et al.* 2012).

Salmonella is an important causative agent of foodborne infections, with a comprehensive host spectrum (Wei *et al.* 2011), where certain cases are so severe it often requires antimicrobial therapy for treatment (Marrero-Ortiz *et al.* 2012). It is habitually isolated from environmental sources that serves as relay for the microbes and co-operate in its dispersal amid various hosts (Abakpa *et al.* 2015).

Salmonella species are becoming progressively resistant, making it more problematic to treat patients with severe infections (Rusul *et al.* 2012). This makes multidrug-resistant *Salmonella* an imperative subject area of exploration and a foremost concern for food safety (Abakpa *et al.* 2015; Levantesi *et al.* 2012; Rusul *et al.* 2012).

Salmonella has the capability to adapt to diverse external conditions, including low pH or high temperature; this allows it to survive outside the host organism (Semenov *et al.* 2007). Undeniably, *Salmonella* is able to attach and adhere to plant surfaces prior to dynamically infecting the interior of various plants, steering to the colonisation of plant organs (Gu *et al.* 2011; Klerks *et al.* 2007) and destruction of the plant immune system (Schikora *et al.* 2012). In addition, *Salmonella* originating from plants retains virulence toward animals (Schikora *et al.* 2012); thus, plants are an alternative host for *Salmonella* pathogens and have a role in its transmission back to animals.

Rooibos (*Aspalathus linearis*) is a flowering shrub-like bush, innate to the Cedarberg Mountains in the Western Cape region of South Africa, where it is comprehensively cultivated (Gouws *et al.* 2014). Rooibos has been traditionally utilised for therapeutic purposes; treating asthma, colic, eczema, headache, nausea and mild depression (Abakpa *et al.* 2015; Gouws *et al.* 2014).

The tea is rich in polyphenols, caffeine-free and comprises a rare source of the dietary dihydrochalcones aspalathin and nothofagin (Gouws *et al.* 2014). Polyphenols have generally shown antimicrobial potential; however, their activity is reliant on the bacterial species and type of polyphenols present. Rooibos tea is consumed largely worldwide; therefore, a need arises for the production of tea that is microbiologically safe to drink and of superior export quality (Bagudo *et al.* 2014; Gouws *et al.* 2014). Internationally, and in South Africa, a microbiological standard has been prescribed which Rooibos export enterprises have to follow. While their regulations differ somewhat; they all have a zero tolerance for the presence of *Salmonella* spp. (Abakpa *et al.* 2015; Agrawal *et al.* 2013; Gouws *et al.* 2014).

Public health intercessions, such as access to safe water, adequate sanitation, hygiene, vaccinations, schooling, health communication, and access to acute medical care can promote improvements in health, and in social and financial development (Abakpa *et al.* 2015; Khan *et al.* 2007). One consequence of the improved availability of antimicrobial agents for the indicative treatment of illness in hospitals, however, has been the development of antibiotic resistance in pathogens, and this poses a health concern (Abakpa *et al.* 2015; Agrawal *et al.* 2013). Antimicrobial resistance is, indeed, a matter of profound importance for public health globally (Bagudo *et al.* 2014; Zakar *et al.* 2012).

Climatic change and urbanisation have impeded Earth's freshwater resources (Zakar *et al.* 2012). These factors cause freshwater to become progressively scarcer worldwide; many countries resort to utilising treated wastewater directly for domestic, manufacturing, and agricultural uses (Hamilton *et al.* 2006). Nevertheless, the detection of residue-level antimicrobials and pathogens have frequently been reported in wastewater emissions and in recycled wastewater (Fatta-Kassinos *et al.* 2011).

Due to extended exposure to antimicrobials remaining in wastewater, some bacteria, such as *Salmonella*, acquire resistance to these antimicrobial compounds (Abakpa *et al.* 2015). In the occurrence of low levels of antimicrobials, bacteria have revealed the capability to attain antibiotic resistance genes (Gal-Mor 2010). Genetically modified crops, with antibiotic resistance marker genes (microbes added intentionally or unintentionally to the food chain that could potentially transfer antimicrobial resistance genes) and food processing technologies that use antibiotics are also matters of concern (Gay and Gillespie 2005; Gouws 2015).

People are, in general, very dependent on antibiotics for the treatment of infectious diseases; however, it should never be considered more than commodities in food and agriculture (Fielding *et al.*, 2012). Although the main focus is to control antibiotic usage, there is little understanding of the situation with regards to antibiotic resistance in disease-causing pathogens.

Meagre medical and veterinary antibiotics, as well as the lack of concern for the practice of infection prevention and control, has put South Africa in a situation whereby most of the foodborne infections caused by pathogens are untreatable (Gouws and Brözel 2000). With the widespread use of agricultural antibiotics, evolution of

disease-causing microbes resulted in many antibiotics losing their efficacy. As microbes generally evolve, they adapt to their environment (Gouws 2015). If antimicrobials inhibit them from growing and spreading, they evolve novel mechanisms to counterattack the antibiotics by altering their genetic makeup (Abakpaa *et al.* 2015). As a result, altering their genetic structure ensures that the progeny of the resilient microbes also are resistant (Capita and Alonso-Calleja 2013). Most microbes reproduce by dividing every few hours, permitting them to evolve rapidly and acclimatise quickly to new environmental conditions. Throughout replication mutations arise; some of these mutations may assist in the survival of an individual microbe when exposed to an antimicrobial (Gouws 2015).

Microbes could acquire genes from each other as well, including the genes that make the microbe drug-resistant. The use of antibiotics, even when they are utilised appropriately, creates a discriminating pressure for resistant organisms (Gouws, 2015). As a result, an alarming escalation in the prevalence of ARBs combined with the meagre success rate of the pharmaceutical industry's antibiotic discovery systems, is one reason for significant distress (Fatta-Kassinos *et al.* 2011; Hamilton *et al.* 2006).

Respiratory and enteric diseases involve an extensive proportion of the affliction of morbidity and mortality in the emerging world; severe respiratory infections and diarrheal illness are the leading two killers of children under 5 years of age worldwide (Fatta-Kassinos *et al.* 2011). Reproductive tract pathogens cause rudimentary infections of the mucosal membranes; nonetheless, if left untreated, infections with these pathogens could lead to pelvic inflammatory diseases, ectopic pregnancies and barrenness, which could also assist the transmission of HIV.

Antibiotic-resistant *Salmonella* has become a problem due to several reasons. There is an urgent need for alternative classes of antimicrobial compounds that can be used in the management of *Salmonella* infections (Khan *et al.* 2007). Some authors have postulated that the antimicrobial resistance of *Salmonella* strains could be related with the expression of certain virulence genes including *invA* (Dione *et al.* 2011).

Antibiotic-resistant serovar Typhi is generally resistant to first-line antibiotics, such as chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole. These strains became prevalent in some Asian countries during the late 1980s to early 1990s and surfaced as a major therapeutic problem (Kumar *et al.* 2011). Fluoroquinolone was then considered as an effective agent for the treatment of antibiotic-resistant typhoid fever (Marrero-Ortiz *et al.* 2012). However, with the increased usage of fluoroquinolone to treat enteric fever, strains of serovar Typhi with diminished susceptibility to ciprofloxacin arose in the Indian subcontinent, southern Asia and sub-Saharan Africa; this was then allied with clinical treatment failure (Kumar *et al.* 2011).

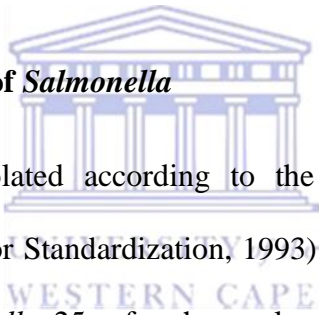
Patients infected with nalidixic acid resistant serovar Typhi strains ought to be treated with ceftriaxone. Ceftriaxone, cefotaxime and cefixime are currently used effectively for the treatment of enteric fever, including nalidixic-resistant and fluoroquinolone-resistant strains (Abakpaa *et al.* 2015, Gal-Mor *et al.* 2010; Kumar *et al.* 2011).

4.3 MATERIALS AND METHODS

4.3.1 Sample Collection of Rooibos Tea

Sixty samples of Rooibos tea were collected from several different processing plants in the Cederberg area (within the Western Cape region), from August to December during 2013-2014. The samples were collected just after fermentation, and drying; several samples were assembled before, and others after, pasteurisation. The dried tea leaves were then packaged into sterile whirl-pak bags and transported to the laboratory. Samples were labelled according to its specific origin, with date of collection, prior to and after pasteurisation. The samples were subjected to a controlled environment until it arrived at the laboratory, where it was stored in a cool arid place for further analysis.

4.3.2 Bacterial Isolation of *Salmonella*



Salmonella strains were isolated according to the Standard ISO-6579 method (International Organization for Standardization, 1993) with some modifications. For the pre-enrichment of *Salmonella*, 25g of each sample was homogenised in sterile bags with 225ml of BPW (Merck Biolab) and incubated at 37°C for 18-24 hours. Following the incubation, 0.1ml of PBW was added to 10ml of RVS broth (Merck Biolab) followed by further incubation at 41.5°C for 24 hours. A loopful of culture was then taken from the selective enrichment RVS broth, streaked onto XLD (Merck Biolab), and incubated at 37°C for 24 hours. Subsequent to incubation the black colonies, an indication of H₂S production, were assumed to be presumptive positives of *Salmonella*. The colonies were then re-streaked onto TSA (Merck Biolab) which was incubated for 24 hours at 37°C.

Several single colonies were inoculated into TSB (Merck Biolab) to characterise each *Salmonella* isolate culture for further identification. The isolates were then subjected to Gram stain analysis to confirm Gram reaction and morphology. The biochemical analysis of each isolate was done, as a confirmatory test using the API 20E (Biomerieux) system for accurate identification and confirmation.

4.3.3 Confirmation of *Salmonella*

4.3.3.1 Biochemical Confirmation of *Salmonella*

Biochemical identification of *Salmonella* isolates was performed by utilising the API 20E system (Biomerieux) for characterisation and confirmation. The API 20E system (Biomerieux) is a plastic strip that consists of 20 microtubes, containing parched substrates (Murray, 1979). The API 20E (Biomerieux) was ultimately developed for the identification of Enterobacteriaceae and it includes various tests such as: o-nitrophenile- β -D-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilisation, hydrogen sulphide production, urease, tryptophan deaminase, indole production, acetoin production by the Voges-Proskauer test, gelatinase and fermentation of glucose, mannose, inositol, sorbitol, rhamnase, sucrose, melibiose, amygdaline and arabinose. One colony representing a presumptive *Salmonella* isolate was re-suspended in deionised water and this was inoculated into the API 20E (Biomerieux) test strips. This was done according to the manufacturer's instructions to identify the Gram negative organism.

4.3.4 Antimicrobial Susceptibility Testing

Each culture tested was streaked onto TSA (Merck Biolab) to obtain isolated colonies. After incubation at 37°C overnight, two well isolated colonies were selected with an inoculating needle loop, and transferred to a tube of 1ml TSB (Merck Biolab) and mixed thoroughly, followed by incubation until turbid (usually 16-24 hours) at 37°C. The inoculums were then set to 0.5 McFarland Standard by adjusting the turbidity to the proper density (Appendix A). Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was then dipped into the suspension and streaked over the entire surface of Muller-Hinton (MH) agar (at most three times), rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum. This was done to perform a disk agar diffusion assay.

The antibiotic susceptibility of the isolates was determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013) Disk agar diffusion assays was done with a panel of eight different antibiotic agents, and this was done in duplicate to confirm the results. The antibiotics tested were as follows: ampicillin (AMP) (10 µg), tetracycline (TE) (30 µg), kanamycin (K) (30 µg), streptomycin (S) (10 µg), nalidixic acid (NA) (30 µg), and sulphafurazole (SF) (300 µg), chloramphenicol (C) (30 µg) and ceftriaxone (CRO) (30 µg) (Oxoid). The disks containing the antibiotics were then placed on the agar plates with a disk dispensing apparatus. Diffusion of the drug in the disk begun immediately; therefore, once a disk makes contact with the agar surface, the disks were not to be moved. After the disks were placed on the plate, it was incubated at 37°C for 16-18 hours. Subsequent to incubation, the zone diameters were measured of complete inhibition (including the

diameter of the disk) and it was recorded in millimeters and inferred based on analysis of zone diameter of test culture provided by CLSI (2013). The zone meters

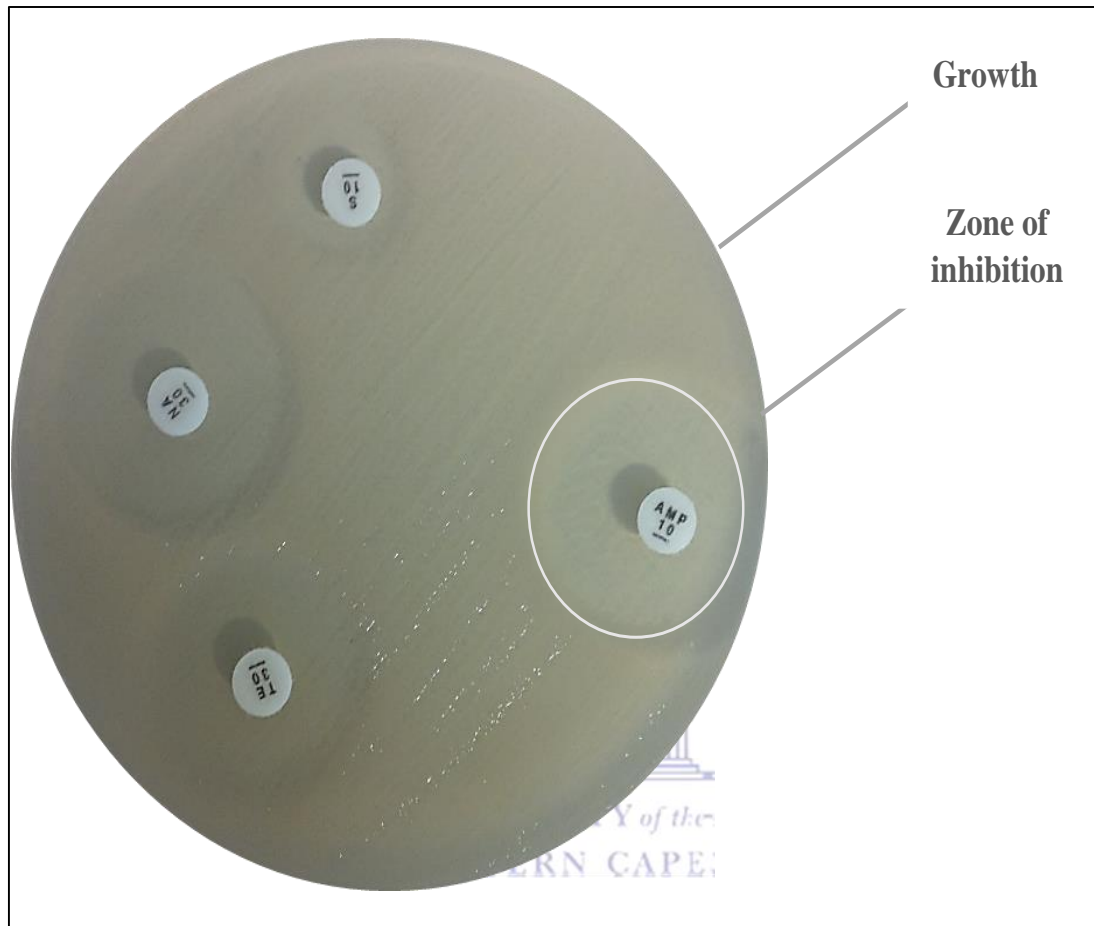


Figure 4.1: The antimicrobial susceptibility disc diffusion test

Figure 4.1 The antimicrobial susceptibility disc diffusion test: disk placement and measurement of inhibition zone diameters on MH agar. Antibiotic disks S 10: streptomycin (10µg), AMP 10: ampicillin (10µg), TE 30: tetracycline (30µg) and NA 30: nalidixic acid (30µg).

The distance was measured from the inner-most colonies (i.e., those closest to the disk) to the center of the antimicrobial disk, and this measurement was doubled to obtain the diameter (Figure 4.1); the measurement was then recorded and interpreted based of

antimicrobial susceptibility. The interpretive categories determined as susceptible, intermediate or resistant were utilised according to CLSI guidelines (CLSI 2013) and diameter of zone of inhibition (Appendix B).

E. coli (ATCC 25922) strain which is sensitive to all the antibiotics was used as a quality control organism and *S. enterica* serovar Typhi (ATCC 14028) was used as a positive control. An isolate was defined as ‘resistant’ subsequent to confirmation of resistance to at least one antibiotic agent tested, and ‘multiple resistance’ was defined as resistance to two or more antibiotic agents (CLSI 2013).



4.4 RESULTS AND DISCUSSION

In this study, a total of 26 *Salmonella* isolates were subjected to antimicrobial susceptibility testing by disk diffusion technique. These were again tested against eight commonly used antibiotics (Agrawal *et al.* 2013); this revealed several resistance patterns. All isolates exhibited multidrug resistance (MDR), each isolate being resistant to two or more antibiotics (Table 4.1). Resistance to streptomycin (10µg) was the most common resistance phenotype observed among all the isolates (Table 4.1). Two isolates (2A and 15A) from Rooibos tea exhibited resistance to six antibiotics tested and one isolate (7A) exhibited resistance to seven of the eight antibiotics tested (Table 4.1). This signifies a great public health concern as some incidences of salmonellosis are severe and need antimicrobial treatment (Marrero-Ortiz *et al.* 2012). Henceforward, these MDR *Salmonella* strains obtained from Rooibos processing farms is a major concern for food safety. The concern is heightened as MDR *Salmonella* isolates have been suggested to be more virulent than non-MDR *Salmonella* isolates (Foley and Lynne 2008).

The detection of these MDR *Salmonella* strains in this study demands attention. The findings show that the isolates have the potential to develop resistance for routinely prescribed antibiotic drugs and proffers significant health vulnerabilities to consumers; therefore, the need for prudent control measures arises. However, it cannot be said with certainty that the antimicrobial resistance genes could have been traced from a definitive source due to small quantity of isolates tested. The mechanisms from which *Salmonella* develops resistance consists of the production of enzymes, that can disintegrate cell permeability to antibiotics, stimulation of antimicrobial efflux pumps,

and production of β -lactamase to vitiate the chemical structure of antimicrobial agents (Foley and Lynne 2008; Sefton 2002).

Table 4.1: Antimicrobial resistance of *Salmonella* strains isolated from Rooibos tea by disk diffusion assay

Isolate Number	Degree of Susceptibility							
	AMP ^a	TE ^a	K ^a	S ^a	CRO ^a	SF ^a	C ^a	NA ^a
2	S	I	R	R	S	R	I	I
2A	R	R	R	R	I	R	I	R
3	S	S	R	R	I	R	S	I
3B	R	I	R	R	S	R	S	I
4	S	I	R	R	S	R	I	I
4A	R	S	R	R	I	R	S	R
7	I	I	R	R	I	R	S	I
7A	R	R	R	R	I	R	R	R
8	S	I	R	R	I	R	S	I
9	I	S	R	R	I	R	I	I
9A	R	I	R	R	I	R	R	I
11	I	S	R	R	I	I	S	S
11B	S	S	R	R	S	R	S	I
12B	I	I	R	R	I	R	S	I
13	S	S	R	R	R	R	I	S
13A	R	I	R	R	S	R	I	I
14	S	I	R	R	I	R	S	I
14A	S	I	R	R	I	R	I	I
15A	I	R	R	R	S	R	R	R
16A	R	I	I	R	I	I	I	I
17	I	S	R	R	S	R	R	S
18	I	I	R	R	I	R	I	I
20	R	I	R	R	I	R	I	I
54 (1)	I	I	I	R	I	R	S	I
54 (6)	S	I	R	R	I	R	I	S
54 (7)	I	I	R	R	I	R	I	I

Degree of susceptibility: R=resistant, I=intermediate, S=susceptible

^aAMP^a: ampicillin (10µg), TE^a: tetracycline (30µg), K^a: kanamycin (30µg) and S^a: streptomycin (10 µg), CRO^a: ceftriaxone (30µg), SF^a: sulphafurazole (300µg), C^a: chloramphenicol (30 µg) and NA^a: nalidixic acid (30µg).

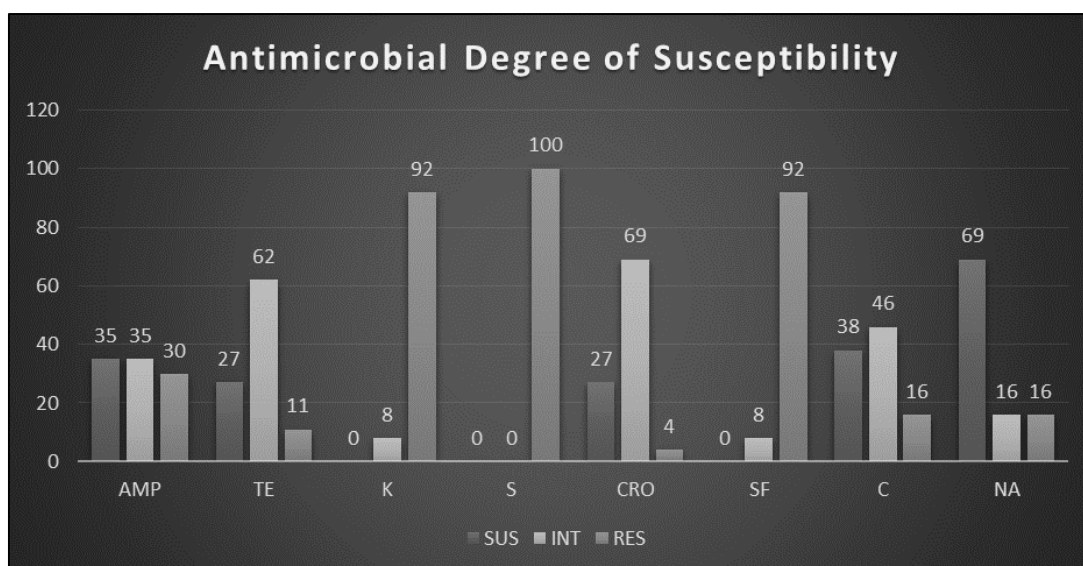


Figure 4.2: Degree of antimicrobial susceptibility of *Salmonella* strains isolated from Rooibos tea by disk diffusion assay

Percent susceptibility of AMP: ampicillin (10µg), TE: tetracycline (30µg), K: kanamycin (30µg) and S: Streptomycin (10 µg), CRO: ceftriaxone (30µg) SF: sulphafurazole (300µg), C: chloramphenicol (30 µg) and NA: nalidixic acid (30µg). Antimicrobial degree of susceptibility RES: resistant, INT: intermediate and SUS: susceptible.

Previously, chloramphenicol and ampicillin (first generation antibiotics), were used as treatment for *Salmonella* infections (Birosova and Mikulasov 2009). However, as depicted in (Figure 4.2), 30 percent of the isolates demonstrated resistance to ampicillin and 16 percent to chloramphenicol; this suggests that it will not remain

effective for much longer. The isolation of highly resistant *Salmonella* strains from herbs has been previously reported (Brockmann *et al.* 2004; Zhao *et al.* 2006), including serovar Typhimurium DT 104; this salmonellosis outbreak was associated with sesame seed consumption (Guérin *et al.* 2001; Little 2001). It was typically resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline. The prevalence of ARB *Salmonella* strains in these herbs seem to be higher than that found for imported foods in general, where values ranged from 8-17 percent for isolates from food samples collected throughout 2001-2005 (Zhao *et al.* 2003, 2006).

In a study of virulence genes by Dione *et al.* (2011) they observed a high prevalence (99.5 percent) of strains carrying *invA*. They discovered significant correlations between the presence of this gene and other virulence genes, as well as antimicrobial resistance to amoxicillin, ticarcillin, sulfamethoxazole, tetracycline, trimethoprim, streptomycin and sulphonamides (Agrawal *et al.* 2013; Thai *et al.* 2012).

Salmonella strains showed the highest susceptibility to Nalidixic acid at 69 percent (Figure 4.2). Kumar *et al.* (2011), reported *Salmonella* strains having a higher susceptibility to chloramphenicol to (95.3 percent) and ampicillin (94.5 percent); however, the results were not comparable in the present study as it was much lower. *Salmonella* strains that were Tetracycline resistant was at 11 percent, as observed in (Figure 4.2); this is one of the most extensively used antibiotics in human and veterinary medicine practice (Carraminana *et al.* 2004; Thai *et al.* 2012; Yan *et al.* 2010). *Salmonella* isolates demonstrated complete resistance to streptomycin (100 percent). Additional reports in other countries (Chen *et al.* 2004; Poppe *et al.* 2001; Yan *et al.* 2010) also revealed that *Salmonella* isolates were generally resistant to this antibiotic.

The ever-increasing resistance of *Salmonella* to universal antibiotics has been routing to the usage of third-generation cephalosporins, in order to combat salmonellosis. As a result, ceftriaxone is now being utilised specifically for treating children (Agrawal *et al.* 2013; Endt *et al.* 2012). However, only 27 percent of the isolates showed sensitivity to ceftriaxone, and 69 percent showed intermediate susceptibility (Figure 4.2); as a result, this antibiotic will not remain effective for treatment of salmonellosis.

In a study by Abakpa *et al.* (2015), the presence of *Salmonella* strains in vegetables and environmental samples from irrigation sites in Nigeria were revealed; farmers irrigated vegetables with untreated wastewater, as well as using untreated animal manure in produce production. *Salmonella* detection from vegetation is usually a sign of faecal contamination; this may possibly be tracked to sewage emissions into wastewater used for irrigation of vegetables. Bagudo *et al.* (2014) found that contamination of natural water increased the intensity of detection, incidence and perseverance of pathogenic microbes in parts involved with sewage discharge. However, its detection in this study is not related to the usage of untreated animal manure as a basis of nutrient replacement in the soil, as no manure or antibiotics is used in the production and processing of Rooibos tea; fresh water is generally used in harvesting of Rooibos.

According to Levantesi *et al.* (2012), plants could also be used as vectors for some pathogens to live in until they reach a human host. Plants are generally not optimal environments for *Salmonella*, which stresses the bacteria and this could amplify the potential of developing resistances to harsh environments, such as those with antibiotics present (Thomas 2014). The antibiotics accumulated inside the plant may also be considered a stress to the internalised *Salmonella*.

It is probable that these isolates could have attained resistant genes to several antibiotics from other enteric bacteria (Marrero-Ortiz *et al.* 2012; Rusul *et al.* 2012). This characterises an immense public health concern as particular cases of salmonellosis are severe and usually require antimicrobial therapy for treatment. Bhullar *et al.* (2012) recently reported a screen of samples from the culturable microbiome of Lechuguilla Cave in New Mexico. This region of the cave was secluded for over four million years and it was revealed that, like surface microbes, these bacteria tested were found to be highly resistant to antibiotics; certain strains were resistant to as many as 14 various commercially available antibiotics currently (Bhullar *et al.* 2012). The implications of the Bhullar *et al.* (2012) study was significant in understanding the occurrence of resistance, and in this case microbiomes that were isolated from human use of antimicrobial agents. This supports a growing awareness that antibiotic resistance is native, prehistoric, and built-in the microbial pan genome (Bhullar *et al.* 2012). Hence, this could be another reason for the high level of antimicrobial resistance within the *Salmonella* strains that was isolated from Rooibos tea, as no antibiotics were utilised during cultivation and processing. The theory of the antibiotic resistome suggests that resistance is the result of active and competitive microbial interactions that ante-cede human use of antibiotics (Wright, 2007, 2010).

A study of soil *Actinomycetes* reported prevalent MDR, even in the absence of evident human sources of antibiotics (Bhullar *et al.* 2012; D'Costa *et al.* 2006). These studies support a proposition that resistance is a primitive and genetically-rich biological phenomenon, profoundly rooted in the microbial pan genome. There is also an increasing amount of evidence suggesting that non-pathogenic environmental

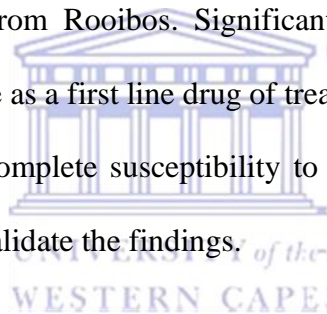
organisms could act as reservoirs of resistance genes which have the potential to be transferred to pathogenic bacteria (Allen *et al.* 2009; Donato *et al.* 2010).



4.5 SUMMARY

Emergent drug resistance in *Salmonella* has compelled scientists to explore alternatively novel classes of antimicrobial agents. Farmers depend mainly on untreated wastewater sources for irrigation purposes, while also using raw animal manure as nutritional sources to vegetables. Moreover, preceding studies report that *Salmonella* can live for lengthy periods of time in the farm environment, as there is constant movement within the farm from wildlife, humans, livestock faeces, soil and vegetation. The alarm is intensified by reports that say MDR *Salmonella* isolates have been described as being more fatal than non-MDR *Salmonella* isolates. The detection of these resistant *Salmonella* strains in this study demands responsiveness. The findings specify that these isolates have the ability to attain resistance for customarily recommended antimicrobial drugs and present significant health risks to consumers; henceforward, the requisite for introduction of prudent control actions. It cannot, however, confirm that the antimicrobial resistance genes occurred from a conclusive source as only a small number of isolates were tested. Consumption of Rooibos tea without adequate decontamination and suitable pasteurisation signifies a serious public health risk. This study presents the first data that validates the potential transmission strains of *Salmonella*-harbouring virulence and resistance dynamics from herbal infusions, causing infections in humans. Better awareness of the factors that possibly influence the development and distribution of resistant zoonotic pathogens, can enhance the implements used by public health experts to regulate antimicrobial resistance, which can restrict possible therapeutic options in acute cases of salmonellosis. With the amplified mindfulness of the health benefits of consumption of Rooibos tea, the deficiency of access to safe water by farmers and the rise in organic

production in South Africa, stern compliance with the treatment of wastewater is encouraged. The crucial consequences ascending from these situations, the chain of transmission of *Salmonella* and its resistance mechanism should be meticulously studied and monitored to lessen the dispersal and danger to human health. This study also supports the argument that antibiotic resistance could be native, prehistoric, and built-in the microbial pan genome; this may possibly be another reason for the high level of antimicrobial resistance within the *Salmonella* strains that were isolated, as no antibiotics were utilised during cultivation and processing of Rooibos. Now, recently, vast arrays of pharmaceuticals (including antibiotics), have been found in the drinking water supplies; this too could have been another reason for the level of resistance found in the *Salmonella* isolates from Rooibos. Significantly, and from the results, no recommendation can be made as a first line drug of treatment for salmonellosis, as no *Salmonella* strain revealed complete susceptibility to any antimicrobial compound. Further trials are needed to validate the findings.



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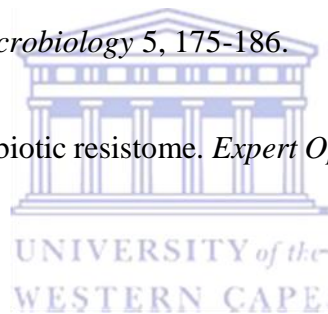
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CHAPTER 5

CONCLUSION

5.1 CONCLUSION

Determining the prevalence of virulence genes of *Salmonella* could be significant in predicting the risk of contracting salmonellosis from food products. This study will therefore lead to a better understanding of the fate of *Salmonella* in the processing environment and it is the first to identify virulence genes in herbal tea. Several studies of virulence determinants in *Salmonella* have focused on clinical and veterinary strains only, however this study focused on virulence genes in *Salmonella* isolated from plant material. To fully understand the scale of virulence in *Salmonella* and to combat this emergent problem, it is also vital to consider consumer exposure to potential virulence genes present in food products. Furthermore, this study provides evidence about the possible public health risks associated with unpasteurised herbal teas. It is therefore crucial to identify any trends in *Salmonella*-related infections by conducting routine virotyping of genes. Currently, no reports have been published about virulence genes in *Salmonella* (isolated from herbal teas) in South Africa. Studies on these virulence factors may provide new insights into the prevention and treatment of salmonellosis. Regardless of the controls that have previously been set into place, *Salmonella* infection that arises from contaminated food remains a great issue, with numerous outbreaks occurring annually worldwide. Detection of *Salmonella* prior to contaminated foods being consumed is therefore an indispensable attribute of safeguarding public health and, parenthetically, preserving the statuses and affluences of food manufacturers and processors. Scrutiny of *Salmonella* in all the various phases of feed-food chain creates a vital component in the investigation of epidemiology of

foodborne salmonellosis, and in the progress and execution of proficient *Salmonella* control stratagems. The reduction in the number of microorganisms in Rooibos tea is, however, challenging due to the subtle nature and flavour properties of the plant. Less abrasive approaches are therefore necessary to control microbial contamination. The results also revealed that unpasteurised herbal teas are potential mechanisms for the transmission of pathogenic *Salmonella* isolates; this could be linked to the prevalence of salmonellosis and other *Salmonella*-related infections. Currently, rooibos tea farmers use steam pasteurisation, whereby water is heated to approximately 180°C to generate steam. Methods such as the use of radiation, *Salmonella*-specific bacteriophages and ozone treatment, however it is more expensive than other available methods. Determining antimicrobial resistance levels for microbes is important in predicting the risk of proliferation of the microbes in the environment. To completely appreciate the scale of antimicrobial resistance and to oppose this emergent problem, it is also essential to consider consumer exposure to resistant strains present in food products. Additionally, this work provides information about the possible public health risks associated with unpasteurised herbal teas. It is also imperative to recognise any trends in antimicrobial resistance by conducting routine screening. Currently, no reports have been published about antibiotic resistance in *Salmonella* isolated from herbal teas in South Africa. The results also showed (and it is of significance to note) that from the results no recommendation can be made as a first line drug of treatment for salmonellosis, as no *Salmonella* strain revealed complete susceptibility to any antimicrobial compound. From this study it was concluded that research in Rooibos is extremely limited and the potential of this food product is so immense, that additional research needs to be carried out.

APPENDIX A

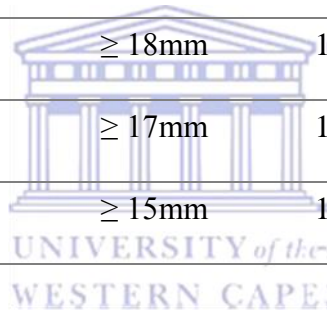
PREPARATION OF TURBIDITY STANDARDS (MCFARLAND TURBIDITY STANDARDS)

Commercially prepared 0.5 McFarland turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland turbidity standard may be prepared by adding 0.5ml of a 1.175 percent (wt/vol) barium chloride dehydrate ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) solution to 99.5ml of 1 percent (vol/vol) sulfuric acid (H_2SO_4). The turbidity standard is then aliquoted into test tubes, identical to those used to prepare the inoculum suspension. Seal the McFarland turbidity standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland turbidity standards may be stored for up to six months in a dark at room temperature (i.e., $22^\circ\text{-}25^\circ\text{C}$); discard after six months or sooner if any volume is lost (mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred; if it has, a fresh turbidity standard should be prepared). Before each use, shake the tube containing the turbidity standard well, so that the fine white precipitate of barium sulfate is mixed in the tube. The accuracy of the density of a prepared McFarland turbidity standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland turbidity standard, the absorbance at a wavelength of 625nm should be 0.08-0.1. Alternately, the accuracy of the McFarland turbidity standard may be verified by adjusting a suspension of a control strain (e.g., *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts of colonies.

APPENDIX B

Table B.1: Diameter of zone of inhibition

Antimicrobial agent	Disk potency	Susceptibility	Intermediate	Resistant
Ampicillin	10µg	≥ 17mm	14-16mm	≤ 13mm
Chloramphenicol	30µg	≥ 18mm	13-17 mm	≤ 12mm
Streptomycin	10µg	≥ 15mm	12-14mm	≤ 11mm
Nalidixic Acid	30µg	≥ 19mm	14-18mm	≤ 13mm
Ceftriaxone	30µg	≥ 23mm	20-22mm	≤ 19 mm
Kanamycin	30µg	≥ 18mm	14-17mm	≤ 13 mm
Sulphafurazole	300µg	≥ 17mm	13-16mm	≤ 12 mm
Tetracycline	30µg	≥ 15mm	12-14mm	≤ 11 mm



Source: CLSI (2013)