Evaluation of the microbial safety of commercially produced tomatoes in South Africa and the development of a novel enrichment broth for the identification of *Escherichia coli* O157:H7

By

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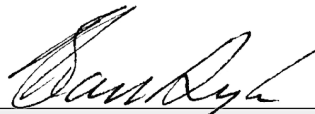**Co-supervisor: Dr. E. M. du Plessis**

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DECLARATION

I hereby certify that this thesis, submitted herewith for the degree of MSc (Agric) Plant Pathology to the University of Pretoria, contains my own independent work, except where duly acknowledged. This work has hitherto not been submitted for any other degree at any other University

Brigitte Nelle van Dyk

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In Loving Memory of Jaco

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PREFACE

The global promotion of fresh fruits and vegetables for improved health and longevity has resulted in a substantial increase in fresh produce demand and consumption. Furthermore, increased shelf lives and improved logistics allow the distribution of fresh produce worldwide. Accompanying this shift in consumer behaviour is the increasing prevalence of foodborne outbreaks linked to the consumption of fresh produce contaminated with pathogens. Traditionally linked to the consumption of undercooked meat and egg products, the presence and health risk posed by these pathogens on fresh produce is cause for great concern. As a result, the past decade's research concerning foodborne pathogens, especially *Salmonella enterica* and *Escherichia coli* O157:H7, has shifted from a veterinary focus to a strong plant pathology perspective.

The research in this dissertation consists of two distinct parts, representing two diverging aspects of the field. The first part was a case study aimed to determine the presence/absence of *E. coli* O157:H7 and *Salmonella enterica* on tomatoes produced in South Africa. The second part aimed at evaluating the utilization of limited glucose release technology to improve the recovery and detection of injured *E. coli* O157:H7 from water samples.

Chapter one reviews the ecology of bacterial foodborne pathogens in the plant environment, as well as the conventional and "omic" methods used for the isolation and detection of these pathogens, with special reference to *E. coli* O157:H7 and *Salmonella enterica*. This chapter elucidates the role fresh produce plays in vectoring enteric pathogens from one warm-blooded

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host to the next, and possible mechanisms whereby pathogens survive this sub-optimal journey. To ensure consumer safety, factors representing risk during production and processing of tomatoes should be tested for the presence of these pathogens. Various conventional methods for the detection of foodborne pathogens are discussed, as well as the specific challenges that exist in this field of research. Finally, the use of novel technologies linked to bioinformatics in the age of culture-independent ecology and detection of foodborne pathogens is discussed.

Various outbreaks have been linked to the consumption of contaminated tomatoes and tomato products, mostly by *Salmonella enterica*. Tomato production in South Africa has increased drastically and although it is consumed in most South African households, no information is available about the microbiological quality of the environments where they are produced and the fruits themselves. Therefore chapter two investigates the microbiological quality of fruit produced by two major tomato producers in the Northern provinces of South Africa. Coliforms were enumerated with 3M Petrifilm and Colilert and dominant species identified using MALDI-TOF analysis. Multiplex PCR specific to *Salmonella enterica* and *E. coli* O157:H7 were used for pathogen detection. Identities were confirmed by sequencing.

Several effective new techniques for pathogen detection have been developed during the past decade, however of all of the techniques lack a detection limit low enough to allow direct testing of foodstuffs since pathogens mostly occur at very low numbers on fresh produce, far outnumbered by resident background microflora and in a physically compromised state. An enrichment step is included prior to the detection of pathogens to allow resuscitation and multiplication of pathogen cells to detectable levels. Sublethal injury also prevents

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resuscitation of cells on solid selective and differential media, leading to detection failure and the underestimation of pathogen numbers in a sample. Chapter three investigated the use of enzyme-limited glucose release technology for increased resuscitation and recovery of sublethally injured *E. coli* O157:H7. EnPresso® B (Biosilta, Finland) was tested as an enrichment broth and incorporated into a solid media and compared to Sorbitol MacConkey Agar for enumeration.

Research presented in this dissertation provides the most extensive study of microbiological quality of tomatoes commercially produced in South Africa to date and the first study describing the application of limited glucose release in the detection of *E. coli* O157:H7 from water. Each chapter has been written as a separate entity and the second chapter will be submitted for publication. It is envisaged that the results will aid the South African tomato industry in continuing the supply of sound safe produce. Furthermore, the contribution to a novel strategy for *E. coli* O157:H7 detection will lay the foundation for future studies in this exciting and important field.

CHAPTER 1

Literature Review: Human enteric pathogens in fresh produce - occurrence, survival, and detection

ABSTRACT

Fresh produce safety is becoming a global concern. In recent years fresh produce-associated outbreaks of infectious diseases caused by human enteric pathogens have increased drastically. This indicates that human enteric pathogens, especially *Salmonella enterica* and *Escherichia coli*, are able to effectively colonize plants and survive environmental factors from "farm-to-fork". In this review the ability of *Salmonella* and *E. coli* O157:H7 to use plants as alternate hosts is assessed. The abiotic and biotic environmental factors influencing human enteric pathogens' survival success are discussed. *Salmonella* and *E. coli* O157:H7 were found to have the genetic abilities to effectively colonize plants and persist in the environment for extended periods of time. Current research suggests that *Salmonella* and *E. coli* O157:H7 rely on the same mechanisms to infect both animal and plant hosts. However, our current understanding regarding the ecology of human enteric pathogens outside the host and the mechanisms they use to survive in the plant environment is lacking. The effective utilization of plants as alternate hosts has major implications on current and future fresh produce safety. To ensure fresh produce safety in the future, pre- and postharvest strategies need to be developed that exclude human enteric pathogens from fresh produce. In the absence of an efficient kill step, surveillance is the most important way to mitigate the risks posed by microbial contamination of fresh produce. Conventional detection methods used for foodborne pathogen detection include traditional culturing, immunology-based methods, and polymerase chain reaction (PCR). Selective enrichment techniques in combination with various conventional and advanced "omics" techniques will potentially allow for better detection of foodborne pathogens and promote surveillance. This will help ensure safe fresh produce in the future.

1 INTRODUCTION

The growing global concern for fresh produce safety in recent years can be attributed to the increasing occurrence of highly publicized cases of outbreaks caused by human enteric pathogens (Harapas *et al.* 2010; Keeratipibul *et al.* 2011; Miller *et al.* 2011; Brilhante São José and Dantas Vanetti 2012; Bennett *et al.* 2014; Lennox *et al.* 2015). This can be partially attributed to the increased consumption of raw and minimally processed food per capita worldwide to promote personal health and wellbeing (Mattson *et al.* 2011; Brilhante São José and Dantas Vanetti 2012; Yeni *et al.* 2014; Lennox *et al.* 2015). Other factors include increased international trade, immunocompromised individuals, and importantly, the ability of human enteric pathogens to use plants as alternate hosts (Beuchat 2002; Berg *et al.* 2014; Fernandes *et al.* 2014; Marvasi *et al.* 2014; Yeni *et al.* 2014; Olanya *et al.* 2015a). Internalization of human enteric pathogens into host tissues yields surface sterilization procedures commonly used ineffective (Schikora *et al.* 2008; Bae *et al.* 2012; Olanya *et al.* 2015a). Also, a lack of knowledge concerning the contamination, mechanisms of internalization, and persistence of human enteric pathogens in plants make the development of effective control strategies difficult (Bartz *et al.* 2015).

In 2011, *Escherichia coli* O104:H4 caused a major food-borne outbreak in Europe, which was associated with fresh produce (Scheutz *et al.* 2011). This outbreak caused thousands of people to become ill, hundreds developed haemolytic uremic syndrome (HUS), and resulted in a death toll of over thirty (Yue *et al.* 2012). Over 500 Shiga toxin producing *E. coli* (STEC) serotypes have been isolated following the isolation of the notorious *E. coli* O157:H7 in 1982 (Yokoigawa *et al.* 1999; Eribo and Ashenafi 2003; Wick *et al.* 2005; Yue *et al.* 2012). Infections caused by STEC may cause symptoms ranging from self-limiting gastroenteritis, to

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bloody diarrhoea and HUS, and even death (Eribo and Ashenafi 2003; Wick *et al.* 2005). According to the US Centres for Disease control and Prevention (CDC), 73 000 people are infected with *E. coli* O157:H7 annually in the US (Mead *et al.* 1999). During a decade (1995-2005), 19 *E. coli* outbreaks occurred that was linked to the consumption of fresh produce (Keeratipibul *et al.* 2011). A spinach and spring mix contaminated by *E. coli* O157:H7 in the US in the same year resulted in 33 people to become ill, 13 requiring hospitalisation, and the development of HUS in two patients (CDC 2012b). During 2013, *E. coli* O157:H7 caused two major fresh produce linked outbreaks in the US. During the first outbreak, contaminated RTE salads resulted in 33 illnesses (CDC 2013b). Contaminated lettuce resulted in 94 people becoming ill later that year (Andrews 2013).

Various serotypes of *Salmonella enterica* are the most prevalent human enteric pathogens associated with foodborne outbreaks worldwide (Yeung *et al.* 2014; Maradiaga *et al.* 2015; Olanya *et al.* 2015a), accounting for most of the foodborne outbreaks in the US (Chen *et al.* 2010; Gu *et al.* 2011; Cheung and Kam 2012; Soares *et al.* 2012; Gu 2014). Diseases caused by different *Salmonella* serovars include gastroenteritis, salmonellosis, and typhoid fever, which may result in death (Chen *et al.* 2010). Annually, an estimated 1.5 billion salmonellosis infections occur worldwide following consumption of contaminated food or water (Schikora *et al.* 2008; Khan *et al.* 2014). Annually, *Salmonella* accounts for 1.2 million foodborne illnesses (Scallan *et al.* 2011; CDC 2015b) in the US and 94 million foodborne illnesses globally (Iwamoto 2015) and costs the US about US$3,7 billion in medical expenses alone (Anonymous 2015). Of these, most salmonellosis outbreaks are associated with the consumption of fresh produce, resulting in about 50 000 infections and 50 casualties annually (Barak *et al.* 2007; Schikora *et al.* 2011; Crowe *et al.* 2015). Between 1998 and 2008, 12 outbreaks caused by *Salmonella* associated with tomatoes were reported in the US alone

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(Barak *et al.* 2005). In 2008, a single outbreak of salmonellosis caused 1442 people to become ill, which led to the hospitalization 273 people and two deaths (Harapas *et al.* 2010). In a multistate outbreak in the US in 2012, cantaloupes contaminated with *Salmonella* Typhimurium and Newport caused 261 people to become ill, of whom 94 patients needed hospitalisation and three deaths (CDC 2012a). Cucumbers contaminated with *Salmonella* Saintpaul caused illness in 84 people in a multistate outbreak in 2013 (CDC 2013a). In a multistate outbreak in 2014, cucumbers contaminated by *Salmonella* Newport resulted in 275 patients falling ill (Angelo *et al.* 2015). Later that year 115 people fell ill after consuming bean sprouts contaminated by *Salmonella* Enteritidis (CDC 2015a).

Salmonella enterica and *E. coli* O157:H7 are able to colonize and internalize in tomatoes, peppers, sprouts, spinach, lettuce, green onions, cucurbits, legumes, nuts, herbs, fruit, and beetroot, and survive the stresses encountered from "farm-to-fork" (Dinu *et al.* 2009; Noel *et al.* 2010; Schikora *et al.* 2011; Gómez☐Aldapa *et al.* 2014; Marvasi *et al.* 2014; Landry *et al.* 2015; Simko *et al.* 2015). Studies have shown that *Salmonella* and *E. coli* O157:H7 effectively internalize and proliferate in plant tissues (Teplitski *et al.* 2009; Zhou *et al.* 2014; Bartz *et al.* 2015; Nugent *et al.* 2015). This raises the question as to whether these pathogens have evolved strategies to use plants as alternate hosts, ensuring their survival outside their hosts and re-introduction into their primary warm-blooded hosts (Teplitski *et al.* 2009; Schikora *et al.* 2011; Brandl *et al.* 2013).

The specific mechanisms used by human enteric pathogens to survive and persist in the environment and within the living tissues of plants are poorly understood (Beuchat 2002; Schikora *et al.* 2008; Teplitski *et al.* 2009; Noel *et al.* 2010; Gu *et al.* 2011; Gu *et al.* 2013; Gu 2014; Simko *et al.* 2015). Effective attachment, colonization and infection of plants

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depend on plant genotype, phenotype, and environmental factors (Teplitski *et al.* 2009; 2014; Dees *et al.* 2014; García and Hirt 2014; Zhou *et al.* 2014; Simko *et al.* 2015). Understanding the biological and ecological factors enabling survival and persistence of human enteric bacteria is crucial in developing pre- and postharvest control strategies to ensure safe produce in the future (Brandl 2006; Dinu *et al.* 2009; Teplitski *et al.* 2009; Noel *et al.* 2010; Mendes *et al.* 2013; Rastogi *et al.* 2013; 2014; Dees *et al.* 2014; Marvasi *et al.* 2014; Bartz *et al.* 2015).

Rapid and reliable detection of pathogens within the production and processing systems play a fundamental role in the management of fresh produce safety in the absence of a kill-step (Geng *et al.* 2011; Park *et al.* 2013; Hirneisen 2015). Rapid detection allows improved monitoring of bacterial contaminants within the produce environment reducing the risk of outbreaks and the economic losses due to the cost of recalls and healthcare (Almeida *et al.* 2014; Beaubrun *et al.* 2014). Furthermore, detection of potential contaminants within the environment where produce is grown and processed can serve as an early warning system (Yeni *et al.* 2014).

Traditional culturing methods are time consuming and laborious and may take up to seven days for confirmed results (Chen *et al.* 2010; Cheung and Kam 2012; Hirneisen 2015). This is a problem since fresh produce has a short shelf life and the time between harvest and consumption is minimal (Yeni *et al.* 2014) and the long periods needed for detection and confirmation of foodborne pathogens may potentially delay the response time to foodborne outbreaks (Yoshitomi *et al.* 2015). Also, traditional culturing methods often fail to detect pathogens if they are in a viable but non-culturable (VBNC) state (Dinu *et al.* 2009; Gilmartin and O'Kennedy 2012; Dinu and Bach 2013). The shortcomings of traditional culturing techniques can be overcome by molecular methods such as the polymerase chain reaction

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(PCR). Although sensitive and rapid, PCR still has shortcomings when applied to detection of foodborne pathogens in a complex food matrix due to the presence of inhibitory substance and non-specific amplification (Bottero *et al.* 2004). Furthermore, conventional PCR methods fail to (1) detect the low numbers of cells usually contaminating fresh produce, and (2) differentiate between viable and dead cells (Cheung and Kam 2012) and therefore detection generally requires a 24 hour enrichment period prior to PCR (Beaubrun *et al.* 2014).

The aim of this review is to provide a summary of the aspects of produce contamination. These include the mechanisms of contamination and survival of enteric pathogens in the produce and environment. Also discussed are the current methods available to detect enteric pathogens from the environment and fresh produce. *Salmonella enterica* and *E. coli* O157:H7 were the focus of this review. It is hoped that the reviewed information will provide insight into aspects of the produce contamination that need to be addressed by research.

2   PLANTS AS ALTERNATIVE HOSTS OF HUMAN ENTERIC PATHOGENS

2.1   <u>Introduction of foodborne pathogens into the plant environment</u>

Infection by *Salmonella* and *E. coli* occurs via the oral-faecal cycle (Viazis and Diez-Gonzalez 2011; Harris 2014). Cattle have been identified as the major reservoirs for *E. coli* O157:H7, which is consistently shed in the faeces of infected cattle (Pearl *et al.* 2006; Yue *et al.* 2012). Swine, cattle, and poultry serve as main reservoirs for *Salmonellae* (Carrasco *et al.* 2012; Arnold *et al.* 2014). Contamination of agricultural crops may occur at any stage during the time from production to consumption (Beuchat 2002; Brandl *et al.* 2013; Harris 2014; Bartz *et al.* 2015). In the agricultural setting, the passive dispersal mechanisms used by

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human enteric pathogens is usually through air and water or by direct contact with a contaminated surface (Barak *et al.* 2011; Bae *et al.* 2012; Lopez-Galvez *et al.* 2014). These pathogens can be brought into contact with plants via untreated manure used as fertilizer, raw sewage and feedlot run-off, contaminated irrigation water or water used for pesticide application, sediment, contaminated soil or vectored by organisms such as livestock, wild animals, birds, insects, slugs, earthworms, and fungi (Beuchat 2002; Sproston *et al.* 2006; Williams *et al.* 2006; Whipps *et al.* 2008; Kroupitski *et al.* 2009a; Harapas *et al.* 2010; Gu *et al.* 2011; Kroupitski *et al.* 2011; Viazis and Diez-Gonzalez 2011; Levantesi *et al.* 2012; Cevallos-Cevallos *et al.* 2014; Farias *et al.* 2014; Gruszynski *et al.* 2014; Lopez-Galvez *et al.* 2014; Bell *et al.* 2015). Studies have shown that human enteric pathogens like *Salmonella enterica* and *E. coli* O157:H7 can survive in soil, sediment and water for extended periods of time, sometimes for up to a year (Baloda *et al.* 2001; Islam *et al.* 2004; Arthurson *et al.* 2011; Brandl *et al.* 2011; Duffitt *et al.* 2011; Ma *et al.* 2011; Cevallos-Cevallos *et al.* 2014). Therefore, water and soil become important secondary reservoirs in the field (Ma *et al.* 2011; Fernandes *et al.* 2014; Bell *et al.* 2015). The ability of these pathogens to survive in the soil and crop debris creates the opportunity for them to infect crops planted in following seasons (Barak *et al.* 2008; Fernandes *et al.* 2014).

2.2 Survival in the plant environment

2.2.1 Genetic factors allowing survival

When human pathogenic bacteria are introduced into the plant environment, they have to adapt to and survive the environmental factors, locate a new host or alternate host, move towards it and colonize it (Harris 2014). Many questions remain regarding the genetic capabilities allowing survival of human enteric pathogens in the agricultural environment for

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extended periods of time (Brandl *et al.* 2013; Bartz *et al.* 2015). Unlike the homeostatic environment inside the warm-blooded host, the environment is highly heterogeneous, with cells being exposed to suboptimal conditions, including limited nutrient availability, osmotic stress, low pH, and a variety of temperature regimes challenges (Gorden and Small 1993; Yokoigawa *et al.* 1999; Duffitt *et al.* 2011; Yue *et al.* 2012; Rastogi *et al.* 2013; Karabıyıklı *et al.* 2014; Delbeke *et al.* 2015b).

Adaptation to the environment is essential for cell survival and persistence and colonisation of plants, which are predetermined by genetic traits that are regulated by differential gene expression (Topp *et al.* 2003; Duffitt *et al.* 2011; Zhang *et al.* 2014b). Gene expression is strictly controlled by various conserved regulons known to occur in enteric bacteria. These include the regulators of the stress response (RpoS alternative sigma factor), and iron acquisition (IroA). Exposure of enteric bacterial cells to physiochemical stresses such as acids, starvation, high osmolarity and temperature extremes trigger the induction of over 50 genes by the alternative sigma factor RpoS (Duffitt *et al.* 2011). RpoS functions as the general regulator during the stress response, and in addition to enabling survival under suboptimal conditions, is also involved in the regulation of biosynthesis pathways, DNA replication and modification, transcription and translation, metabolism, mobile elements, pathogenesis and virulence, antibiotic resistance, and transport and binding proteins (Beales 2006; Vidovic *et al.* 2012). When subjected to stress, the *mar* locus is activated, resulting in increased antibiotic resistance. Heat shock proteins, chaperones preventing protein denaturation, are produced in response to increased temperatures and certain chemicals. This conserves the integrity enzyme structures and allows metabolic processes to continue. Cold shock proteins, encoded by CspA, result in phenotypic changes of the cell, allowing increased survival and resilience to stresses (Beales 2006).

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Quorum sensing (QS) determines gene expression in a cell density-dependent manner (Surette *et al.* 1999; Cloak *et al.* 2002; Daniels *et al.* 2004; Williams 2007). QS allows intercellular communication regarding cell densities and metabolic status by utilising freely diffusible, low-molecular weight molecules known as autoinducers (AIs) (Surette and Bassler 1998; Surette *et al.* 1999; Daniels *et al.* 2004; Yoon and Sofos 2008). AIs are constituently expressed, and as cell numbers increase AIs accumulate. The intra-species QS molecule autoinducer 1 (AI-1) and the universal interspecies QS molecule autoinducer 2 (AI-2) are N-acylhomoserine lactone (AHL) derivatives that are continuously secreted and perceived by both Gram positive and –negative bacteria (Cloak *et al.* 2002; González Barrios *et al.* 2006; Yoon and Sofos 2008). Once the intracellular concentration surpasses a threshold level, gene expression various regulons are either up- or down regulated. Co-opted regulators like the two-component regulon PhoP-PhoQ in *Salmonella*, decreases cell sensitivity to QS molecules. These may function to repress the expression of high cell-density dependent genes in the absence of sufficient QS molecules (Toth *et al.* 2006). AI-2 is LuxS-dependent (Surette *et al.* 1999) and dictates the expression of genes responsible for chemotaxis, biofilm formation, flagella synthesis, motility and production of virulence factors (Xavier and Bassler 2003; González Barrios *et al.* 2006; Yoon and Sofos 2008). Although *Salmonella* lacks AHL synthase, it does have a functional AHL receptor encoded by *sdiA* (Cox *et al.* 2013). AI-2 and AHL perception makes interspecies communication possible. During over-population or nutrient limitation, AI-2 signals the initiation of swarming and biosurfactant production (Daniels *et al.* 2004; Barak *et al.* 2009). The ability to change phenotypically enables survival in the environment. Becoming mobile or remaining sessile, aggregating or remaining solitary at the correct times and under the correct conditions determines the fate of cells.

Activation and deactivation of the Rcs phosphorelay system allows the transition between motile and sessile states and *vice versa*, respectively, in response to environmental cues, allowing the correct state (biofilms versus swarmers) to occur at the correct time. In a study on *Salmonella* conducted by (Barak *et al.* 2009) genes for motility and biofilm formation were found to be differentially expressed. The Rcs phosphorelay system in *Salmonella* positively regulates biofilm formation by activating biosynthesis pathways for LPS and colonic acid production, while suppressing motility and swarming (Wang *et al.* 2007). Genes encoding the type three secretion system (T3SS) on the *Salmonella* Pathogenicity Island 2 (SPI 2) is induced by the Rcs system while genes encoding motility and virulence on the *Salmonella* Pathogenicity Island 1 (SPI1) are suppressed. The T3SS is necessary for survival both inside and outside the host (Berger *et al.* 2010).

Polysaccharides function in adhesion, virulence, antimicrobial resistance and biofilm formation and are therefore important for survival in the environment (Toth *et al.* 2006; Barak *et al.* 2007; Danhorn and Fuqua 2007; Castelijn *et al.* 2012). Conserved enterobacterial polysaccharides include lipopolysaccharides (LPS), the O-antigen capsule, cellulose, and extracellular polysachharides (EPS) (Toth *et al.* 2006). LPS are essential for full bacterial pathogenesis and virulence (Harshey and Matsuyama 1994; Toguchi *et al.* 2000) by contributing to antimicrobial resistances, biofilm formation, surface migration during swarming, and host defence manipulation (Harshey and Matsuyama 1994; Toguchi *et al.* 2000; Kim *et al.* 2003; Kim and Surette 2005). During attachment to plant surfaces, *Salmonella* and *E. coli* both produce cellulose, poly-β-1,6-N-acetyl-D-glucosamine (PGA), colonic acid, and the O-antigen capsule (Solomon *et al.* 2005; Brandl 2006; Barak *et al.* 2007; Noel *et al.* 2010). Research showed that the genes *stfC, bcsA, misL,* and *yidR* are upregulated during survival on postharvest lettuce (Kroupitski *et al.* 2013). Adhesion to plant tissue and

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biofilm formation is mediated by *bcsA, misL,* and *yidR,* that encode a cellulose synthase catalytic subunit, a SPI3 autotransporter adhesion, and an ATP/GTP-binding protein, respectively.

The Rcs system is induced upon exposure to low temperatures, solid surfaces, osmotic stress, and an increase in nutrient availability. By inducing colonic acid production and suppressing flagellar biogenesis, a sessile lifestyle is favoured. Biofilms are biological structures composed of a community of microorganisms that adhere to one another through a matrix of extracellular polymers and collectively adhere to a solid surface (Solomon *et al.* 2005; Lapidot *et al.* 2006; Lapidot and Yaron 2009). Biofilms can form on various substrates, both living and inert. Biofilm formation is important for environmental survival and host colonization, increasing cells' resistances towards physiochemical stresses, protection from predation, consortial metabolism, and opportunities for horizontal gene transfer (HGT) (Castelijn *et al.* 2012; Jahid *et al.* 2015). Biofilms allow the survival of human enteric pathogens in the environment, forming on soil particles, in irrigation pipelines, and on plant surfaces in the rhizo- and phyllosphere (Solomon *et al.* 2005; Lapidot and Yaron 2009; Jahid *et al.* 2015). Nutrient availability, temperature and relative humidity determine biofilm formation (Bae *et al.* 2012). Flagella, fimbriae, adhesins and agglutinins are important in biofilm formation and surface attachment.

Fimbriae (or pili) are extracellular organelles of Gram-negative bacteria important for attachment to eukaryotic cells and inert surfaces (Sauer *et al.* 2000; Toth *et al.* 2006; Holden *et al.* 2008). Fimbrial structures include thin aggregative fimbriae (Tafi) in *Salmonella* and curli in *E. coli* (Barak *et al.* 2005; Barak *et al.* 2007; Barak *et al.* 2009). Chaperone-ushered fimbrial adhesins of animal pathogenic enterobacterial are responsible for glycosylation-

pattern recognition on host cell surfaces, thereby conferring affinity towards a certain host or host tissue (Sauer *et al.* 2000). The function of fimbrial adhesins in phytopathogenicity is still unclear, albeit being encoded by animal- and phytopathogenic enterobacteria alike (Holden *et al.* 2008).

Agglutinins are a class of surface-exposed adhesin proteins involved in biofilm formation and cell-cell interactions (Danhorn and Fuqua 2007). All share conserved consensus motifs and functions, but little sequence similarities. Plant-specific agglutinins include *Pseudomonas fluorescence*'s LapA (large adhesin protein A), involved in biofilm formation (Danhorn and Fuqua 2007), and *Pectobacterium atrosepticum*'s HecA, required for biofilm formation for cell attachment, aggregation and virulence (Toth *et al.* 2006; Holden *et al.* 2008). The agglutinins SiiE and BapA of *Salmonella* are important for adherence to animal cells. Homologous to both are present in plant-associated bacteria, suggesting SiiE and BapA may facilitate attachment of *Salmonella* to the plant, however, their role in plant cell attachment is yet to be determined (Holden *et al.* 2008). Biofilm formation by *Salmonella* relies on the production of thin aggregative fimbriae (Tafi), cellulose and colonic acid (Solomon *et al.* 2005; Brandl 2006; Barak *et al.* 2007). During biofilm formation and attachment of *Salmonella* to plant surfaces, AgfD regulates the expression of *agfA* and *bcsA*, resulting in Tafi and cellulose biosynthesis (Barak *et al.* 2005; Barak *et al.* 2007). Low temperatures, nutrient limitation and low osmolarity induce the formation of the rdar morphotype in *Salmonella*, which is associated with Tafi, colonic acid, and cellulose production (Solomon *et al.* 2005; Lapidot *et al.* 2006; Gu *et al.* 2011).

The RcsB- and RcsAB-binding boxes form part of the Rcs system in *Salmonella* and *E. coli*, respectively (Wang *et al.* 2007). High osmolarity causes the RcsAB-binding box in *E. coli* to associate with OmpR (an osmoregulator), suppressing the Rcs system, thereby allowing

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flagella motility. Mobility enables chemotaxis in cells, allowing the movement towards, or away from a chemical signal, used for host identification and nutrient location (Kroupitski *et al.* 2009b). *Escherichia coli* and *Salmonella* may also undergo a dimorphic transition, resulting in hyper flagellated swarmer cells when challenged by adverse environmental conditions (Harshey and Matsuyama 1994). Swarmer cells are multinucleate and filamentous (Duffitt *et al.* 2011). Swarming motility enables a bacterial colony to move as a group, allowing rapid surface migration and colony expansion (Harshey and Matsuyama 1994; Kim *et al.* 2003). Differentiation into a swarming state causes major changes in gene expression (Kim *et al.* 2003). Swarmer cells become anabolic, experiencing a global metabolic shift. This shift results in the activation of stress-related biosynthetic pathways, including LPS synthesis (Kim and Surette 2005). EPS, LPS and flagella are essential in swarming motility (Kim *et al.* 2003; Barak *et al.* 2009). EPS can absorb great amounts of water, and together with LPS are able to protect cells from desiccation and act as biosurfactant during swarming (Kim and Surette 2005).

Iron acquisition from the environment is essential for full virulence (Toth *et al.* 2006) and proliferation of *Salmonella enterica* in tomatoes (Nugent *et al.* 2015). The ability of bacteria to scavenge iron from the environment dictates its success. Gram-negative bacteria strictly regulate the acquisition of iron using universal regulators. Bacteria have evolved iron-chelating molecules called siderophores, which bind iron quickly and efficiently in the environment (Hantke *et al.* 2003; Toth *et al.* 2006). Enterobactins are produced by most animal and plant enteric pathogens. Salmochelins are the most important catecholate siderophores produced by various *Salmonella* and pathogenic *E. coli* strains. The *iroA* locus of *Salmonella* and *E. coli* encodes Iro proteins that are responsible for the glycosylation, export, and transport of salmochelin. IroN is an outermembrane receptor for salmochelin, and other *Salmonella* siderophores (Hantke *et al.* 2003). Hemophores bind to heme and heme-

bound proteins. Hemophore genes are conserved in animal and plant enteric pathogens, indicating that these genes have been retained throughout evolution due to the essential function they serve in survival (Toth *et al.* 2006). Bacteria also acquire iron directly by binding to heme-binding proteins, followed by the reduction of the iron to which the proteins are bound (Toth *et al.* 2006).

Secretion systems are essential for successful colonization and invasion of host tissue. Six secretion systems have been described for Gram-negative bacteria (Toth *et al.* 2006). General virulence factors that ensure survival in the environment are secreted by the type one-secretion system (T1SS). These include siderophores, toxins, and adhesins. In *Salmonella* the *Salmonella* pathogenicity island 4 (SPI4) encodes the adhesin SiiE and the T1SS that secretes it (Gerlach *et al.* 2007). The type two secretion (T2SS) system is a pseudopilus complex which shares many similarities with the type IV pilus system and is responsible for the secretion of proteins and virulence factors into the environment (Korotkov and Hol 2008). Although common in enteric bacteria, *Salmonella* does not possess a T2SS and the T2SS of *E. coli* O157:H7 is prophage-encoded. The T2SS of *E. coli* O157:H7 secretes Shiga-like toxins (*stx* gene), also encoded by the prophage genome. Most pathogenic Gram-negative bacteria have type three secretion systems (T3SS). T3SSs have been the focus of most research conducted regarding host-pathogen interactions. T3SS are responsible for secreting effector proteins, both Avr proteins and helper proteins, directly into their host cells, thereby modifying the environment and overcoming host defences (Hornef *et al.* 2002; Espinosa and Alfano 2004; Schikora *et al.* 2008; Shirron and Yaron 2011) allowing colonization, multiplication and persistence in host tissue (Üstün *et al.* 2012). The T3SS is, however, also the basis for effector triggered immunity (ETI) in the innate immunity of plants and animals (Hornef *et al.* 2002; Nürnberger and Brunner 2002; Alfano and Collmer 2004; Ausubel 2005;

Dangl and McDowell 2006; Katagiri and Tsuda 2010). *Salmonella* has two T3SSs, encoded independently by the SPI1 and SPI2 that play a role in infection of both animals and plants (Holden *et al.* 2008; Schikora *et al.* 2008; Üstün *et al.* 2012).

2.2.2 Biotic factors affecting survival

The size and composition of bacterial populations in the plant environment is dependent on environmental factors including pH, mineral, nutrients, water availability, bacterial and host species, genotype and phenotype (Danhorn and Fuqua 2007; Berger *et al.* 2010; Mendes *et al.* 2013; Rastogi *et al.* 2013; Williams *et al.* 2013; 2014; Karabıyıklı *et al.* 2014). Under field conditions, human enteric pathogens encounter various natural inhabitants as well as phytopathogenic bacteria and fungi (Whipps *et al.* 2008; Rastogi *et al.* 2013; Simko *et al.* 2015). Interactions with these native organisms may facilitate increased survival or exclude human enteric pathogens from the environment (Mendes *et al.* 2013; Cálix-Lara *et al.* 2014). Plant exudates utilized, niche preferences, and resistance to antimicrobial compounds of human enteric bacteria determine the outcome of cohabitation with other organisms (Lindow and Brandl 2003; Cooley *et al.* 2006; Brandl *et al.* 2013; Cálix-Lara *et al.* 2014).

Various organisms have been shown to increase the survival of *Salmonella* and *E. coli* in the plant environment. This occurs if there is no competition for the same nutrients or ecological niche, or if cohabitation of a specific niche by both organisms favours one another. Organisms from various kingdoms have been shown to interact with human enteric bacteria and promote their survival (Brandl *et al.* 2011; Brandl *et al.* 2013; Mendes *et al.* 2013; Simko *et al.* 2015).

Diverse interactions between fungi and oomycetes and bacteria exist in the environment. These interactions may prove to be beneficial or detrimental to both fungal and bacterial

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survival (Brandl *et al.* 2011). Many bacteria are able to antagonize fungi through the production of biocides. Other bacteria may parasitize fungi, or *vice versa*. Other interactions may prove mutualistic. The fungus *Aspergillus niger* has been shown to facilitate the survival and distribution of *Salmonella* in the plant environment. *Aspergillus niger* successfully colonises soil and plants and forms part of the natural microflora communities within the plant environment. *Aspergillus niger's* cell walls are mainly composed of chitin. The interaction between fungal chitin and bacterial cellulose allows initials attachment of *Salmonella* cells to *Aspergillus niger's* cell walls. The chitin-cellulose interaction occurs rapidly, followed by biofilm formation on the hyphal surfaces of *Aspergillus niger*. The biofilms formed on *Aspergillus niger* can cover entire hyphae and after 24hours branching occurs. *Aspergillus niger* can therefore act as a vector for *Salmonella*, promoting survival and dispersal within the plant environment (Brandl *et al.* 2011). Infection of Romaine lettuce by the oomycete *Bremia lactucae*, the causal agent of downy mildew, was shown to facilitate the proliferation of *E. coli* O157:H7 and *Salmonella enterica* on lettuce leaves, especially in necrotic lesions (Simko *et al.* 2015).

Bacterial epiphytes, endophytes and phytopathogens all play differential roles in the survival of *E. coli* and *Salmonella* in the plant environment (Mendes *et al.* 2013; Rastogi *et al.* 2013). *E. coli* O157:H7 *and Salmonella enterica* benefit from associating with pectinolytic bacteria such as *Pectobacterium carotovorum* and *Dickeya dadantii*, helping them gain access to nutrients and a protected environment (Brandl *et al.* 2013; Meng *et al.* 2013). The colonisation of tomatoes by *Xanthomonas campestris* pv. *vesicatora* and *Xanthomonas perforans* favour the colonization and internalization of *Salmonella* in the phyllosphere by manipulating host resistances, thereby allowing internalization through stomata and the

acquisition of nutrients from the apoplast (Barak *et al.* 2008; Potnis *et al.* 2014). *Wausteria paucula* increases the persistence of *E. coli* O157:H7 in the soil six-fold (Cooley *et al.* 2006).

Pseudomonads, especially *Ps. fluorescence* and *Ps. chloraphis*, have proven to outcompete and antagonize *Salmonella* and *E. coli* on fresh produce (Fett 2006; Liao 2008; Olanya *et al.* 2015b). Pseudomonads, *E. coli* and *Salmonella* seem to compete for the same niches and nutrients (Olanya *et al.* 2015b). The ability of Pseudomonads to grow rapidly at low temperatures and successfully manipulate host plant defences gives them the upper hand in the competition, excluding human enteric pathogens from the environment. Another competitor that *Salmonella* and *E. coli* fail to overcome is *Enterobacter asburiae*. *Ent. asburiae* is able to suppress *Salmonella* by ten-fold and *E. coli* O157 by 20- to 30-fold during seedling colonization (Cooley *et al.* 2006). In a recent study, a naturally occurring epiphyte of tomato plant leaves, *Paenibacillus alvei* strain TS-15, was shown to be extremely antagonistic towards *Salmonella enterica* on tomato plants *in situ* (Allard *et al.* 2014). Lactic Acid Bacteria (LAB) also antagonise *E. coli* O157:H7 and *Salmonella enterica* by competing for nutrients and secreting antimicrobial compounds both *in vitro* and *in situ* (Cálix-Lara *et al.* 2014).

Protozoa play an important role in the persistence of both *E. coli* and *Salmonella* in the environment. During feeding, protozoa may ingest *E. coli* and *Salmonella* cells. These cells are harboured within the protozoa where they are protected against desiccation, and vectored to new locations. *Acanthamoeba polyphaga* can accommodate more than ten *E. coli* O157 cells at a time (Barker *et al.* 1999). Species from the genera *Acanthamoeba* and *Tetrahymena* have been shown to ingest *Salmonella* cells via endocytosis from the environment and

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maintain them within vesicles (Brandl *et al.* 2005). Mechanisms and environmental factors facilitating exocytosis are still unknown.

Different microorganisms therefore interact differently with human enteric pathogens. Some may be commensal, while others may be antagonistic. The existence of SdiA, a LuxR-type protein enables *Salmonella* and *E. coli* to perceive AHL QS signals of other microbiota (Teplitski *et al.* 2009; Teplitski *et al.* 2012) allowing interspecies communication. The manner in which *Salmonella* and *E. coli* interact with these organisms can determine whether they will persist in the environment or not. The antagonistic effect of some natural microflora against foodborne pathogens demonstrates the possibility of developing biological control agents to effectively prevent the carriage of foodborne pathogens on fresh produce (Zheng 2014).

2.3   Overcoming plant host immunity and establishment

All bacteria, unlike fungi and nematodes, are unable to directly enter plants. Active internalization occurs through natural openings (stomata, lenticels, lateral root emergence sites etc.) and wounds caused by biological agents and physical damage (insects, herbivores, wind, farm implements, hail etc.) (Tyler and Triplett 2008; Deering *et al.* 2012; Simko *et al.* 2015). *Salmonella enterica* and *E. coli* are able to actively colonize the surfaces of plants (Barak *et al.* 2005; Barak *et al.* 2007; Barak *et al.* 2009; Arthurson *et al.* 2011; Gu *et al.* 2011; Gu *et al.* 2013). Colonization of plants is dependent on cultivar and age (Noel *et al.* 2010; Barak *et al.* 2011; Han and Micallef 2014). Wounds enable easy access to the plant and increase the persistence of enteric pathogens within the plant (Harapas *et al.* 2010). Colonization of plant surfaces and apoplastic intercellular spaces requires biofilm formation

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by *Salmonella* and *E. coli* (Barak *et al.* 2005; Barak *et al.* 2007; Schikora *et al.* 2008; Kroupitski *et al.* 2009b; Barak *et al.* 2011; Kroupitski *et al.* 2011) and the production of other polysaccharides, for example the O-antigen capsule (Barak *et al.* 2007; Lapidot and Yaron 2009). Plant surface characteristics determine biofilm distribution. *Salmonella* and *E. coli* biofilm formation occurs preferentially around wounds and natural openings (Kroupitski *et al.* 2009a). Cellulose and curli production during attachment to plant surfaces are important but not critical (Lapidot *et al.* 2006; Noel *et al.* 2010). However, cellulose and TaFi are essential for biofilm formation by *Salmonella* exposed to the phyllosphere via irrigation water. Other factors contributing to colonization success may include the presence of native biofilm-forming plant- associated bacteria (Rastogi *et al.* 2013).

In the phyllosphere, *Salmonella* has been shown to preferentially colonize the bases of type 1 trichomes (Barak *et al.* 2011). Internalization occurs through open stomata, dependent on light and chemotaxis towards photosynthates (Kroupitski *et al.* 2009a). *Salmonella*, but not *E. coli*, is able to suppress MAMP-induced stomatal closure, however the effector responsible remains unknown (Melotto *et al.* 2006; Kroupitski *et al.* 2009a; Kroupitski *et al.* 2011; Kroupitski *et al.* 2013; Meng *et al.* 2013).

The colonization of leaves may lead to systemic infection since direct inoculation of *Salmonella* onto tomato leaves caused the whole phyllosphere to become infected (Barak *et al.* 2008; Barak *et al.* 2011). Following direct inoculation of leaves with *Salmonella*, *Salmonella* translocated to the midrib veins, finally entering the xylem (Gu *et al.* 2011; Gu *et al.* 2013). The inoculation of leaves also led to infection of flowers, fruit and seed. Vertical transmission of *Salmonella* has shown to be possible, in which case seeds infected with

Salmonella may result in vertical transmission the next generation crop (Lewis Ivey *et al.* 2014).

The ability of the pathogen to colonize the root positively correlates with its ability to internalize. Lateral root emergence sites are the preferred sites for colonization in the rhizosphere (Mendes *et al.* 2013). Roots are easily accessible from contaminated soil and irrigation water and plant root exudates serve as a rich nutrient source. Internalization occurs actively through wounds or the lateral junctions where emerging roots cause cracks in the root surface from where access to the vascular system is gained (Dong *et al.* 2003; Tyler and Triplett 2008).

Virulence-promoting effector molecules are secreted by the T3SS of Gram-negative bacteria (Sperandio *et al.* 1999; Alfano and Collmer 2004; Espinosa and Alfano 2004; Block *et al.* 2008; Üstün *et al.* 2012). Effector molecules are composed of virulence-promoting proteins and helper proteins that assist during target molecule location in the host cell (Jones and Dangl 2006). Enzymatically active effector proteins modify the host to ensure survival, proliferation, and persistence within host tissue (Alfano and Collmer 2004; Block *et al.* 2008; Block and Alfano 2011). Effector proteins usually mimic or target host molecules functioning in signalling pathways. This enables the pathogen to disable PAMP triggered immunity (PTI), or even effector triggered immunity (ETI) (Nimchuk *et al.* 2003; Jones and Dangl 2006; Katagiri and Tsuda 2010).

Salmonella triggers JA-dependent host responses in *Arabidopsis thaliana* (Schikora *et al.* 2008). The MAPK signalling cascade was activated in response to *Salmonella* resulting in the activation of MPK3 and MPK6. In the presence of *Salmonella*, the SA-regulated PR1

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gene was also upregulated. However *Salmonella* is able to suppress the defences and infect the plant (Meng *et al.* 2013; García and Hirt 2014). *Salmonella* also only induces partial stomatal closure (Kroupitski *et al.* 2009a). It is hypothesized that this is either due to the plant PTI not recognizing *Salmonella*, or that *Salmonella* has effectors that suppress PTI, thereby preventing stomatal closure thereby enabling *Salmonella* to colonize the substomatal space.

Salmonella and *E. coli*'s ability to effectively invade plants as alternate hosts, and their ability to persist in the environment for extended periods of time causes great concern for the future of fresh produce safety (Barak and Schroeder 2012). Even though colonization seems to be a rare event, the scale on which fruits, vegetables and seedlings are produced create sufficient opportunities for pathogen contamination (Barak *et al.* 2008; Barak *et al.* 2011). The increase in foodborne outbreaks associated in the past decade may, in part, be attributed to the ability of human enteric pathogens to survive in a non-host environment (Schikora *et al.* 2011; Hernández☐Reyes and Schikora 2013). To meet the increasing demand for fresh produce, agricultural practices are intensified and an increase in the prevalence of fresh produce-associated foodborne outbreaks can be expected.

Salmonella and *E. coli* occur either as biofilms on the surface of plants, or reside in the intercellular spaces of mesophyll cells (Schikora *et al.* 2008). The use of traditional sanitizers in a wash-step will therefore not remove all the bacteria present because it is unable to penetrate tissues and biofilms (Schikora *et al.* 2008; Bae *et al.* 2012) and may in fact facilitate cross-contamination (Wang and Ryser 2014). In the absence of an effective "kill-step" in the fresh produce supply chain, ensuring produce safety relies on good agricultural practices to ensure the exclusion of human enteric bacteria from the plant environment and subsequent

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contamination (Schikora *et al.* 2008; Harapas *et al.* 2010; Barak and Schroeder 2012; Schikora *et al.* 2012), and monitoring pathogens throughout the entire supply chain (Yeni *et al.* 2014). The methods used for isolation and identification of specifically *Salmonella enterica* and *E. coli* O157:H7 will be discussed in the following section.

3 CONVENTIONAL METHODS FOR THE DETECTION OF FOODBORNE PATHOGENS FROM ENVIRONMENT AND FRESH PRODUCE

Reliable methods to monitor the presence or absence of foodborne pathogens in food and agricultural environment are an important part of Food Safety Management Schemes to help assure the safety of the consumer (Garrido *et al.* 2013; Hirneisen 2015). In the fresh produce industry where shelf life is short and the scale of production large, the use of culturing methods alone is not a viable option (Yeni *et al.* 2014). Detection should aim to be rapid, sensitive, specific, and adhere to international test standards (Malorny *et al.* 2003; Saroj *et al.* 2008; Gordillo *et al.* 2011; Beaubrun *et al.* 2014; Delbeke *et al.* 2015a).

Traditional methods for the detection of foodborne pathogens rely on enrichment and selective plating, followed by serological and biochemical testing (Shelton *et al.* 2004; González-Escalona *et al.* 2012; Lee *et al.* 2015). The main drawbacks of culturing are that it is time-consuming and laborious (Suo *et al.* 2010; González-Escalona *et al.* 2012; Khan *et al.* 2014; Lee *et al.* 2015). Standard methods usually take between four and seven days for isolation and identification of target species (Schrank *et al.* 2001; Khan *et al.* 2014). Two to three days are spent on isolating potential colonies by culturing or negative confirmation, followed by an additional five to seven days for confirmation by biochemical and serological testing (Saroj *et al.* 2008; Chen *et al.* 2010; Yeung *et al.* 2014). To increase rapidity,

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molecular and immunological methods are utilized following enrichment to increase target pathogen numbers to detectable limits (Garrido *et al.* 2013; Delbeke *et al.* 2015a).

3.1   Enrichment

Culture and colony based methods are still the most used techniques due to their reliability, accuracy and relatively low cost compared to newer techniques (Velusamy *et al.* 2010). Culture methods rely on the cultivation of bacterial cells to form visible colonies on a medium that allow their detection. Culture methods are an effective means of enumerating the number of foodborne pathogens in a sample. The main technical drawbacks of culturing techniques is the lack of selectivity that results in overgrowth of background microflora (Kim and Bhunia 2008) and the occurrence of foodborne pathogens in environmental samples existing in a dormant VBNC or physiologically stressed state (Gilmartin and O'Kennedy 2012). As a result these cells may fail to resurrect on culture media and pathogen numbers may be underestimated, or pathogens may not be detected at all (Dinu *et al.* 2009; Gilmartin and O'Kennedy 2012). Non-selective pre-enrichment for resuscitation and selective enrichment steps are therefore implemented before bacteria are cultured on selective and differential media followed by biochemical and serological confirmation (Baylis *et al.* 2000; Saroj *et al.* 2008). Rapid methods have been developed for the detection of foodborne pathogens, but their detection limits remains high, usually between $1x10^2$ to $1x10^3$ cells (or CFU) analyte per unit food (Gehring *et al.* 2012). Therefore, enrichment is possibly one of the most important tools used for pathogen detection (Reissbrodt *et al.* 1996) since almost all detection methods rely on an enrichment step to improve detection limits (Asperger *et al.* 1999; Vimont *et al.* 2006; Khan *et al.* 2014). Enrichment aims at increasing pathogen cell numbers to a detectible level and the resuscitation of stressed or dormant cells (Kim and Bhunia 2008).

3.1.1 Function of Enrichment

It has often been witnessed that foodborne pathogens in fresh produce are present in very low numbers (Hepburn *et al.* 2002; Johannessen *et al.* 2002), often far below the detection limit of different techniques. Since the infective dose is often very low (de Boer and Heuvelink 2000; Hepburn *et al.* 2002), varying between 10 to a 100 cells, detection of low cell numbers is extremely important to ensure consumer safety (Asperger *et al.* 1999; Zhu *et al.* 2011; Cheung and Kam 2012). Except for occurring in very low numbers in the environment, many foodborne pathogens are in a physiologically stressed or injured state (Wu 2008). An enrichment step is often required to increase cell numbers for isolation and detection, resuscitate stressed cells, and remove contaminants and may well determine detection success (Busse 1995; Shelton *et al.* 2004; Kim and Bhunia 2008; Tsai *et al.* 2012; Beaubrun *et al.* 2014).

In most food safety laboratories, two enrichment steps are utilized before the isolation and detection of pathogens from environmental and food samples (Xiao *et al.* 2010; Cheung and Kam 2012; Rambach 2014). These steps include non-selective pre-enrichment and selective post-enrichment (Xiao *et al.* 2010; Tsai *et al.* 2012; Yue *et al.* 2014). Non-selective pre-enrichment is used for the primary resuscitation and multiplication of pathogens from the sample (Hirneisen 2015). It consists of a broth with a high nutrient value allowing cell resuscitation and multiplication (Tsai *et al.* 2012). Following this step, an aliquot is transferred to a selective post-enrichment broth (Rambach 2014). The choice of selective agent, the incubation temperature, and the time of incubation all influence the recovery success (D'Aoust *et al.* 1992). The main drawback of the enrichment step is that it is time-consuming, costly, laborious, and requires space (Kim and Bhunia 2008; Zhu *et al.* 2011).

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Enrichment may, however, be an inexpensive method if it is designed to optimally recover multiple organisms simultaneously (Shelton *et al.* 2004; Xiao *et al.* 2010).

The aim should be to optimize the enrichment technique to (1) increase the concentration of target bacteria for easy detection with subsequent methods, (2) adequately suppress background flora, and (3) recover cells from complex food matrices (Asperger *et al.* 1999; Kim and Bhunia 2008; Xiao *et al.* 2010; Fedio *et al.* 2011; Gehring *et al.* 2012; Tsai *et al.* 2012; Almeida *et al.* 2014). Optimizing growth and selectivity is essential for effective isolation of pathogens (Liamkaew *et al.* 2012).

3.1.2   *Factors influencing enrichment procedure*

When choosing the enrichment broth and cultivation conditions to be used, the sample matrix and type and level of background microflora should be considered and adjustments made accordingly (Asperger *et al.* 1999).

The sample matrix has a great impact on enrichment and recovery of target pathogens as a result of the modification of media composition in the presence of food (Beckers *et al.* 1987), the interactions and adherence of the target with the food matrix (Gnanou-Besse *et al.* 2010), the uneven distribution of the target in the sample, and the presence of inhibitory compounds and natural microflora (Taskila *et al.* 2012). Different food components and conditions may also inhibit PCR (Saroj *et al.* 2008) and immunological detection due to antigen breakdown (Hahm and Bhunia 2006).

Fresh produce supports a consortium of bacteria (Fedio *et al.* 2012) that influence the efficiency of target recovery (de Boer and Heuvelink 2000; Weagant and Bound 2001). The

sample source, matrix and physical conditions determine the types and levels of background microflora (BM) present (Vold *et al.* 2000). BM are co-enriched with the target pathogen (Cheung and Kam 2012), and usually occur at far higher numbers and better physical condition than the target species itself (Zhang *et al.* 2012). During enrichment these dominant species are co-enriched and may suppress target organism growth (Duffy *et al.* 1999; Vold *et al.* 2000; Vimont *et al.* 2006) in the phenomenon known as the Jameson effect (Jameson 1962). This effect describes the suppression of growth of target organisms in batch culture when the dominant Gram-negative species reaches stationary phase (Mellefont *et al.* 2008; Al-Zeyara *et al.* 2011). Suppression occurs as a result of inhibitory compounds produced (Mellefont *et al.* 2008; Cálix-Lara *et al.* 2014), the production of the same by-product, or the competition and depletion of essential nutrients (Gnanou-Besse *et al.* 2010). In *Salmonella* this effect occurred due to the induction of the RpoS global regulator when the redox potential in the media is lowered (Komitopoulou *et al.* 2004). Different mechanisms may underlie this effect depending on the species involved (Al-Zeyara *et al.* 2011). A measure of selectivity is incorporated in the enrichment procedure to minimize the effect of BM and enhance the recovery of the target organism (de Boer and Heuvelink 2000). Inhibition of BM is achieved by the incorporation of selective chemical agents and modification of incubation temperatures (D'Aoust *et al.* 1992; Dogan *et al.* 2003; Al-Zeyara *et al.* 2011). It should however be kept in mind that selective conditions may result in a decreased rate of recovery of sub-lethally injured cells (Hara-Kudo *et al.* 2000; Foster *et al.* 2003) which is often the case in environmental samples due to UV irradiation, desiccation, and temperature variations (Hepburn *et al.* 2002). As a result a two-step enrichment method is often employed, where samples are pre-enriched in a non-selective broth to allow resuscitation (Liao and Fett 2005), followed by selective enrichment to reduce competitors (Reissbrodt *et al.* 1996; Hara-Kudo *et al.* 2000).

The use of selective agents is very important in enrichment broths since they can exclude many BM that can outcompete the target pathogens to avoid false results (Kim and Bhunia 2008). The choice and concentration of selective agent is very important for optimum target recovery (Hepburn *et al.* 2002). Gram-negative bacteria are intrinsically resistant to antibiotics with hydrophobic properties, including the novobiocins, rifamycins, actinomycin D, macrolides and fusidic acid (Scheutz and Strockbine 2005). Inorganic inhibitors and antibiotics commonly used include novobiocin, vancomycin, cefsulodin, cefixime, bile salts, potassium tellurite, acriflavine, cycloheximide, fosfomycin, nalidixic acid, sodium citrate, and sodium chloride (Blackburn and McCarthy 2000; Restaino *et al.* 2001; Kim and Bhunia 2008; Xiao *et al.* 2010; Fedio *et al.* 2011). Since the target cells may be in a physiologically poor state or sub-lethally damaged, the selective agent can easily select against these cells, delaying or preventing the growth and recovery of the target organism and contributing to isolation and detection failure (Rocelle *et al.* 1995; Restaino *et al.* 2001; Hepburn *et al.* 2002; Kim and Bhunia 2008).

The inhibiting effect of the selective agents can be overcome by supplementing the broth with growth promoting substances. These include glucose, mannitol, sodium sulphite, sodium pyruvate, casamino acids, lactose and yeast extract (McDonald *et al.* 1983; Xiao *et al.* 2010; Fedio *et al.* 2011). Other molecules that have been shown to stimulate growth and resuscitate injured cells are iron-chelating siderophores (for iron acquisition), antioxidants that limit the effect of ROS, like the ROS-degrading enzymes catalase and Oxyrase®, pyruvate, ascorbic acid, and thiol compounds (McDonald *et al.* 1983; Czechowicz *et al.* 1996; Reissbrodt *et al.* 2002; Wu 2008). For the recovery of injured cells, compounds such as Tween™ 80 (Polyoxyethylene sorbitan monooleate) and $MgSO_4$ that assist in the repair of damaged cell membranes may be useful (Murthy and Gaur 1987).

During enrichment, lag-times are determined by the cultivation conditions and the physiological state the target cells are in (Jasson *et al.* 2009). Factors include pH, temperature, salt and nutrient concentration, osmolarity, and oxygen concentration. Growth of bacteria during enrichment cause changes in the cultivation conditions. Changes that influence lag-time and growth rates during enrichment include osmotic stress as a result of changes in osmolarity, acidic or alkaline stress as a result of changes in pH due to the production of metabolites, and oxidative stress as a result of the evolution of reactive oxygen species (ROS), and thermal stress caused by shifts in cultivation temperatures (Taskila *et al.* 2012). The addition of salts, buffers, antioxidants and ROS-degrading enzymes, and the adjustment of temperature may help mitigate osmolarity, pH, oxidative, and thermal stresses, respectively.

Following an enrichment step, foodborne pathogens are traditionally detected by culturing on selective and differential plating media, molecular methods targeting various unique genetic regions in the target genome, or immunological methods aimed at antigens expressed on the target's surface (Lee *et al.* 2015). The detection method used will also determine the appropriate enrichment medium. The medium components should not (or only minimally) interfere with detection directly, nor cause the suppression of marker gene or antigen expression (Hahm and Bhunia 2006; Taskila *et al.* 2012).

3.2 Conventional Detection Methods of Foodborne Pathogens

3.2.1 Selective Plating: Enumeration and Isolation

Plating on solid media remains the most used method of selectively enumerating and isolating target pathogens both prior to, and after enrichment, usually following capture by

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immunomagnetic separation (IMS) (Gordillo *et al.* 2011; Delbeke *et al.* 2015a). Selective and differential media that selects for and differentiates the target species from background microflora is used to isolation and detect foodborne pathogens by culturing (Tzschoppe *et al.* 2012; Delbeke *et al.* 2015a). Media are developed that differentiate between bacteria based on defining biochemical characteristics. Differentiation is based on enzymatic activities of various species and strains to yield a distinctive reaction, linked to changes in pH (in the case of sugar fermentation and the production of organic acids) or the cleavage of a chromogen/fluorogen that releases a dye (Perry and Freydiére 2007). For the detection of *E. coli,* chromogenic and fluorogenic reactions are based on the hydrolysis of substrates by *lacZ* (β-galactosidase) gene that cleaves lactose into glucose and galactose (Pitkänen *et al.* 2007) and β-glucuronidase (GUD) (Manafi 2000), encoded by the *uidA* gene found in a high percentage of *E. coli* strains, with the exception of *E. coli* O157 strains (Frampton and Restaino 1993). Direct plating for enumeration purposes has the advantage over Most Probable Number (MPN) methods by being much more cost-effective and yielding isolated target cells (Brichta-Harhay *et al.* 2007).

The ability of most *E. coli* strains to ferment lactose is utilised by Levine's Eosin Methylene Blue (EMB) agar, where the two indicator dyes methylene blue and Eosin Y is absorbed by the cells at a low pH, turning the colonies black. Unlike most other lactose fermenters, *E. coli* produces strongly acidic organic acids and exhibits flagellar movements. These properties, in combination with the metachromatic properties of the dyes, result in colonies with a metallic sheen (Levine 1943). This property however is not unique to *E. coli*, with certain *Citrobacter* and *Enterobacter* species yielding false positives.

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Typical *E. coli* O157:H7 strains do not ferment sorbitol within 24 hours and are β-glucuronidase (GUD) negative (Ratnam *et al.* 1988; de Boer and Heuvelink 2000). The most commonly used selective and differential solid media for *E. coli* O157:H7 detection is Sorbitol MacConkey (SMAC) agar, taking advantage of this serotype's inability to ferment sorbitol (March and Ratnam 1986; Fedio *et al.* 2011; Fedio *et al.* 2012). However, there are many sorbitol non-fermenters such as some *Hafnia alvei* and *Salmonella* strains, resulting in false positives (Park *et al.* 2011), while certain *E. coli* O157:H7 strains are sorbitol fermenters, resulting in false negatives (Gunzer *et al.* 1992). Selectivity is increased by the addition other non-fermentable sugars such as rhamnose (Chapman *et al.* 1991) or gentiobiose (Park *et al.* 2011). Addition of selective agents cefixime and potassium tellurite is also common (Zadik *et al.* 1993). SMAC, however commonly used, does not support the growth of stressed and injured cells (Blackburn and McCarthy 2000). CHROMagar® O157 is a media that is also extensively used for the isolation of *E. coli* O157:H7, where the target produces pink colonies, whereas other *E. coli* serotypes generally produce blue colonies (Bettelheim 1998a). Rainbow™ Agar O157 (Biolog) was developed and contains two chromogenic subtrates for β-galactosidase (blue-black) and β-glucuronidase (red). Since *E. coli* O157:H7 is glucuronidase-negative, grey or steel black colonies are yielded on Rainbow™ Agar O157, while other *E. coli* colonies can be anything from pink to purple, blue, or black (Bettelheim 1998b).

Salmonella enterica is most often grown on Xylose Lysine Deoxycholate (XLD) or Xylose Lysine Tergitol™-4 (XLT) agar, the only difference being the selective agents Sodium deoxycholate and Tergitol™-4 being used, respectively. For the conventional detection of *Salmonella* on media such as XLD, XLT4, and Hektoen Enteric Agar (HEA) the generation of H_2S and inability to ferment lactose by *Salmonella* species are used (Cooke *et al.* 1999).

These media are based firstly on the pH drop caused by *Salmonella*'s ability to ferment xylose, followed by the reversal of the pH drop by the decarboxylation of Lysine. Secondly, *Salmonella*'s ability to reduce iron is utilised. The ferric ammonium citrate together with sodium thiosulfate results in the reduction of iron and the production of hydrogen sulphate under alkaline conditions, causing the colonies to be red with a black centre. Fluorogenic and chromogenic substrates are based on the presence of caprylate (C_8) esterase activity (Cooke *et al.* 1999), presence of α-galactosidase activity, and absence of β-galactosidase activity (Perry *et al.* 1999) that is specific to *Salmonella,* unlike most other *Enterobacteriaceae*.

Selective plating, either traditional or chromogenic has the drawbacks of being time-consuming, delayed results, overgrowth of BM yielding false negatives, and confirmation of presumed positive colonies by molecular, serological, and biochemical testing (Bettelheim 1998a; Manafi 2000; Kim *et al.* 2006; Delbeke *et al.* 2015a; Lee *et al.* 2015). To overcome these drawbacks, molecular methods involving PCR has gained popularity due to its rapidity and accuracy (Gordillo *et al.* 2011; Khan *et al.* 2014; Delbeke *et al.* 2015a).

3.2.2   The Polymerase Chain Reaction

The polymerase chain reaction (PCR) has been utilized for the rapid detection and serotyping of foodborne pathogens from various sample sources due to its rapidity, ease, sensitivity, and specificity; reducing the need for time-consuming and unreliable selective plating (Meng *et al.* 1996; Gordillo *et al.* 2011; Almeida *et al.* 2014; Beaubrun *et al.* 2014; Khan *et al.* 2014; Lee *et al.* 2015). The PCR has many applications; it can detect single pathogens conventionally, or multiple pathogens and/or virulence genes simultaneously by multiplexing (Kim *et al.* 2006; Fujioka *et al.* 2013), or do all of the above in addition to quantification in real-time (Chen *et al.* 2010). The greatest drawback of PCR is that it amplifies all DNA in a

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sample, and therefore does not differentiate between viable and non-viable cells (Dinu and Bach 2013). Multiplexing allows the detection and differentiation of various pathogens or pathotypes by amplifying different target sequences at once by combining different sets of primers in the same reaction mixture (Chandra *et al.* 2013; Fujioka *et al.* 2013) allowing faster screening of samples, limiting workspace and labour, and therefore reducing costs (Garrido *et al.* 2013; Delbeke *et al.* 2015a). Multiplexing further has the advantage that an internal amplification control (IAC) can be included to rule out false negatives (González-Escalona *et al.* 2012). Real-time PCR has the advantages of being faster, having smaller chances for cross-contamination (and therefore false positives), increased specificity and sensitivity, potential for automation, and the possibility for quantification (Fitzmaurice *et al.* 2004; Fujioka *et al.* 2013; Delbeke *et al.* 2015a). Multiplex PCR for the detection of multiple pathogens usually test for a combination of *Salmonella enterica, E. coli* O157:H7, and *Listeria monocytogenes* (Kim and Bhunia 2008; Garrido *et al.* 2013; Delbeke *et al.* 2015a).

Suitable PCR-targets for the detection of *E. coli* O157:H7 include virulence genes encoding *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2)*, eaeA* (intimin), *hlyA* (enterohemolysin), *fliC*$_{h7}$ (flagellar H7 gene), and *rfbE* (O157 LPS) (Desmarchelier *et al.* 1998; Boerlin *et al.* 1999; Bottero *et al.* 2004; Bischoff *et al.* 2005; Sarimehmetoglu *et al.* 2009). Furthermore, primers specific for the dysfunctional *uidA* gene, which contains a G residue instead of a T residue at position 92, is also valuable for detecting *E. coli* O157:H7 (Cebula *et al.* 1995). *Salmonella enterica* is detected most often by targeting *invA* (*Salmonella* invasion protein) (Rahn *et al.* 1992; Malorny *et al.* 2003; González-Escalona *et al.* 2012; Khan *et al.* 2014). Other targets include *fimA* (major fimbrial subunit), *spv* (*Salmonella* Plasmid Virulence), *stu* (enterotoxin), *fliC* (flagellin), *hila* (invasion transcriptional activator) (Chen *et al.* 2010), *sifB* (secreted

effector protein SifB) (Almeida *et al.* 2014), *fimW* (*Salmonella* type I fimbriae regulator) (Zhang *et al.* 2014a), and *siiA* (type 1 secretion-related protein) (Hassena *et al.* 2015).

Conventional PCR has the limit that it cannot distinguish between live and dead cells (Dinu and Bach 2013). Detection of live cells only can be done applying reverse transcriptase PCR (RT-PCR). The short half-life of mRNA allows it to be the perfect target for identifying viable cells (Yaron and Matthews 2002). mRNA chosen for detection in RT-PCR should be target specific, constitutively expressed, and have a short half-life once cells perish. Of multiple targets for *E. coli* O157:H7, *rfbE* was the best target for RT-PCR (Yaron and Matthews 2002). Combining RT with real-time PCR allows potential quantification of target organisms in the sample. The detection of viable cells only can also be accomplished by treating the sample with propidium monoazide (PMA) prior to PCR. Binding of PMA to dead cells' DNA via photolysis will allow only live cells to be detected. This method allows the quantification of organisms in a sample and can detect cells in the VBNC state as well (Dinu and Bach 2013).

3.2.3 Immunological Methods

Immunological methods are based on the specificity of antigen-antibody interactions (Yeung *et al.* 2014). The antigens targeted by immunological detection methods for *E. coli* O157:H7 include the somatic (O157) and flagellar (H7) antigens, as well as Shiga toxins 1 and 2 (de Boer and Heuvelink 2000; Fu *et al.* 2005; Fu *et al.* 2010).

The enzyme-linked immunoassay (ELISA) is the most widely used immunoassay for the detection on *E. coli* O157:H7 and Shiga toxins (de Boer and Heuvelink 2000). ELISA for the detection of *E. coli* O157:H7 and Stxs is performed using polyclonal antibodies for capture

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and monoclonal antibodies for detection. Because the detection limit for these tests are generally high, they are performed following an enrichment step. Presumptive results can be obtained within one day instead of the two required when using conventional culturing techniques. Additionally, ELISA allows the screening of a high number of samples at once.

Immunomagnetic separation (IMS) as a valuable method for targeted sample purification, and enhanced detection of microbes by plating and PCR (Scotter *et al.* 2000; Tims and Lim 2003; Fedio *et al.* 2011; Geng *et al.* 2011). Immunomagnetic separation (IMS) relies on capturing organisms from a system using para-magnetic particles coated with antibodies specific to the target organism's antigens. The target adheres to the surface of the particles to form a complex that is removed from the system by the application of a magnetic field. IMS removes the target from potentially interfering food debris and BMs and increases the concentration of target cells prior to detection (Bennett *et al.* 1996). The use of IMS increases the sensitivity, specificity, and rapidity of detection methods. Performing IMS prior to enrichment cultivation serves as a pre-concentration step that may reduce the time needed for enrichment by reducing interference from sample components and BMs (Taskila *et al.* 2012). IMS may not be successful prior to enrichment if the cells occur in a stressed state, which may reduce antigenic reactions. Therefore, incorporating a short pre-enrichment step prior to IMS allows the resuscitation of cells and the regain of antigenicity. The most used commercial IMS system is the Dynabeads anti-*E. coli* O157 immunocapture system (Dynal, Oslo, Norway).

Immunological methods for *E. coli* O157:H7 show cross-immunoreactivity with antigenically related organisms such as *Escherichia hermanii, Salmonella* O30, *Hafnia alvei,* and *Citrobacter freundii* (de Boer and Heuvelink 2000; García-Aljaro *et al.* 2005). Consequently

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immunological methods allow rapid screening of foodstuffs and environmental samples for identification of negative and presumptive samples, but require confirmation by isolation and characterization (Yeung *et al.* 2014).

Conventional detection techniques are relied upon heavily in the food industry to ensure product safety. However, each still has its drawbacks and suffers from relatively high detection limits and relies heavily on culture techniques. Fresh produce is a living system, each product having its own microbiome of which human pathogens may be part (Berg *et al.* 2014). With advances in technology and bioinformatics, studying foodborne pathogens' evolution, ecology, and adaptation to stress is possible as part of the plant's microbiome is almost possible in a culture-independent manner. In the following section a brief overview of theses techniques is given.

4   APPLICATION OF FOODOMICS FOR ENSURING SAFE PRODUCE

4.1   Introduction to Foodomics

The "Central Dogma Theory of Genetics" explains how DNA from the genome is translated to RNA that form part of the organism's transcriptome. RNA is then transcribed to proteins to make up a proteome. These proteins facilitate biochemical pathways during metabolism, and all the various small intermediate and end product chemicals, or metabolites, make up the metabolome (Davies 2010). In systems biology, characterizing and quantifying different molecules that form part of various "omes" of living organisms using various techniques coupled to bioinformatics, is called "omics" technologies. Whereas microorganisms were traditionally studied singularly using culturing and immunological techniques, "omics"

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techniques allow us to study the composition and activities of entire communities from various environments in a largely culture-independent manner (Schneider and Riedel 2010). When applying "omics" techniques to studying and monitoring the quality, safety, nutrition, and microbiology of food to ensure consumer health and confidence, "foodomics" is a term commonly used (Giacometti and Josic 2013; Ibáñez *et al.* 2013). "Omics" techniques help researchers to study the contamination, survival, and persistence of foodborne pathogens in fresh produce in an informative, predictive, and comparative manner (Cevallos-Cevallos and Reyes-De-Corcuera 2012) by allowing the rapid and accurate detection, identification, and quantification of pathogens and their toxins (Gašo-Sokač *et al.* 2010). Furthermore, "omics" techniques help monitor changes in food quality, and microbial ecology and behaviour as a result of processing, cleaning, and sanitation processes (Giacometti and Josic 2013).

4.2   Genomics and Transcriptomics

For a long time microbial diversity in various environments were studied by culturing and identifying microbes with biochemical and immunological tools, however, with the invention of culture-independent sequencing techniques, it is estimated that less than 1% of all microorganisms can be cultured using standard methods (Fournier *et al.* 2014; Samarajeewa *et al.* 2015). Genomics tools has allowed detection and identification of pathogens directly from samples (Fournier *et al.* 2014) and allowed the study of microbial ecology in different environments without cultivation (Schneider and Riedel 2010). Various molecular techniques have been developed to study microbiomes independent of culturing (Simon and Daniel 2011; Su *et al.* 2012), including denaturing/temperature gradient gel electrophoresis (DGGE/TGGE)), 16S rRNA sequencing, single-strand-conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), DNA Microarray, terminal restriction

fragment length polymorphism (T-RLFP), and quantitative PCR (qPCR) (Su *et al.* 2012; Samarajeewa *et al.* 2015). Metagenomics studies started by cloning environmental DNA directly into a phage vector, followed by sequencing, bypassing a culture step (Pace *et al.* 1985). Since then, advances in PCR and sequencing technologies has allowed studying environmental ecology and metabolism (Simon and Daniel 2011). Next generation sequencing (NGS) techniques such as 454 pyrosequencing have allowed high-throughput, large-scale characterization of various diverse microbial consortia at a highly reduced cost compared to conventional techniques (Samarajeewa *et al.* 2015).

NGS techniques applied to studying the fresh produce microbiome, either by sequencing conserved marker genes (e.g. 16S rRNA) or shotgun sequencing of community DNA helps to identify and quantify all the operational taxonomic units (OTUs) in a sample (Ercolini 2013), including those of human pathogens and spoilage organisms (Simon and Daniel 2011; Rastogi *et al.* 2013). NGS techniques by direct nucleic acid extraction has the benefit of preventing enrichment bias, reliable identification of all OTUs in a sample, and the screening of multiple samples in one run (Ercolini 2013). Currently, the main drawbacks of metagenomic approaches in the food industry relates to the high cost of sequencing, especially the initial cost of buying equipment and training. Furthermore, large numbers of sequences are generated for each sample that requires skilled bioinformaticians to analyse and convert to usable data. Still, the ability to identify pathogens from sequence data, independent of culturing is the way to go (Ercolini 2013). Unfortunately, the low numbers of cells usually present in food and limitations to DNA extraction and concentration techniques still limit application of sequencing. Targeted sequencing used to detect possible foodborne pathogens and associated microbiota in a specific sample should be designed to complement culturing procedures, directly from enrichment cultures (Bergholz *et al.* 2014). For detection purposes,

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metatranscriptomics that relies on the isolation, detection, and sequencing of mRNA transcripts, is crucial. Since DNA has a long half-life and may persist in the environment for years after the target organism has expired. RNA molecules on the other hand have short half-lives and the detection of intact mRNA relates directly to viable and/or metabolically active cells.

Transcriptomic studies relying on the extraction and sequencing of mRNA can give a good indication of the diversity and activities of the fresh produce microbiome (Knief 2014). Transcriptomics applied to the study foodborne pathogens to fresh produce allows the identification of various genes involved in the attachment and survival of foodborne pathogens on plants (Rastogi *et al.* 2013). Furthermore, these studies can help identify other microorganisms that are conducive or suppressive to the colonization of plants by foodborne pathogens (Mendes *et al.* 2013) that can be used for risk assessment and the use of beneficial microorganisms to prevent contamination of produce. Microarrays with sequences complementary to mRNA transcripts are the most commonly used technology for gene expression profiling (Davies 2010) and can allow rapid screening of a sample for expression linked to the presence and activities of target organisms, allowing the rapid and sensitive identification and comparison of viable organisms in a sample without the need for culturing (Fournier *et al.* 2014).

4.3   Metabolomics

Simply defined, metabolomics is the systematic study of all chemical constituents of a biological system (Cevallos-Cevallos and Reyes-De-Corcuera 2012; Castro-Puyana and Herrero 2013). Metabolomics usually aims to study all the low molecular weight (<1500 Da)

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chemical compounds in a sample that result from the metabolic pathways within the biological system (Ibáñez *et al.* 2013). Metabolomic studies may be targeted (profiling) or untargeted (fingerprinting) (Cevallos-Cevallos and Reyes-De-Corcuera 2012; Castro-Puyana and Herrero 2013) to either study a specific group of chemicals in the sample (Cevallos-Cevallos *et al.* 2009), or determine the inherent biological characteristics of the target (Davies 2010), respectively. Metabolomics techniques may further be used to detect biomarkers that indicate the presence or abundance of a specific living organism or its activity within a sample. In food safety studies, mass spectrometry (MS) techniques are used mostly for microbial fingerprinting that aims to detect biomarkers for rapid pathogen detection in samples (Ibáñez *et al.* 2013). Metabolic profiling and biomarker detection of foodborne pathogens are usually carried out by GC-MS to analyse the volatile organic compounds (VOC) associated with pathogen growth (Castro-Puyana and Herrero 2013). VOC-based metabolic fingerprinting allowed the detection of *Salmonella*-caused pork spoilage (Xu *et al.* 2010). In a different study, SESI-MS-mediated VOC-based metabolic fingerprinting of *E. coli* strains, including *E. coli* O157:H7, six core peaks were identified that can be used as biomarkers for the detection of *E. coli* strains from contaminated samples (Zhu and Hill 2013). In an earlier study, the metabolic profiles of *Salmonella* and *E. coli* O157:H7 were evaluated by GC-MS following a short (18 hour) incubation in nonselective media (Cevallos-Cevallos *et al.* 2011). Although fingerprints were successfully detected, no unique biomarkers were identified that can be used for accurate identification of either pathogen from the sample. Nonetheless, further studies into metabolic fingerprinting and biomarker identification may in the future contribute to rapid, accurate, and sensitive identification of pathogens in a sample that will reduce the risk of possible foodborne outbreaks.

4.4   Proteomics: Foodborne Pathogen Detection with specific reference to MALDI-TOF MS

Proteomics involve the study of the proteome, i.e. involving all proteins expressed within a living organism (Schneider and Riedel 2010). Since proteins are encoded on organisms' genome, related organisms express similar proteins that can be used for taxonomic purposes. The increased sensitivity and accuracy of MS techniques, coupled to advances in bioinformatics tools and computing power allows the characterization and identification of various proteins (Schneider and Riedel 2010; Lou *et al.* 2015). Analysing biomarker protein patterns unique to specific genera, species, and strains using MS techniques, especially MALDI-TOF MS, allow researchers to rapidly and accurately identify bacteria (Liu *et al.* 2007; Christner *et al.* 2014; Santos *et al.* 2015). MALDI-TOF MS can be applied to whole cells, excluding the need for prior protein extraction steps and still obtain protein profiles required to accurate identification (Holland *et al.* 1996). MALDI-TOF MS detects protein profiles by combining the mass-to-charge ratio (*m/z*) and signal intensity of proteins to yield a peak, with similar proteins yielding similar peaks (Carbonnelle *et al.* 2011; Christner *et al.* 2014). MALDI-TOF MS analysis starts by crystalizing a sample in a matrix. The matrix is then bombarded with a UV laser that vaporises the sample to release ions of different sizes. The ions are then passed through an acceleration grid before moving through a flight tube and ultimately hitting a detector. The detector determines the mass and quantity of the ions, creating mass spectra (Giebel *et al.* 2010). Matching the specific pattern of conserved peaks (biomarkers) yielded by MALDI-TOF MS analysis to a database allows accurate chemotaxonomic classification of the organism in question (Lay Jr 2000).

The matrix choice depends on the biomolecule(s) to be identified. The matrix has two functions; firstly, the absorption of energy from the laser, and secondly, the isolation of biopolymer molecules (Lou *et al.* 2015). Matrices used include sinapinic acid (SA), α-cyano-

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4-hydroxycinnamic acid (CHCA), 3,5-dimethoxy-4-hydroxycinnamic acid, ferulic acid, 2-(4-hydroxyphenylazo)benzoic acid (HABA), and 2,5-dihydroxybenzoate (DHB) (Carbonnelle *et al.* 2011; Chui *et al.* 2015; Lou *et al.* 2015). SA, CHCA, and ferulic acid are used when the molecules in question are proteins, while DHB is used when studying glycopeptides and – proteins (Giebel *et al.* 2010). CHCA was successfully used in a universal sample preparation method to fingerprint a diverse range of bacteria (Liu *et al.* 2007). Reproducibility is often a concern when using MALDI-TOF MS for identifying organisms (Lay Jr 2000; Lou *et al.* 2015). Since protein expression is highly dependent on the environment the microorganism is exposed to, experimental conditions should be carefully controlled (Carbonnelle *et al.* 2011; Veloo *et al.* 2014). Conditions of special importance are growth media, temperature, and culture age (Lay Jr 2000; Keys *et al.* 2004; Veloo *et al.* 2014). Microorganisms should preferably (and depending on each organism's growth requirements) be grown the same prior to MALDI-TOF MS. Culture age is also important since each growth phase is characterised by the expression of unique proteins, usually as a function of population density and nutrient availability/depletion. Direct MALDI-TOF MS analysis of intact bacteria is accurate, rapid, and cost-effective (Lou *et al.* 2015; Santos *et al.* 2015). The main drawbacks are problems with reproducibility and that the identification of bacteria still relies on culturing (Christner *et al.* 2014). However, with improvements, MALDI-TOF MS will prove invaluable in foodborne pathogen detection and characterisation (Chui *et al.* 2015; Santos *et al.* 2015).

Knowledge about how foodborne pathogens colonize plants, interact with native microbiota, and how they survive processing and sanitation processes will prove invaluable in predictive microbiology allowing the design of strategies to increase food safety measures by changing the environment in ways that counter the contamination and persistence of foodborne pathogens on fresh produce (Brul *et al.* 2006). Furthermore, "omics" techniques will

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facilitate the sensitive, rapid, and accurate detection of foodborne pathogens in samples to facilitate monitoring of produce safety throughout the supply chain.

5   CONCLUSIONS

The ability of *Salmonella* and *E. coli*'s to effectively invade plants as alternate hosts, and their ability to persist in the environment for extended periods of time causes great concern for the future of fresh produce safety. Even though colonization seems to be a rare event, the great scale on which fruits, vegetables and seedlings are produced create opportunities for bacterial colonization. The increase in fresh produce-associated foodborne outbreaks associated in the past decade may, in part, be attributed to the ability of human enteric pathogens to survive in a non-host environment. To meet the increasing demand for fresh produce, agricultural practices are intensified and an increase in the prevalence of fresh produce-associated foodborne outbreaks can be expected.

For the prevention and control of foodborne pathogens, economical, rapid, and accurate methods have to be deployed. To ensure food safety, various techniques have been developed for foodborne pathogen isolation and detection. However, all techniques have their drawbacks and where one is superior in one aspect, it may have a major shortcoming in another. Traditional enrichment and selective and differential media enables the determination of cell viability, but lacks sensitivity and is laborious and time consuming. Immunological methods are rapid and can differentiate between viable and non-viable cells, but are inhibited by food matrices and low cell concentrations. PCR methods are rapid and highly sensitive, but food matrices interfere with detection of target sequences and PCR is

unable to differentiate between viable and non-viable cells unless cells are treated with propidium monoazide (PMA) (Dinu and Bach 2013).. The solution lies in using a combination of all conventional techniques, drawing on the strengths of all. Enrichment allows pathogen recovery, plating tests viability, immunological methods allow targeted separation, and PCR can be used for identification. Real-time PCR allows faster, more accurate, sensitive and specific detection of foodborne pathogens and shows great potential for automated high-throughput detection. Enrichment still precedes most detection methods, however highly selective one-step enrichment techniques have still to be developed. There is a definite trend towards the development of selective multipathogen enrichment techniques, coupled with multiplex real-time PCR detection and identification. Furthermore, progress of "omics" techniques will prove invaluable in studying the ecology of foodborne pathogens in fresh produce, enhance the sensitivity and rapidity of pathogen detection, and contribute to the development of specialised media.

As the scale of fresh produce production and consumption increases, so does the incidence of foodborne outbreaks. To ensure future produce safety, good agricultural practices and pathogen detection techniques must be optimised to prevent contamination and allow quickly, reliable, and cost-effective surveillance.

6   OBJECTIVES OF THIS PROJECT

The major increase in tomato production in South Africa has increased the potential for foodborne outbreaks. Also, very little is known about the microbiological safety of South African fresh produce in general. Therefore there is a need to (1) determine the current safety

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status of commercially produced tomatoes in South Africa; (2) identify potential contamination sources in the supply chain; (3) develop strategies to mitigate risks to ensure continued tomato safety.

This project focused on a) determining the microbial safety of commercially produced tomatoes in South Africa, and b) evaluating limited glucose release for the enhanced recovery of injured *Escherichia coli* O157:H7 from water.

The overall aims of this project are to achieve the following goals using conventional culturing methods, biochemical detection and enumeration techniques, and MALDI-TOF, multiplex PCR and Sequencing for isolate identification and identity confirmation.

(1)   To isolate and identify the human enteric pathogens *Salmonella enterica* and *Escherichia coli* O157:H7 on tomatoes and in the tomato supply chain;

(2)   To assess the quality of the irrigation water used in tomato production;

(3)   To identify potential point sources of enteric pathogen contamination in tomato production

(4)   Evaluate the potential of limited glucose release technology for the enrichment of injured *Escherichia coli* O157:H7 from water

(5)   Evaluate the potential of limited glucose release technology for culture-based enumeration of injured *Escherichia coli* O157:H7 from water

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CHAPTER 2

Microbiological status of commercially produced tomatoes in South Africa

ABSTRACT

Tomatoes have been implicated in various microbial disease outbreaks and are considered a potential vehicle for foodborne pathogens. Trace-back studies mostly implicate contamination during production and/or processing. The microbiological quality of commercially produced tomatoes was thus investigated from the "farm-to-market" focussing on the impact of contaminated irrigation and washing water, facility sanitation and personal hygiene. A total of 905 samples were collected from three large-scale commercial farms from 2012 - 2014. The farms differed in water sources used (surface vs. well) and production methods (open field vs. tunnel). Total coliforms and *Escherichia coli*, and prevalence of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were determined. Dominant coliforms were identified using MALDI-TOF MS. No pathogens or *E. coli* were detected on any of the tomatoes tested throughout the study despite the high levels of coliforms ($4.2 - 6.2$ log cfu/g) present on the tomatoes at market level. The dominant species associated with tomatoes belonged to the genera *Enterobacter, Klebsiella,* and *Citrobacter*. Water used on farm for irrigation purposes were found to be "not-fit-for-purpose" according to national agricultural irrigation standards, with high *E. coli* titres resulting from either highly contaminated source water (3,19 log MPN/100mL river water), or improper storage of source water (1,72 log MPN/100mL stored well water). *Salmonella* Typhimurium was detected on two separate occasions on the contact surface in the processing facility of the first farm in 2012. Contact surface coliform counts ranged between $2.9 - 4.8$ log cfu/cm^2. Risky areas identified in this study were water used for irrigation purposes and poor sanitation practices in the processing facility. Implementation of effective food safety management systems in the

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fresh produce industry to ensure product safety to the consumer is therefore of utmost importance.

1   INTRODUCTION

In the past three decades, foodborne disease outbreaks associated with fresh produce has increased annually (Sivapalasingam *et al.* 2004; Lynch *et al.* 2009; Crowe *et al.* 2015). Fresh produce associated outbreaks have increased in the US from 0.7% in 1970, to 6 % in the 1990s (Sivapalasingam *et al.* 2004), 13% in the early 2000s (Doyle and Erickson 2008) and 46% in 2014 (CDC 2015b) of all reported foodborne outbreaks. Fresh produce production has similarly increased to meet the growing world demand (Pollack 2001; Olaimat and Holley 2012). The expansion of the fresh produce industry is characterised by increased production intensities, centralized production and distribution systems, ready-to-eat (RTE) food products, expanded global trade of fresh produce, and surveillance efficiency (Beuchat 2002; Lynch *et al.* 2009). On the other hand, the consumer profile has also changed, with an increasing number of individuals who are disproportionately susceptible to foodborne illnesses due to age or suffering from conditions resulting in a immunocompromised state (Kobayashi 2006; WHO 2008). These factors have not only contributed to the increased incidence of foodborne illnesses, but also to the geographical range and number of people affected in a single outbreak (Jacobsen and Bech 2012) as witnessed during the 2011 outbreak of *E. coli* O104:H4 in Germany. This outbreak was caused by contaminated sprout seeds imported from Egypt and resulted in 53 confirmed deaths, with hundreds of people developing haemolytic uremic syndrome (HUS) (EFSA 2012). This suggests that fresh produce serves as a major vehicle for enteric pathogen dissemination (Holden *et al.* 2008; Warriner *et al.* 2009). In the US alone, the economic burden of foodborne outbreaks in 2013 was estimated to be between $4.8 billion to $36.6 billion (Hoffmann *et al.* 2015), not only in monetary value due to recalls and product

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destruction (Anonymous 2011), but also the impact these outbreaks have on the health and welfare of consumers and their perceptions of the safety of the food system (Olaimat and Holley 2012). *Salmonella enterica* and *E. coli* O157:H7 have been implicated in various tomato-associated foodborne outbreaks (Hedberg *et al.* 1999; CDC 2007; Greene *et al.* 2008; CDC 2015a).

Contamination of tomatoes can occur at any point during the farm-to-fork continuum (Beuchat 2002; Holden *et al.* 2008; Olaimat and Holley 2012; Bartz *et al.* 2015). The common areas where contamination occurs are during production in the field, processing, and at the consumer end (Carrasco *et al.* 2012; Doyle and Erickson 2012; Jacobsen and Bech 2012; Shenge *et al.* 2015). Due to the absence of a "kill-step" when tomatoes are consumed raw it is of utmost importance to restrict contamination throughout the supply chain (Doyle and Erickson 2012; Olaimat and Holley 2012; Pagadala *et al.* 2015). Pre-harvest sources of contamination include feedlot run-off, untreated manure, contaminated soil and water, and the presence of animals and birds in the field (Beuchat 2002; Holden *et al.* 2008; Warriner *et al.* 2009; Jacobsen and Bech 2012; Cevallos-Cevallos *et al.* 2014; Lopez-Galvez *et al.* 2014; Bell *et al.* 2015). Postharvest contamination and cross-contamination are usually due to contaminated harvesting equipment and containers, contaminated flume water, the presence of pathogens on surfaces in the packinghouse, the hands of workers, retailers, and in the kitchens of consumers (Beuchat 2002; Warriner *et al.* 2009; Zhou *et al.* 2014). Of all these potential sites of contamination, the production and processing environments are suspected of being the primary source of tomato contaminants due to the wide distribution of outbreaks (Hanning *et al.* 2009; Lopez-Galvez *et al.* 2014). The close association of enteric pathogens with their plant hosts, and specifically the adhesion and internalisation of *Salmonella* in

tomato fruits, causes common sanitation practices to be ineffective at killing or inactivating these pathogens (Hanning *et al.* 2009; Bae *et al.* 2012; Bartz *et al.* 2015; Nugent *et al.* 2015).

According to FAOSTAT (2014), South Africa produced 564740 tons tomatoes in 2012, a 34% increase since 1992, mostly supplying local markets (DAFF 2013). Despite increases in exports, a mere 3% of tomatoes produced in South Africa in 2012 were exported to other countries (DAFF 2013; FAOSTAT 2014). Tomato is the most consumed vegetable after potatoes in the country with 12kg per capita consumption (DAFF 2013). Tomatoes are consumed by many South Africans, mostly as part of a tomato and onion stew (73,1%), but is also consumed raw (26,9%) in salads and as wedges (Labadarios *et al.* 2005). It is thus of utmost importance that commercially produced tomatoes are safe to eat, since contaminated tomatoes can affect the consumer, particularly South Africans, where 19,1% of the population have increased susceptibility to foodborne illnesses due to their positive HIV/AIDS status (WHO 2008).

In this study we investigated the current microbiological safety of commercially produced tomatoes in South Africa given the challenge of contaminated water sources, vulnerable public and lack of information pertaining to the safety of tomatoes produced in South Africa. The presence of *Salmonella* Typhimurium and *E. coli* O157:H7 in the pre- and postharvest environments from the field to the market was investigated. The microbial quality of the water used for irrigation and washing, the soil and tomato fruit were determined and the sanitation levels of contact surfaces and personal hygiene of hands were evaluated by enumerating generic *E. coli* and coliforms to test for faecal contamination and effectiveness of sanitation practices, respectively. All samples were processed following a multi-assessment

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approach of traditional and molecular methods and confirmation of organism identity using sequencing. The results were interpreted in the context of source of contamination exceeding regulatory standards and comparative management practices using a diagnostic tool. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was used to rapidly identify coliforms and other members of the *Enterobacteriaceae* family that occur within the tomato production and supply chain environment in South Africa.

2   MATERIALS AND METHODS

2.1   <u>Study areas and sampling design</u>

A longitudinal study of three commercial tomato farms was done in the tomato-production regions during the tomato-growing season. The study was conducted from 2012 to 2014. The first two farms are located 67 km apart in the Limpopo Province and owned by the same company. On these farms, tomatoes are grown in the open field year-round. On Farm 1, water used for irrigation is pumped from a river frequently contaminated by an upstream sewage plant (Matlala 2012; Moloto 2012; Erasmus 2014). On Farm 2, water is pumped from a large dam fed by surface water and adjacent to a cattle pasture. On both farms the water is stored in storage dams for use in drip irrigation. The third farm, owned by a second company, is situated in the Gauteng Province where tomatoes are grown in tunnels in mulched raised beds and well water, pumped and stored in an uncovered storage dam, prior to use in drip irrigation. The locations of the farms within South Africa are shown in Figure 1. A comparison of the farms and their practices is given in Table 1. Each farm had their own processing facility where tomatoes are washed, dried, sorted, and packed and dispatched to

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the informal markets, national fresh produce markets or directly to retailer-distribution centres. Company 1 washes fruit in flumes with water containing the disinfectant Sporekill® (ICA International Chemicals, Stellenbosch). Company 2 washes fruit with pressurised sprays using untreated water.

A total of 905 samples were collected and analysed during this study. Each farm was visited three times throughout one growing cycle. The visits took place four weeks and two weeks before harvest and at harvest. Farm 1 was visited in 2012, Farm 2 was visited in 2013 and Farm 3 was visited in 2014. Samples were obtained from the field, processing facility and the retail vendors, fresh produce market or informal market depending on the farms' dispatch systems. Samples included tomatoes, soil, water, and swabs taken from contact surfaces and hands of pickers and packers. Coliform and *E. coli* counts were performed for all water samples from all three farms, and all samples from Farms 2 and 3, to determine basic sanitary levels and potential faecal contamination, respectively. All samples were also tested for the presence of *E. coli* O157:H7 and *Salmonella* Typhimurium due to their reported association with disease outbreaks in other countries (FAO and WHO 2008). The samples were transported in cooler boxes for transport back to the Plant Pathology laboratories of the University of Pretoria and were processed within 24 h of collection. A summary of sampling procedures, sample sizes and processing is given in Table 2.

2.2 Sample collection

2.2.1 Farm sampling.

All samples were collected in triplicate. Water was collected in 1 L and 100 ml volumes from each site. Water used for irrigation purposes was collected from the irrigation water source, on-farm storage dam, and irrigation pipe outlet. Each tomato sample consisted of five fruits aseptically picked from three plants growing adjacent to each other. Tomatoes were harvested aseptically from the same plants throughout the study on respective farms and transported in brown paper bags in a cooler box. On Farms 1 and 2, plants sampled were chosen at random diagonally throughout the field (approx. 100 m^2). On Farm 3, tomatoes are grown in tunnels (approx. 50 m long, grouped together in rows of ten and containing three rows of plants). Tomatoes were sampled at random from three different middle-rows in the tunnels. From the tomato rhizozone, 50 g soil was collected from each of the same three plants sampled for the fruit, and was pooled to form a 150 g soil sample. Soil and tomato samples were collected in each field at each field visit. On Farm 3, a large commercial composting management system is operated that provides the soil amendments used on the farm. Three 50 g compost samples were collected from each heap and pooled to form a 150 g compost samples. Three compost heaps were sampled to test for the presence of indicator and foodborne pathogens. Contact surfaces included the hands of three tomato pickers and three harvesting crates and were sampled using transport swabs (Copan, Cape Town, South Africa). The pickers' hands and harvesting crates were swabbed in 25 cm^2 and 50 cm^2 areas, respectively, using standard swab sampling procedures (Sanderson *et al.* 2002).

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2.2.2 Processing facility sampling.

Samples were collected at the beginning, middle, and end of each day during each visit. Three samples of five tomatoes were collected entering the processing facility, prior to washing, and three samples of packaged tomatoes leaving each facility. Water used in the flume of the processing facilities of Farms 1 and 2, stored water, as well as water used in the facility was collected three times a day, six hours apart. On Farm 3, water was collected once a day from the sprayer used for washing. Water was collected in 1 L and 100 ml volumes in triplicate. Contact surfaces in the processing facilities on all three farms were sampled using transport swabs (Copan) and collected in triplicate, three times a day. After arriving at the packinghouse, the tomatoes are washed by flume (Company 1) or sprayers (Company 2), and then brushed, air-dried, and sorted according to size and ripeness, and packed by hand. The contact surfaces tested thus included the crates in which the tomatoes arrived, the rollers, the brushers, the conveyers, and packers' hands. The packers' hands and other contact surfaces were swabbed in 25 cm^2 and 50 cm^2 areas, respectively.

2.2.3 Retail and market sampling

Tomatoes were purchased following every farm visit. Tomatoes from Farm 1 are only distributed to the Tshwane fresh produce market. Three boxes of tomatoes originating from Farm 1 was bought at the market the day following the farm visit ensuring that the same batch of fruit from the same sampled field was collected using producer code numbers and harvesting and dispatch dates. Tomatoes originating from Farm 2 are similarly distributed to a local informal market and the Tshwane fresh produce market. Three packs were purchased from the informal market close to the farm, and three boxes were purchased at the Tshwane

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fresh produce market. Tomatoes from Farm 3 are distributed to various retailers, and three packs were bought at a retail outlet close to the University of Pretoria.

2.3   Sample processing

Farm 1 served as a trial study and after its conclusion, the protocol was reviewed and amended slightly to include the enumeration of both *E. coli* and coliforms on Petrifilm *E. coli*/Coliform count plates (3M Microbiology, Johannesburg, South Africa) and applied to all non-water samples analysed from Farms 2 and 3 onwards. In the preliminary study, serial dilutions were performed and plated in duplicate on eosin methylene blue agar (EMB) (Merck Biolab, Johannesburg) to enumerate *E. coli.* As per manufacturer's instructions, 100 ml water samples were used to determine the most probable number (MPN) *E. coli* and Coliforms using Colilert-18 (IDEXX Laboratories Incorporated, Westbrook, ME, USA) reagents heat-sealed in Quanti-Tray/2000 (IDEXX) (Noble *et al.* 2006) and incubated at 37 °C for 48 h. The trays were inspected for chromogenic reactions and fluorescence, indicating the presence of coliforms and *E. coli,* respectively. The 1 L water samples were vacuum-filtered through a 0.45 μm nitrocellulose membrane (National separations, Johannesburg) and transferred to 90 ml buffered peptone water (BPW) (Merck Biolab) for overnight enrichment in BPW (Noble *et al.* 2006; Hamner *et al.* 2007; Mushi *et al.* 2010). The protocol followed for the isolation of *E. coli* O157:H7 and *Salmonella* from tomatoes was a variation to the guidelines presented in the US Food and Drug Administration's (FDA) bacteriological analytical manual (BAM) (Andrews *et al.* 2011). For every sample consisting of five tomatoes, tomatoes were halved, and homogenized in a blender for two minutes. Ten grams and 25 g homogenized puree were added to 90 ml and 225 ml BPW, respectively. The 10 g and 25 g samples were used for ten-fold serial dilutions and enrichment, respectively. Ten grams of rhizozone soil was measured

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from every sample, added to 90 ml BPW, and vortexed for 2 min prior to enumeration or enrichment. Swabs were added to 9 ml BPW for enumeration and enrichment. For enumeration, 1 ml aliquots of all the samples were serially diluted and plated on 3M™ Petrifilm™ *E. coli*/Coliform count plates and incubated at 37 °C for 21 ± 3 h. After incubation, plates expressing counts of 30 – 300 colony-forming units (cfu) were counted. Dominant coliform colonies and colonies testing positive for *E. coli* were picked from the plates and purified for identification. All samples were enriched at 37 °C for 21 ± 3 h with shaking (150 rpm). After enrichment, a 750 µl aliquot was preserved in 60% glycerol at -20 °C.

2.4   DNA extraction, multiplex polymerase chain reaction, and sequencing

Following enrichment, DNA was extracted directly from the enrichment broths using the Quick-gDNA™ MiniPrep (Zymo Research Corp., Pretoria) kit according to the manufacturer's instructions. DNA concentrations were determined using Qubit™ 2.0 Fluorometer (Invitrogen™ Life technologies, Johannesburg). Three sets of primers were used for the multiplex reaction (Table 3). These included primers targeting the *S. enterica* serovar Typhimurium long polar fimbriae D, the *E. coli* O157:H7 mutated β-glucuronidase gene, and a positive internal control, amplifying a region of the universal 16S ribosomal DNA. The multiplex PCR was set up using 14.9 µl ddH$_2$O, 0.3 µl 5X MyTaq™ reaction buffer (Bioline, United Kingdom), 0.3 µl of each primer at their optimal concentrations [30 pmol UidAa and UidAb, 50 pmol SLDF and SLDR, 10 pmol 27 and 1492 (Whitesci, XXIDT, Iowa, USA)], 0.3 µl MyTaq™ DNA Polymerase (Bioline), and 3.0 µl DNA to obtain a final volume of 25 µl. The multiplex PCR was conducted under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of DNA denaturation at 94 °C for 30 s, primer

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annealing at 55 °C for 45 s, and strand extension at 72 °C for 90 s and final extension at 72 °C for 7 min. PCR amplicons were visualised under UV light on 1% (w/v) agarose gel containing 0,1% (v/v) Ethidium Bromide with the VilberLourmat Gel Documentation System (VilberLourmat, Marne La Valle, France) and digitally imaged using E-capt software (VilberLourmat). *E. coli* O157:H7 (ATCC 35150) and *Salmonella* Typhimurium (ATCC 14028) were used as positive controls.

For samples that tested positive for *Salmonella* Typhimurium, a 100 µl aliquot of the preserved enriched sample was transferred to 10 ml tryptone soy broth (TSB) (Merck) and shake incubated (150 rpm) for 24 h at 37 °C. A 100 µl aliquot was then transferred to 10 ml Rappaport-Vassiliadis *Salmonella* enrichment broth (Merck) and shake incubated for 24 h at 37 °C. Thereafter the culture was streak-plated onto *Brilliance Salmonella* agar base (Oxoid, Thermo Scientific, Johannesburg) supplemented with *Salmonella* selective supplement (Oxoid) and incubated at 37 °C for 24 h. Single colonies presumed positive were transferred to TSB and shake incubated for 24 h at 37 °C. DNA was extracted and the concentration measured as described before. A PCR reaction followed by gel electrophoresis was performed to confirm the presence of *Salmonella* as described previously. The amplified DNA was sent for confirmation to the DNA sequencing facility at the University of Pretoria. *Salmonella* Typhimurium homology studies were performed against the NCBI database for sequence similarity using the BLAST (Basic Local Alignment Search Tool) program.

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2.5 MALDI-TOF MS identification of coliforms

The identification of dominant coliform cultures obtained from Petrifilms that were purified and identified using MALDI-TOF MS (BrukerDaltonics, Bremen, Germany) is described below. Single pure colonies grown on standard 1 nutrient agar (Merck) were transferred onto MALDI plates (Sigma-Aldrich, USA) in duplicate and covered with 1 µl cyano-4-hydroxycinnamic acid in an organic solution (50% acetonitrile, 2.5% trifluoroacetic acid). The preparation was crystallized by air-drying at room temperature. Screening is automated without user interference. Flex Control software (BrukerDaltonics) recorded spectra set for bacterial identification. MALDI Biolayer 3.0 software (BrukerDaltonics) matched generated peak lists against the reference library, using an integrated pattern-matching algorithm.

2.6 Food safety risk assessment

Possible risk areas and control activities were identified on the separate farms using a diagnostic tool developed by Kirezieva *et al.* (2013), which can be used by companies to assess the food safety problems they can expect to experience in a specific context and decisions they can make to mitigate these risks. The diagnostic tool aims to assess the riskiness of a production system's output by taking into account the system design, operation, and the specific context in which the system functions. The tool, in the form of a questionnaire, mainly asks questions relating to (1) the contextual factors i.e. that are in place and difficult to change, and (2) the control and assurance activities i.e. measures that are in place to reduce the risk of product contamination and cross-contamination within the specific context of the food safety management system (FSMS). The contextual factors describe the microbiological risk inherent to the characteristics of the product (initial materials and the

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final product) and production system (cultivation system and the source and storage of irrigation water used). Each factor is assigned a rating out of 3, with 1 posing the lowest (or absence of) and 3 posing the highest risk of the factor being associated with microbial contamination. Control activities aim to prevent the contamination of the product and include water control (activities ensuring the safety of water used for irrigation for example monitoring and treatment) and irrigation method (aimed at reducing the contamination of edible parts). Questions that evaluate assurance activities aim to assess the performance of the FSMS by enquiring about auditing and accreditation schemes the company adheres to, customer complaints received, and internal food safety performance indicators such as product sampling and testing, judgment criteria used for test result analyses and the number of product non-conformities experienced. Each control and assurance activity is given a rating out of 4, with 1 being the lowest (or absence of) and 4 being the highest performance in their respective functions of minimising risk. The data obtained from the questionnaires was then presented in spider diagrams to compare the functioning of the FSMSs of the different farms to determine the overall riskiness of the tomatoes produced by each farm.

2.7   Statistical analysis

The differences in indicator bacteria from production to marketing were investigated using SAS version 9.3 statistical software (SAS Institute, Cary, NC, USA). The input for irrigation water was three farms at source, storage dam, and irrigation outlet and the data obtained from Colilert-18 was used. The colony counts obtained from farms two and three from the tomatoes, soil, and contact surfaces were transferred to LOG 10 (X+1) before the performance of analysis of variance (ANOVA). Replications over time were used as block replicates.

Student's t-LSD (Least significant difference) was calculated at a 5% significance level to compare means of significant source effects.

3   RESULTS

3.1   Indicator organisms from tomatoes

Figure 2 shows the total number coliforms recovered from tomato samples throughout the study. No *E. coli* (<10 cfu/g) was detected in any of the tomato samples tested. Coliform numbers increased on tomatoes throughout the growing season with no coliforms being detected at four weeks prior to harvesting of either Farm 2 or three. During the final growing phase (two weeks prior until harvest), tomatoes grown in the open field had significantly less coliforms (<1.0 – 2.0 log cfu/g) than tomatoes grown in tunnels on Farm 3 (3.8 – 5.6 log cfu/g). As a result, tomatoes entering the processing facility on Farm 2 had significantly lower coliforms (mean 0.5 log cfu/g) than tomatoes entering the processing facility on Farm 3 (mean 4.8 log cfu/g). The time of day (beginning, middle, or end) had no effect on coliform numbers. Washing and packing increased coliform numbers significantly on tomatoes on Farm 2 (mean 2.2 log cfu/g on packed tomatoes), while washing and packing caused an insignificant decrease in coliform numbers on tomatoes packaged on Farm 3 (mean 4.7 log cfu/g). At market level, tomatoes sampled from the retailer originating from Farm 3 had the highest number of coliforms (<1.0 – 6.6 log cfu/g), followed by tomatoes sourced from Farm 2's local market (1.9 – 6.2 log cfu/g), and the Tshwane market (1.9 – 4.9 log cfu/g).

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3.2   Indicator organisms from water

Figures 3 and 4 show the levels of *E. coli* and coliforms detected using Colilert-18 in the water for irrigation and postharvest washing, respectively. The source of irrigation water had a significant effect on the final *E. coli* numbers, bur not coliform numbers, at the irrigation point. *E. coli* was detected in the irrigation source water from all three farms. The *E. coli* numbers in the irrigation water sourced from the river on Farm 1 were significantly higher ($P < 0.001$) at the source (3.19 log MPN/100ml), during storage (2.07 log MPN/100ml) and irrigation points (2.09 log MPN/100ml) than the irrigation water sourced from a dam on Farm 2 (source: 0.46 log MPN/100ml, storage: 0.82 log MPN/100ml, irrigation point: 0.55 log MPN/100ml) and borehole on Farm 3 (source: 0.07 log MPN/100ml, storage: 1,72 log MPN/100ml, irrigation point: 1.01 log MPN/100ml). The *E. coli* numbers in the irrigation water did not differ significantly between Farms 2 and 3. The coliform numbers in the water sourced from the river on Farm 1 (3.12 log MPN/100ml) was not significantly higher than from the dam on Farm 2 (2.85 log MPN/100ml), both of which were significantly higher than borehole water of Farm 3 (1.37 log MPN/100ml). Coliform numbers in the irrigation water of Farms 2 (3.34 log MPN/100ml) and 3 (3.37 log MPN/100ml) did not differ significantly but were, however, found to be significantly higher than Farm 1 (2.85 log MPN/100ml). Significant differences in *E. coli* numbers were found in the water used to wash the tomatoes between all three farms. The wash water source of Farm 3 contained significantly higher ($P < 0.001$) numbers of *E. coli* (mean 1.3 log MPN/100ml) than Farm 1 (mean 0.25 MPN/100ml) and Farm 2 (mean 0.08 log MPN/100ml). *E. coli* was detected in low levels (mean 0.05 log MPN/100ml) in the flume water of Farm 1 at the end of the day. Otherwise, no *E. coli* was detected in any of the other flume water samples taken from Farm 1 or 2, irrespective of low levels detected in the source water. Significant differences ($P < 0.001$) were observed in coliform numbers of the wash water source between Farm 1 (mean 2.03 log MPN/100ml) and

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Farms 2 (mean 3.38 log MPN/100ml) and 3 (mean 3.12 log MPN/100ml) throughout the study.

3.3   Indicator organisms from soil

No *E. coli* was detected (<10 cfu/g) in any of the soil or compost samples analysed in this study using the described methods. There were no significant differences ($P = 0.5$) in soil coliform numbers between Farms 2 and 3, and no significant changes in coliform numbers throughout the test period. On Farm 2, soil coliform numbers ranged from 3.02 log to 5.23 log cfu/g. On Farm 3, coliform numbers ranged from 3.4 log cfu/g to 4.51 log cfu/g during the last month of the tomato growing cycle.

3.4   Indicator organisms from contact surfaces

Table 4 shows the contact surface coliforms counts obtained from the processing facilities. No *E. coli* (<1 cfu/cm^2) was detected on any of the contact surfaces tested. All colonies testing positive for β-glucuronidase activities on the Petrifilm count plates were isolated as potential *E. coli* and subsequently identified as *Citrobacter freundii* (n = 3), *Raoultella ornithinolytica* (n = 2), *Enterobacter asburiae* (n = 1), and *Klebsiella oxytoca* (n = 1) by MALDI-TOF MS. *Yersinia enterocolitica* was isolated once on the rollers in Farm 3's packinghouse. In the field, hands of pickers and harvesting crates were swabbed. At the first sampling occasion on Farm 2, no coliforms were detected on any of the hands sampled in the field. At the second and third sampling occasions, significantly higher ($P = 0.01$) coliform numbers were detected on the hands of pickers on both farms. The highest levels detected were 3.2 log cfu/cm^2 and 4.16 log cfu/cm^2 on Farms 2 and 3, respectively. No coliforms were detected on harvesting

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crates on Farm 2 at two sampling occasions, except during the first occasion when 3.33 log cfu/cm^2 was detected. On Farm 3 significantly higher ($P = 0.010$) coliform levels were detected, the highest at 4.37 log cfu/cm^2. In the packinghouses (Table 4), coliforms on the packers' hands differed significantly ($P = 0.0003$) between sampling occasions and times, regardless of the farm where samples were taken. The crates used in the packinghouse did not differ significantly ($P = 0.300$) between farms, sampling occasions, and sampling time. Coliforms on the rollers differed significantly ($P = 0.001$) between sampling occasions and time of day on both farms. Coliform numbers on the brushers in the packinghouse of Farm 2 did not differ significantly between sampling dates and time of day. Coliform numbers differed significantly ($P = 0.002$) between date and time of day on the brushers of Farm 3.

3.5 Human pathogens in the tomato supply chain

No *E. coli* O157:H7 was detected in any of the samples tested. No *Salmonella* Typhimurium was detected in any of the tomato, water, or soil samples. *Salmonella* Typhimurium was detected on two contact surfaces at the packinghouse of Farm 1. One sample originated from the weighing unit, the other from a roller.

3.6 MALDI-TOF MS identification of dominant coliform species in the tomato supply chain

Table 5 lists the species, number of isolates and their source, common habitat and clinical significance identified during this study. Figure 6 illustrates the various sources the species were identified from. From tomatoes, the dominant coliforms isolated on Farm 1 were identified as *Enterobacter cloacae, Citrobacter freundii, Klebsiella pneumoniae* and

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Enterobacter aerogenes. On Farm 2, the dominant tomato coliforms were identified as *Enterobacter asburiae, Enterobacter cloacae, Citrobacter freundii,* and *Klebsiella oxytoca.* On Farm 3, the dominant coliforms were identified as *Enterobacter cowanii* (55%)*, Enterobacter asburiae, Klebsiella oxytoca, Klebsiella pneumoniae,* and *Pantoea agglomerans.* Thirteen dominant coliforms were isolated from all three farms' water and identified as of *Enterobacter cloacae, Citrobacter freundii, Klebsiella pneumoniae, Escherichia vulneris, Enterobacter cowanii, Enterobacter asburiae, Klebsiella oxytoca, Pantoea ananitis, Serratia fonticola,* and *Kluyvera georgiana.* The dominant coliforms identified from the soil on Farm 1 consisted of *Enterobacter cloacae, Serratia marcescens, Klebsiella pneumonia,* and *Pseudomonas mendocina*. On Farm 2, the dominant coliforms were identified from the soil as *Leclercia adecarboxylata, Klebsiella oxytoca,* and *Citrobacter freundii.* The dominant coliforms identified on Farm 3 were *Enterobacter cowanii, Citrobacter freundii,* and *Pseudomonas monteilii.* From the contact surfaces of Farm 1, dominant coliforms were isolated and identified as *Enterobacter cloacae, Citrobacter freundii, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*. Dominant isolates from the contact surfaces of Farm 2 were identified as *Citrobacter freundii, Raoultella ornithinolytica, Klebsiella oxytoca, Enterobacter amnigenus,* and *Enterobacter cloacae.* Of the six dominant isolates identified on Farm 3, four were *Enterobacter amnigenus,* and two belonged to the genus *Buttiauxella.* Compost produced from vegetable waste on Farm 3 contained *Enterobacter asburiae, Enterobacter cloacae, Escherichia vulneris* and *Pseudomonas mendocina* as dominant coliforms.

3.7 Food safety risk assessment

The diagnostic tool (Figure 5) assessment was completed and risks identified for all three farms. For contextual factors, Farms 1 and 2 scored higher (greater risk of microbial contamination) than Farm 3. Since all three farms cultivate tomatoes (final product risk 2/3) from seedlings (initial material risk 3/3), all three farms experienced the same inherent risk due to the inherent characteristics of tomatoes. Because Farm 3 obtains irrigation water from deep wells but fails to store the pumped water properly by storing the water uncovered on the surface before use, it's water supply had a lower risk (2/3) for crop contamination than surface water (3/3) used by Farms 1 and 2. Furthermore, Farms 1 and 2 cultivate tomatoes in an open field in soil (3/3), whereas Farm 3 cultivates tomatoes in a protected system (tunnel) but in soil (2/3). For control activities, Farm 3 performed slightly better than Farms 1 and 2. Water testing is on an *ad hoc* basis for all three farms and none of the farms treat the irrigation water prior to use, they were all assigned 2/4 for water control activities. However, all three farms reduces the risk of tomato contamination via irrigation by applying drip irrigation, Farms 1 and 2 on the surface (3/4) and Farm 3 under plastic mulch (4/4). All three farms scored well for assurance activities. Since all three farms are certified for either HACCP or ISO 22000 and audited yearly by a single third party to retain their certification, they received a score of 3/4 for FSMS evaluation. All three farms scored 3/4 for microbiological complaints received from customers, since complaints received were restricted, indicating good performance. All three farms lack regular and comprehensive sampling and microbiological testing procedures to assess microbiological performance and therefore only scored 2/4. Since microbiological testing is rarely done, no microbiological judgment criteria exist (1/4). However, very few nonconformities were experienced by all three farms (3/4), indicating only specific problems within the functioning of the FSMS.

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4   DISCUSSION AND CONCLUSIONS

No *β*-glucuronidase negative *E. coli* O157:H7 was detected in any samples tested during this study and no *Salmonella* Typhimurium was detected in any of the fruit, water, soil or compost samples analysed in this study. Two samples out of 905 tested positive for *Salmonella* Typhimurium, however none were associated with tomatoes or the environment in which they were grown. The samples that tested positive for *Salmonella* Typhimurium in this study were from the packinghouse of Farm 1 in 2012. It was not possible to link contamination to a specific source at the time of testing due to limitations in sampling size and the time delay between sampling and final results. During this entire study period (2012 - 2014), no foodborne disease detection and subsequent product recall or outbreaks linked to tomatoes were reported in South Africa. It is however important to note that outbreaks are generally unreported, especially in rural areas, due to the lack of a well-functioning surveillance and reporting system (Niehaus *et al.* 2011). Furthermore, fresh produce in South Africa, like many other countries, is not standardly tested for foodborne pathogen presence. During the same time in the US, tomatoes were responsible for six outbreaks: five due to *Salmonella enterica* and one due to *E. coli* O157:H7 (CDC 2015a). Tomatoes were also voluntarily recalled on one occasion following the detection of *Salmonella* (Anonymous 2014). Therefore, the detection of *Salmonella* Typhimurium on the packinghouse contact surfaces represents a hazard and should be managed through effective good hygienic practices in the packinghouse of Farm 1.

In South Africa, fresh water is scarce and farmers usually rely on river and dam water that are often contaminated with waterborne pathogens (CSIR 2010). The high *E. coli* counts

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obtained from the river water used for irrigation on Farm 1 was considered a concern if used directly in irrigation, since the water pump is located downstream from a sewage treatment plant that regularly overflows and contaminate the river with improperly treated waste, a regular occurrence in South Africa (Oberholster and Ashton 2008). Cattle, the main reservoirs for *E. coli* O157:H7, and wildlife graze on the shore of the dam from which irrigation water is sourced on Farm 2, therefore posing a potential hazard to the dam water as a result of runoff. The water source identified as the lowest risk by the diagnostic tool was well water used on Farm 3, however, the detection of *Escherichia coli* and *Escherichia vulneris*, an opportunistic pathogen that grows optimally at 37°C (Brenner *et al.* 1982), from well water indicates potential faecal contamination of the source (Jackson *et al.* 1998). After being pumped from the various sources, water is stored in uncovered storage dams on all three farms that are frequented by waterfowl that can acts as vectors for various pathogens through the faeces (Beuchat and Ryu 1997). The water supply risks identified of the various scenes described above correlated with the *E. coli* and coliform numbers detected during the study. However, no *E. coli* O157:H7 or *Salmonella* Typhimurium was detected in any water samples throughout the study. As shown by the elevated *E. coli* numbers recovered from the storage dam on Farm 3, water storage facilities should preferably be covered to prevent contamination or the water should be treated prior to use. The use of drip irrigation on all three farms mitigates the probability of tomato fruit contamination through contaminated irrigation water (Markland 2012). Drip irrigation applied under plastic mulch, such as used on Farm 3, further reduces the risk of splash-dispersal of potential pathogens introduced by irrigation water and contamination of the fructoplane. Plastic mulch has been suggested to increase the risk of rain splash dispersal of *Salmonella* Typhimurium onto tomato plants (Cevallos-Cevallos *et al.* 2012), however, since the tomatoes on Farm 3 are grown in covered tunnels, plants are never exposed to rain and splash-dispersal risks are low. Our study showed

that *E. coli* counts in water used for irrigation are above (>1 cfu/100ml) the national regulation requirements for water used for food crop irrigation purposes (DWAF 1996). Water was thus according to our study not fit-for-purpose despite the continued certification to GLOBAL G.A.P. Improved water sanitation processes should therefore be investigated to treat water prior to use for irrigation purposes to ensure zero-risk (Allende and Monaghan 2015).

The tomatoes in this study were at a lower risk for in-field contamination because none of the fruit came into direct contact with the soil or irrigation water. This was supported by the fact that no detectable levels (<10 cfu/g) of *E. coli* were found in any of the fruit samples, regardless of the presence of *E. coli* in the irrigation water. Farm 3 grows tomato crops in a protected cultivation system (tunnels), as opposed to the open field production on Farms 1 and 2. The total coliforms isolated from the tomatoes throughout the study ranged from <1 – 6.6 log cfu/g. Coliform numbers were higher on tunnel tomatoes than open field tomatoes, possibly as a result of environmental conditions. Farms 1 and 2 are located in the Limpopo Province of South Africa where the climate is hot and semi-arid with a low annual rainfall of 550 mm and low relative humidity. Farm 3 is located in the Gauteng Province of South Africa and experiences a more temperate oceanic climate with higher relative humidity and annual rainfall of 713 mm. Greater fluctuations in temperature, higher light intensities (and UV radiation), and other environmental factors such as low relative humidity associated with open field cultivation on Farms 1 and 2 may contribute to lower coliform numbers due to decreased survival in the phyllosphere (Fonseca *et al.* 2011).

The rhizozone is more sheltered than the phyllosphere and may support the survival of pathogens (Warriner and Namvar 2010). Still, even though *E. coli* was detected in the

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irrigation water, none were detected in the rhizozone. Survival of *E. coli* and foodborne pathogens in the rhizozone is influenced by various biotic and abiotic factors, the most important being the utilization of plant root exudates and soil type, ability to attach and colonise the plant roots, and the interaction with the native microbial community (Berg and Smalla 2009). No foodborne pathogens (*E. coli* O157:H7 or *Salmonella* Typhimurium*)* or *E. coli* were detected in the compost and at the time of testing was considered fit-for-purpose. *Escherichia vulneris* is a common plant endophyte and its source in the compost heaps is most likely from vegetable and not animal waste. Organic fertilization practices are often described as high risk compared to chemical fertilization by creating opportunities for contamination and increasing soil organic matter (Leifert *et al.* 2008). On the other hand, well-managed organic production systems may have a protective effect on produce safety by increasing the soil's biological activity leading to the competitive exclusion of foodborne pathogens by native microflora (Leifert *et al.* 2008; Pagadala *et al.* 2015).

Washing and packing operations in the packinghouse of Farm 2 contributed to the significant increase in tomato coliform numbers from processing facility entry to dispatch. The detection of *E. coli* in the water source used to wash the tomatoes indicates low levels of faecal contamination of the water source and is therefore unsuitable for use. Contamination of the water possibly occurred during storage and should be sanitized prior to use. Furthermore, a negative temperature differential between the water and the tomatoes that facilitates the infiltration of bacteria, including *Salmonella,* from the water or tomato surfaces into the core tissue and limits the effectiveness of sanitation agents should be prevented by cooling the tomatoes prior to the wash step (Zhuang *et al.* 1995). No *E. coli* was detected in any of the tomato samples tested following the wash step, however, the low levels of *E. coli* in the water

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may have contaminated tomatoes at levels under the detection limit and tomatoes not tested due to limitations in sampling size.

The coliforms most commonly isolated from all sources throughout the tomato supply chain were from the genera *Enterobacter, Klebsiella* and *Citrobacter* (Figure 4). *Citrobacter freundii, Enterobacter cloacae, Klebsiella oxytoca* and *Klebsiella pneumoniae* were all identified from tomatoes tested in this study. These species all form part of the human microbiome and may come from a faecal source and cause nosocomial infections (Leclerc *et al.* 2001). Other opportunistic human pathogens identified from tomatoes included *Enterobacter aerogenes, Enterobacter asburiae, Enterobacter cancerogenus* and *Pantoea agglomerans.* However, they are also regularly isolated in high numbers from environmental and fresh produce samples (Abadias *et al.* 2008; Falomir *et al.* 2010) and are therefore not reliable indicators of faecal contamination (Little *et al.* 1999). Furthermore, recent microbial ecology studies have shown that many potentially opportunistic human pathogens form part of the natural microbiomes of both humans and plants (Ottesen *et al.* 2013), suggesting that their ingestion may enhance overall human health in immunocompetent persons by diversifying the human gut microbiome and stimulating the immune system (Hanski *et al.* 2012; Berg *et al.* 2014). However, for a growing number of immunocompromised individuals in the population, the presence of these coliforms on tomatoes may represent a food safety risk either by causing nosocomial infections or conferring potential antibiotic resistances to normal gut microflora and/or pathogens (Berg *et al.* 2014). Further analysis of antibiotic susceptibility and profiles can indicate the potential origin of these isolates, and the potential risk they pose to consumer health. In the absence of the faecal indicator *E. coli,* even high numbers of *c*oliforms in fresh produce can be expected (Little *et al.* 1999). The presence of

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faecal coliforms is not necessarily an indication of faecal contamination and therefore should be acceptable when producers adhere to good agricultural and hygienic practices (National Advisory Committee on Microbiological Criteria for Foods, 1999).

None of the samples collected throughout the study were tested specifically for *Yersinia spp.* However one isolate identified with MALDI-TOF analysis was confirmed as *Yersinia enterocolitica* isolated from the contact surface of the processing facility of Farm 3. Since samples were not specifically analysed for *Y. enterocolitica*, the presence of this potential pathogen in the tomato production chain is unknown and should in future be further investigated. *Y. enterocolitica* is a known food-borne pathogen, usually linked to the consumption of pork and milk, but also reportedly related to fresh produce caused disease outbreaks (Whipps *et al.* 2008; Huovinen *et al.* 2010). In 2011, an outbreak of *Y. enterocolitica* O:9 caused 21 people to become ill in Norway and trace-back studies linked the outbreak to contaminated RTE salad mix (MacDonald *et al.* 2012). Most biotypes are non-pathogenic, however, and not infrequently isolated from environmental sources (Weagant and Feng 2001). The isolation of *Y. enterocolitica* from Petrifilm is strange considering the species is considered lactose-negative. However, *Y. enterocolitica* is a biochemically diverse species (Bercovier *et al.* 1980) and atypical lactose-fermenting strains of *Y. enterocolitica* have been isolated in the past, where lactose fermentation is considered to be plasmid related (Cornelis *et al.* 1978).

Contact surfaces can be a source of contamination and therefore it is of utmost importance that they be regularly cleaned to prevent cross-contamination of tomatoes (Beuchat and Ryu 1997). In South Africa, the general hygiene requirements are addressed in the National

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Health Act (No. 61 of 2003) (DOH 2004). Under the Act, Regulation 918 of 1999 (DOH 2002) requires that any surface that comes into direct contact with food should be cleaned and washed immediately prior to and after handling of food. After cleaning and washing with the purpose of disinfection, work surfaces may have no residues of cleaning materials that may pollute the food and a sample should not contain more than 100 viable microorganisms per cm^2 of a given surface, as taken and analysed in accordance to the swab technique prescribed by South African Bureau of Standards (SABS) Standard Test Method 763. All the facilities have a daily cleaning schedule and packs large volumes of fruit in the facility. The high numbers of coliforms found on the contact surfaces during our studies in the packing facilities exceed the prescribed hygiene requirement. In addition to high coliform numbers, finding *Salmonella* Typhimurium on contact surfaces in the processing facilities of Farm 1 indicate insufficient hygiene and poor general sanitation practices. Sanitation practices and schedules in these facilities should therefore be amended to comply with the required limit thereby reducing food safety risk.

Limitations in the use of 3M Petrifilm was identified in this study and suggests the possibility that *E. coli* numbers are underestimated when target numbers are low (Schraft and Watterworth 2005). All blue colonies were isolated and identified in this study, even when Petrifilm coliform counts were too numerous or few to count to prevent false negatives. *Citrobacter freundii, Raoultella ornithinolytica, Enterobacter asburiae* and *Klebsiella oxytoca* all yielded blue colonies (without gas formation) on the Petrifilm plates confirming only blue colonies accompanied by gas formation are to be considered as presumptive *E. coli* (AOAC 2000). For freshwater testing, the Colilert-18 method should be the preferred method of testing since it is able to accurately quantify and detect various levels of generic *E. coli* and

coliforms. The use of the Colilert-18 method for samples other than water should be further investigated, as applied successfully with tomato fruit rinsate (Shenge *et al.* 2015), hands and soil (Julian *et al.* 2015). When the levels of *E. coli* are too low to enumerate thereby preventing detection, enriched samples should be streaked on suitable media to confirm presence/absence. Limitations in our study were the use of primers only for typical (*β*-glucuronidase negative) *E. coli* O157:H7 and *Salmonella* Typhimurium. Therefore the possibility exists that *β*-glucuronidase positive *E. coli* O157:H7, non-O157 Shiga Toxigenic *E. coli* (STEC) and other serotypes of *Salmonella enterica* went undetected. In a recent study linking the microbial quality of water and onions produced in South Africa, *Salmonella* spp. were identified in 22% of the water sources and eight (15.6%) *E. coli* isolates tested positive for at least one virulence gene (du Plessis *et al.* 2015). The protocol in future studies should therefore be amended to include detection of pathogenic *Yersinia enterocolitica* biotypes*,* all *Salmonella enterica* serotypes and all STEC.

The diagnostic tool functioned well to identify the microbial risk of contextual factors (product, irrigation water and production system) and shortcomings in the FSMS of each individual farm. Areas of concern identified by the diagnostic tool were the water supply and storage, the lack of water control activities (supported by the *E. coli* numbers detected) and the absence of a structured and comprehensive sampling scheme and the accompanying microbiological judgment criteria to evaluate these results (supported by the high coliform counts obtained from the packinghouse contact surfaces of Farms 2 and 3, and the detection of *Salmonella* Typhimurium on the contact surfaces of Farm 1). The tool is most useful as an aid for producers to self-assess the food safety risks they can expect to experience and the

effectiveness of activities in place to mitigate these risks and communicate the importance of control and assurance activities.

The greatest risks to the microbiological quality of tomatoes produced in South Africa were identified as the irrigation and wash water sources and storage and the inadequate sanitation of contact surfaces in the packinghouses. No foodborne pathogens were detected in any of the tomato samples tested despite the use of contaminated irrigation water. Also, no *E. coli* was enumerated from tomatoes to indicate faecal contamination. However, the presence of contaminants at any stage in production presents a potential hazard to consumer health, especially in South Africa where many are burdened by HIV/AIDS. To ensure quality and safe tomatoes in the future, all potential sources of contamination should be prevented or minimised and good agricultural and hygienic practices (GAPs and GHPs) as well as the necessary food safety interventions must be included throughout the supply chain. Helping producers understand these risks and the control activities available to mitigate these risks and improve the functioning of their FSMS with the aid of a diagnostic tool should help promote the future safety of tomatoes supplied to the South African market. Understanding the biological and ecological factors enabling survival and persistence of human enteric organisms and the more effective detection and monitoring of pathogens are crucial in developing pre- and postharvest control strategies to ensure safe produce in the future.

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Table 1. Comparison of the cultivation- and processing practices between the three farms assessed in this study

	Farm 1	Farm 2	Farm 3
Certification status	GLOBAL G.A.P., HACCP, *"Farming for the future"**	GLOBAL G.A.P., HACCP, *"Farming for the future"*	GLOBAL G.A.P., ISO 22000, *"Farming for the future"*
Production System	Open field cultivation	Open field cultivation	Tunnels with plastic mulch
Growing Media	Soil amended with on-farm produced organic compost from plantwaste	Soil amended with on-farm produced organic compost from plantwaste	Soil amended with on-farm produced organic compost from plantwaste , covered with plastic mulching
Irrigation Water Source	River, water pumped directly from river to storage dam	Artificial earth-fill dam with open channel spillway fed by natural river	Groundwater
Irrigation Water Storage	Uncovered concrete dam	Uncovered earthen dam	Uncovered plastic tarp dam
Irrigation Method	Drip irrigation	Drip irrigation	Drip irrigation, sub-mulching

*"*Farming for the future*" is a commercial standard required by one of the retailers

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Table 2. Sampling and processing procedures used during this study.

Sample Type	Sample Size	Sample collection per farm visit	Total	Processing	Analysis
Tomato fruit	5 fruit (pooled) average mass 182 g, halved and homogenised for 2 min in blender	Field n = 3 Packinghouse: Entry n = 3x3, Exit n = 3x3 Market/Retail n = 3	117	Enumeration: 10 g purée mixed with 90 ml BPW* Enrichment[d]: 25 g purée mixed with 225 ml BPW	Enumeration: 3M Petrifilm *E. coli*/Coliform count plates[a]; Pathogen Detection: Multiplex PCR[b]
Soil	150 g (pooled)	50 g from each plant tested, 3 field samples	27	Enumeration and enrichment: 10 g from pooled sample mixed with 90 ml BPW	Enumeration: Petrifilm *E. coli*/Coliform count plates; Pathogen Detection: Multiplex PCR
Water	3 x 100 ml 1 l	Irrigation source Storage dam Irrigation points Wash water	162	Enumeration: 100 ml samples: Colilert-18 (Quanti Tray/2000) Enrichment: Filtered 100 ml sample mixed with 90 ml BPW	Enumeration: Colilert-18 (Quanti Tray/2000)[c] Pathogen Detection: Multiplex PCR
Swabs	1 swab	Hands: 25 cm² Contact surfaces: 50 cm²	590	Enumeration and Enrichment: Swab added to 9 ml BPW	Enumeration: Petrifilm *E. coli*/Coliform count plates; Pathogen Detection: Multiplex PCR
Compost	150 g (pooled)	3x50g from each heap, 3 heaps tested	9	Enumeration and Enrichment: 10 g from pooled sample mixed with 90 ml BPW	Enumeration: Petrifilm *E. coli*/Coliform count plates; Pathogen Detection: Multiplex PCR

*BPW: Buffered Peptone Water
[a] AOAC 991.14, Detection limit: 10 cfu/g or 1 cfu/cm²
[b] Multiplex PCR, Detection limit: 10^3 cfu after 24 h enrichment
[c] ISO 9308-2:2012, Detection limit: 1 MPN/100ml
[d] Enrichment conditions for all samples: 24 h incubation at 37°C.

Table 3. Multiplex primers used for the detection of *Salmonella* Typhimurium and *Escherichia coli* O157:H7

Target	Primer	Sequence (5'-3')	Product Size (bp)
***E. coli* O157:H7**	UidAaF	GCG AAA ACT GTG GAAA TTG GG	272
	UidAbR	TGA TGC TCC ATA ACT TCC TG	
Salmonella enterica	SLDF	CCT GTG AAT GCC CTG ATG AT	787
	SLDR	TTG CCG GTG GTA CTG ATA GG	
16S Universal Primer (IAC)	27F	GAG TTT GAT CCT GGC TCA G	1500
	1492R	TAC GGY TAC CTT GTT ACG ACT T	

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Table 4. Coliform counts from the packinghouse contact surfaces of Farm 2 and 3.

Sample		n^a	ND^b	$<10^2$	$10^2 - <10^3$	$10^3 - <10^4$	$10^4 - <10^5$	$10^5 - <10^6$	$\geq 10^6$	Mean ± SD^c
Farm 2	Hands	27	3	2	3	5	11	2	1	3.42 ± 1.71
	Crates	27	1	0	3	10	10	2	1	3.83 ±1.16
	Rollers	27	-	1	3	6	7	7	3	4.4 ± 1.22
	Brushers	27	-	1	0	13	11	2	0	4.15 ± 0.67
Farm 3	Hands	27	3	2	2	4	12	4	0	3.5 ± 1.82
	Crates	9	-	-	1	2	6	0	0	4.04 ± 0.78
	Rollers	27	-	3	1	6	16	1	0	3.7 ± 1.35
	Brushers	27	-	3	3	4	15	2	0	3.73 ± 1.33

[a] Number of samples tested
[b] Not detected: <1 cfu/cm^2
[c] log cfu/cm^2

Table 5. MALDI-TOF identification of isolates prevalent in the South African tomato supply chain

MALDI-TOF Identification	Number of isolates				Habitat	Opportunistic Human Pathogen	Reference
	Tomatoes	**Water**	**Soil**	**Contact Surfaces**			
Acinetobacter baumanii	-	-	-	1	Plant endophyte. May be of faecal origin.	Yes	Berg *et al.* 2002; Berg *et al.* 2005; Falomir *et al.* 2010; Berg *et al.* 2014
Buttiauxella spp.	-	-	-	2	Most commonly isolated from molluscs and aquatic sources; also from soil and vegetables	No	Leclerc *et al.* 2001; Izumi *et al.* 2004
Citrobacter freundii	10	2	2	11	Ubiquitous in nature. May be of faecal origin. Regularly isolated from vegetal sources.	Yes	Leclerc *et al.* 2001; Izumi *et al.* 2004
Enterobacter aerogenes	3	-	-	-	Ubiquitous in nature. May be of faecal origin. Regularly isolated from vegetal sources.	Yes	Leclerc *et al.* 2001; Berg *et al.* 2014
Enterobacter amnigenus	-	-	-	5	Usually occurs in aquatic environments. Isolated from vegetal sources. Protective effect against *Salmonella* on tomatoes.	Yes	Leclerc *et al.* 2001; Izumi *et al.* 2004
Enterobacter asburiae	10	1	-	-	Ubiquitous in nature and plant endophyte. Antagonistic towards *Salmonella* and *E. coli* O157:H7.	Yes	Leclerc *et al.* 2001; Cooley *et al.* 2003; Izumi *et al.* 2004
Enterobacter cancerogenus	1	-	-	-	Plant endophytes.	Yes	Leclerc *et al.* 2001; Falomir *et al.* 2010
Enterobacter cloacae	16	2	3	13	Ubiquitous coliform isolated from various environmental and vegetal sources. May be of faecal origin.	Yes	Leclerc *et al.* 2001; Izumi *et al.* 2004; Falomir *et al.* 2010
Enterobacter cowanii	5	1	2	-	Implicated as a novel species causing bacterial wilt in India. Identified in fresh pre-cut spinach.	No	Takahashi *et al.* 2006; Sarkar and Chaudhuri 2015
Escherichia coli	-	24	-	-	Faecal contamination indicator. A large and diverse group that is mostly harmless.	Yes	Leclerc *et al.* 2001; Falomir *et al.* 2010
Escherichia vulneris	-	1	-	-	Plant endophytes. May be of faecal origin.	Yes	Leclerc *et al.* 2001; Izumi *et al.* 2004
Klebsiella oxytoca	3	1	1	3	Ubiquitous in nature. May be of faecal origin. Isolated from vegetal sources.	Yes	Leclerc *et al.* 2001; Falomir *et al.* 2010
Klebsiella pneumoniae	5	2	1	5	Ubiquitous coliform regularly isolated from vegetal sources.	Yes	Leclerc *et al.* 2001; Izumi *et al.* 2004; Falomir *et al.* 2010; Berg *et al.* 2014
Leclercia adecarboxylata	-	-	2	-	Isolated from aquatic environments.	No	Leclerc *et al.* 2001

Table 5 continued.

MALDI-TOF Identification	Number of isolates				Habitat	Opportunistic Human Pathogen	Reference
	Tomatoes	Water	Soil	Contact Surfaces			
Pantoea agglomerans	1	-	-	-	Ubiquitous in nature. Most commonly associated with plants as endophyte and pathogen.	Yes	Leclerc *et al.* 2001; Berg *et al.* 2002; Shi *et al.* 2009; Falomir *et al.* 2010; Berg *et al.* 2014
Pantoea ananatis	-	1	-	-	Ubiquitous coliform most often associated with plants as a pathogen, endophyte and epiphyte.	Yes	Leclerc *et al.* 2001; Coutinho and Venter 2009; Shi *et al.* 2009
Providencia rettgeri	-	-	-	1	Entomopathogenic bacteria that is regularly isolated from soil and water environments. Causal agent of travellers' diarrhoea.	Yes	Yoh *et al.* 2005
Pseudomonas aeruginosa	-	-	-	3	Ubiquitous in nature, it is found in aquatic and terrestrial environments. Isolated from various vegetal sources. May be of faecal origin.	Yes	Izumi *et al.* 2004; Falomir *et al.* 2010; Berg *et al.* 2014
Pseudomonas fulva	-	-	-	3	Most commonly associated with the plant environment.	Yes	Izumi *et al.* 2004; Almuzara *et al.* 2010; Estepa *et al.* 2015
Pseudomonas mendocina	-	-	1	-	Isolated from green pepper.	No	Estepa *et al.* 2015
Pseudomonas monteilii	-	-	1	-	Isolated from green pepper.	No	Estepa *et al.* 2015
Pseudomonas putida	-	-	-	2	Most commonly associated with the plant environment as an endophyte.	No	Berg *et al.* 2002; Izumi *et al.* 2004; Estepa *et al.* 2015
Pseudomonas stutzeri	-	-	-	2	Endophytes of *Physalis ixocarpa* Brot.	No	Marquez-Santacruz *et al.* 2010
Raoultella ornithinolytica	-	-	-	5	Common environmental coliform.	Yes	Leclerc *et al.* 2001
Serratia fonticola	-	1	-	-	Occurs naturally in aquatic environments. Isolated from various environmental and vegetal sources.	No	Leclerc *et al.* 2001; Berg *et al.* 2002; Izumi *et al.* 2004
Serratia marcescens	-	-	2	-	Entomopathogenic. Commonly isolated from environmental and vegetal sources.	Yes	Leclerc *et al.* 2001; Berg *et al.* 2014
Yersinia enterocolitica	-	-	-	1	Environmental strains commonly isolated from water and most lack virulence determinants of pathogenic biotypes associated with food- and waterborne outbreaks.	Yes	Leclerc *et al.* 2001

Figure 1. Map of South Africa indicating the locations of tomato farms assessed in this study
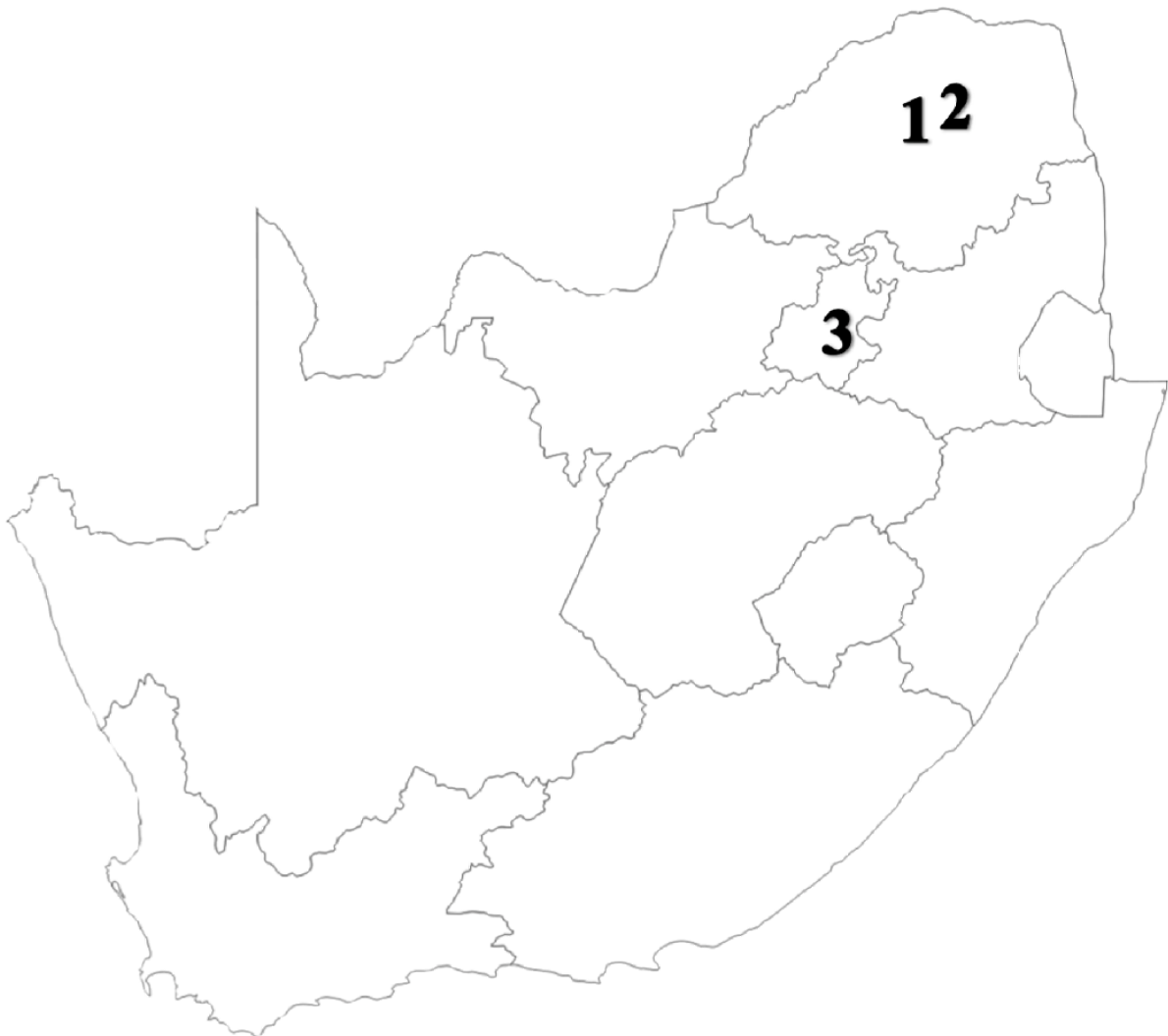
1. Farm 1, Company 1, Limpopo Province: February 2012

2. Farm 2, Company 1, Limpopo Province: August 2013

3. Farm 3, Company 2, Gauteng Province: February 2014

Figure 2. Mean coliform counts of 117 tomato samples collected from Farm 2 and Farm 3 between 2013 and 2014. Capital letters indicate significant differences in coliform numbers at the 95% confidence level ($P < 0,0001$). Error bars indicate standard error from mean.
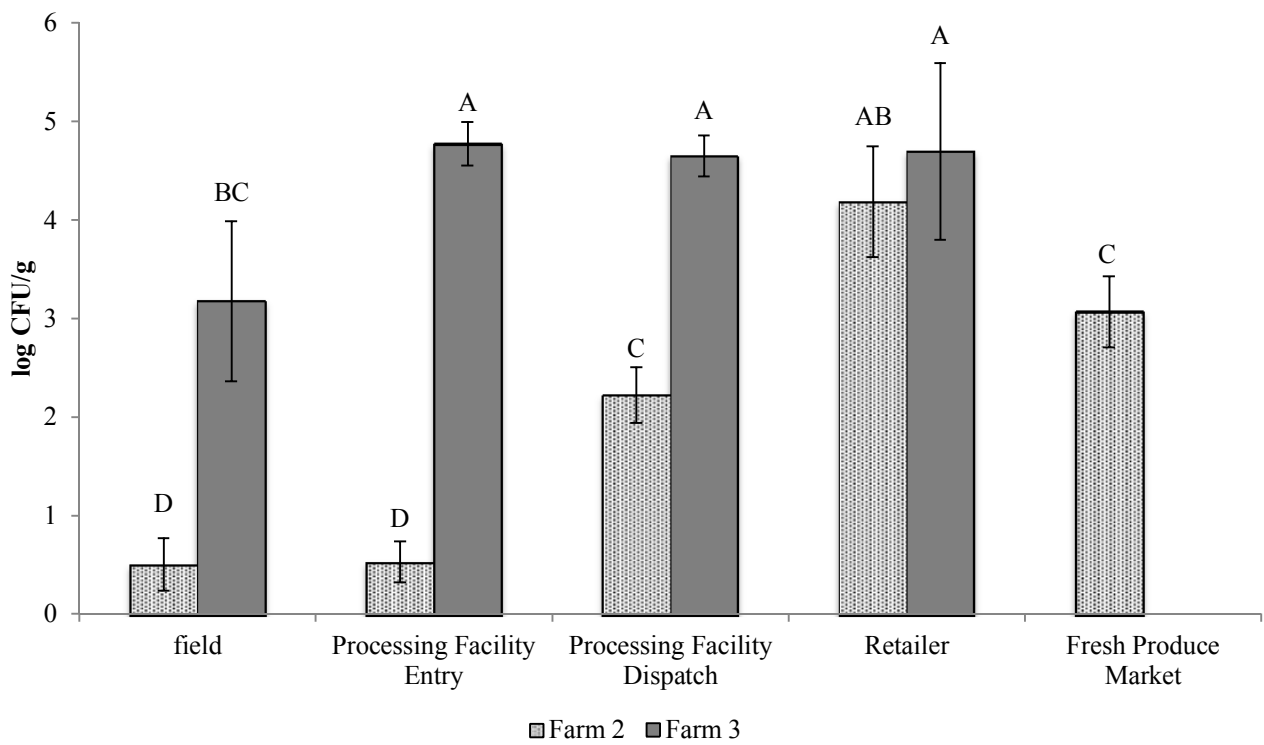
Figure 3. Mean levels of *Escherichia coli* and coliforms in 99 water samples collected from all three farms between 2012 and 2014. Capital letters indicate significant differences in *E. coli* numbers at the 95% confidence level ($P < 0{,}0001$). Lower case letters indicate significant differences in coliform numbers at the 95% confidence level ($P < 0{,}0001$). Error bars indicate standard error from mean. The source of the irrigation water indicated on the graph was a river, dam, and well, for Farm 1, Farm 2, and Farm 3, respectively.
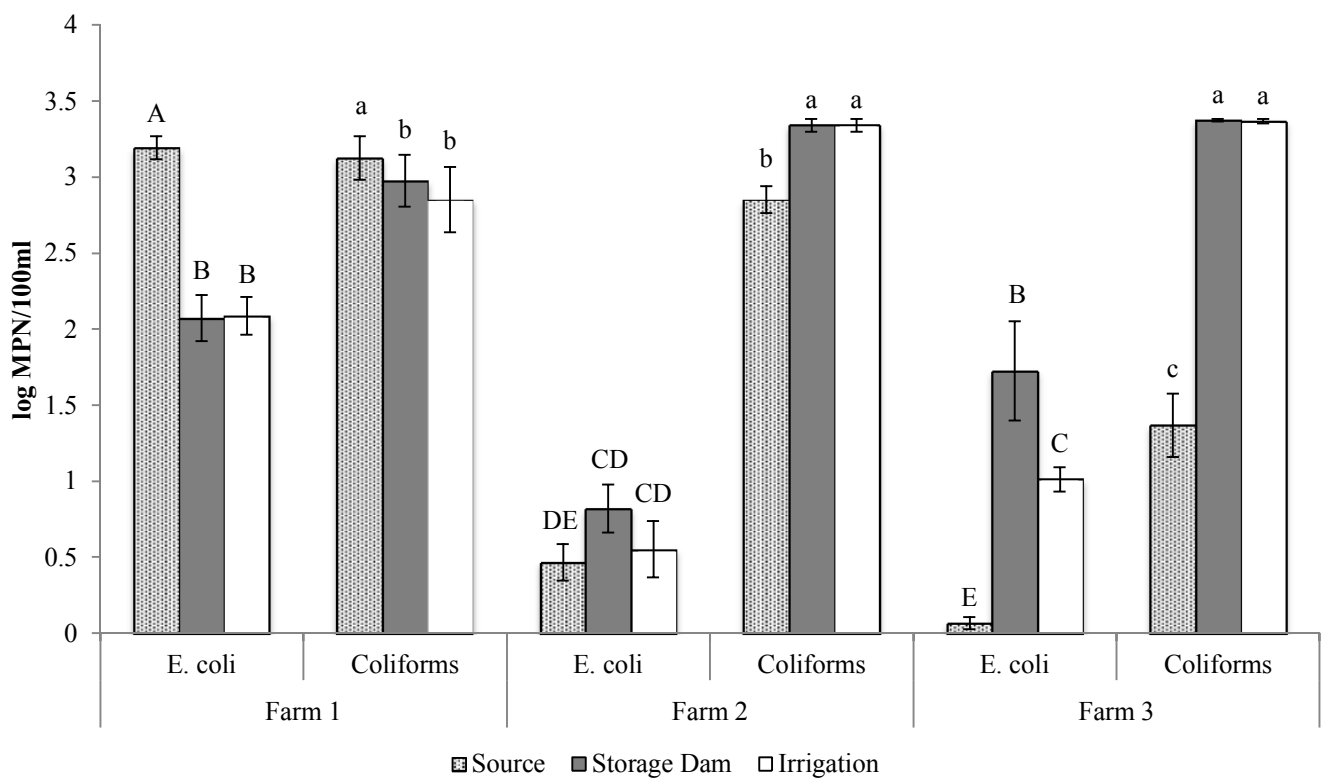
Figure 4. Mean levels of *Escherichia coli* and coliforms in 63 wash water samples collected from all three farms' packinghouses between 2012 and 2014. Capital letters indicate significant differences in *E. coli* numbers at the 95% confidence level ($P < 0,0001$). Lower case letters indicate significant differences in coliform numbers at the 95% confidence level ($P < 0,0001$). Error bars indicate standard error from mean.
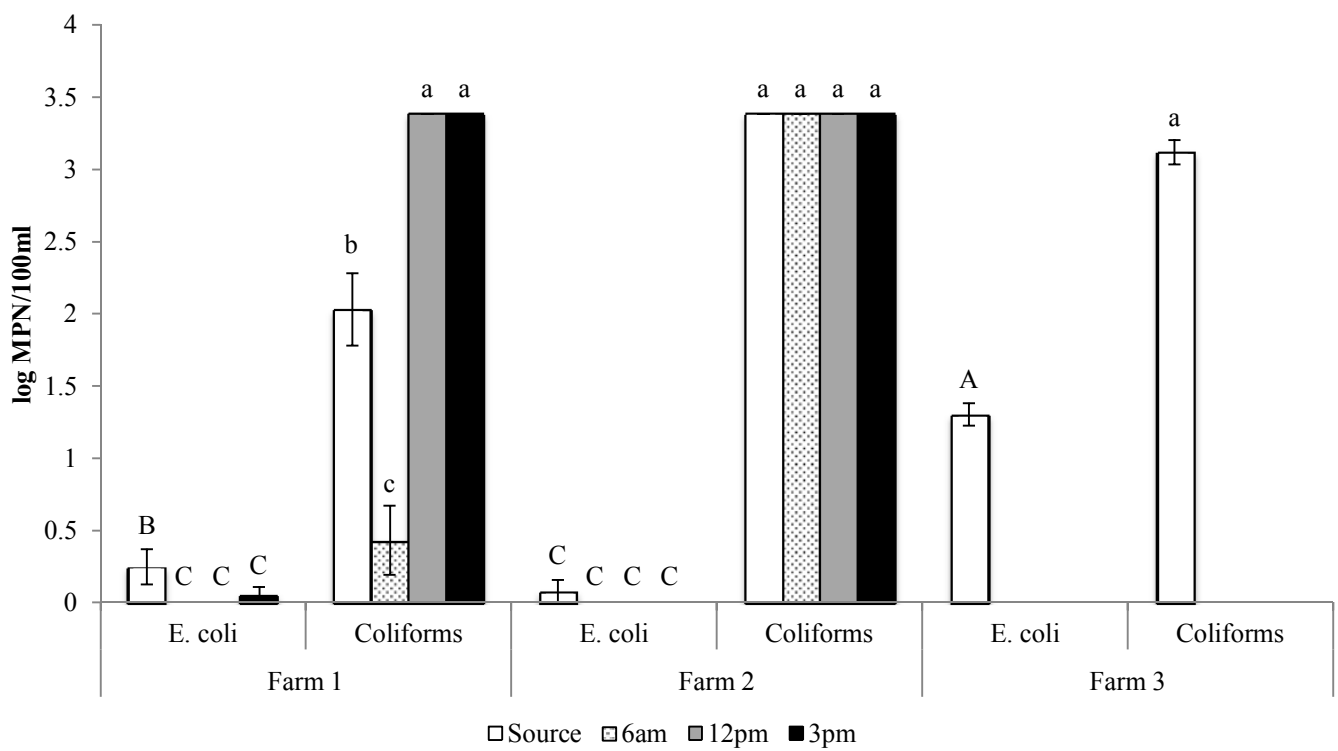
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Figure 5. Diagnostic tool assessment representing the three tomato production farms. The darker areas indicate the risk for microbial contamination of contextual factors and increased area indicates increased risk, whereas light areas indicate the performance of control and assurance activities of the FSMS to mitigate risk and increased area indicates increased performance.
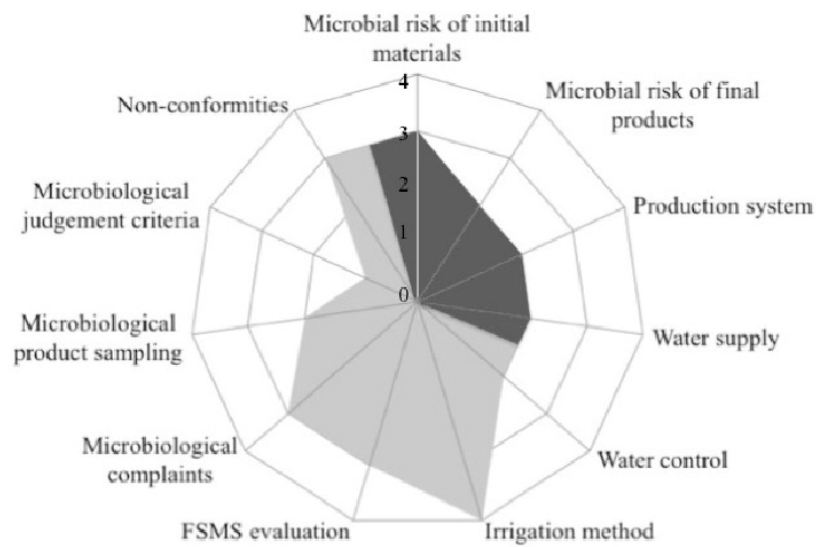
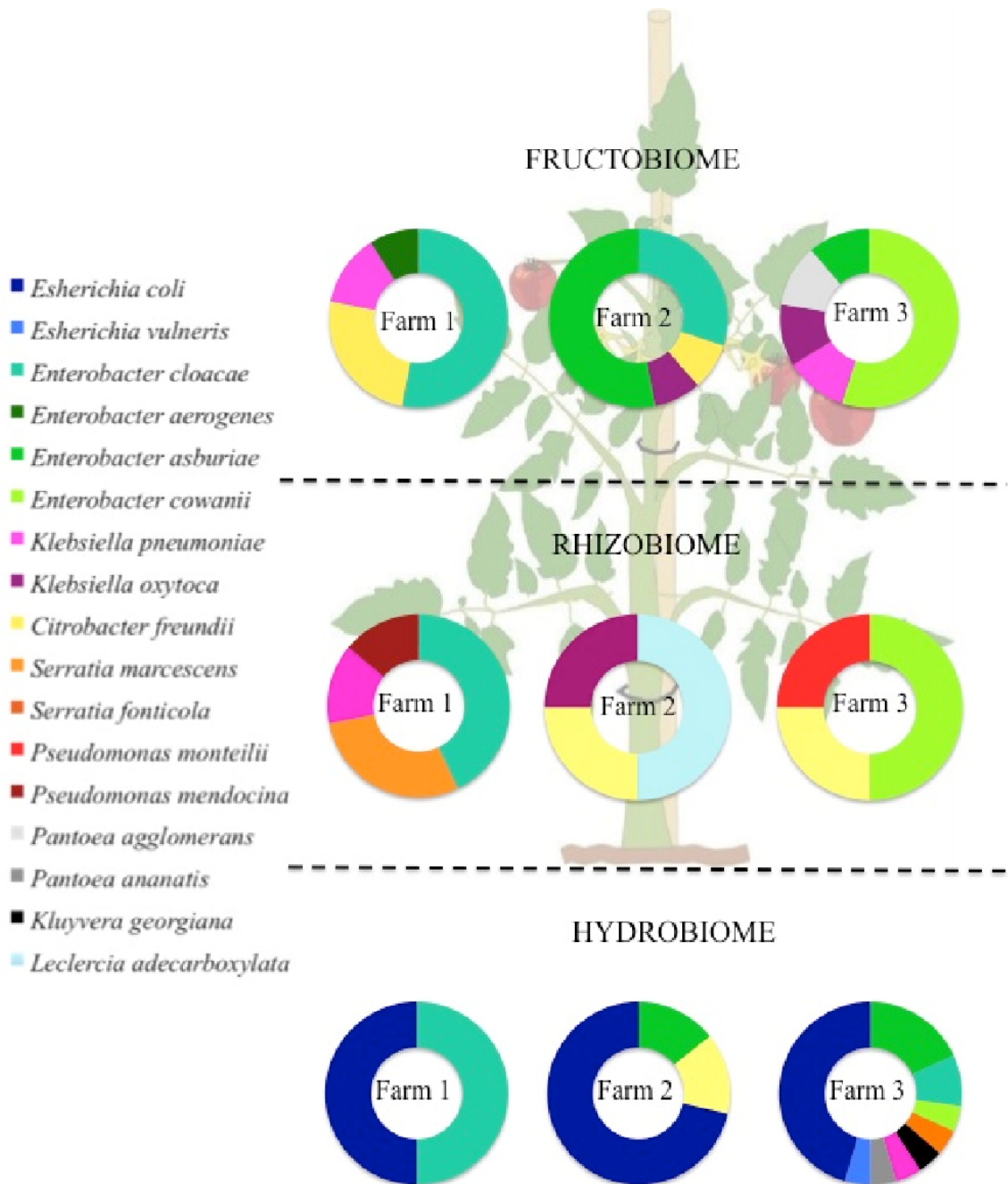## Farm 1

Farm 2

Farm 3

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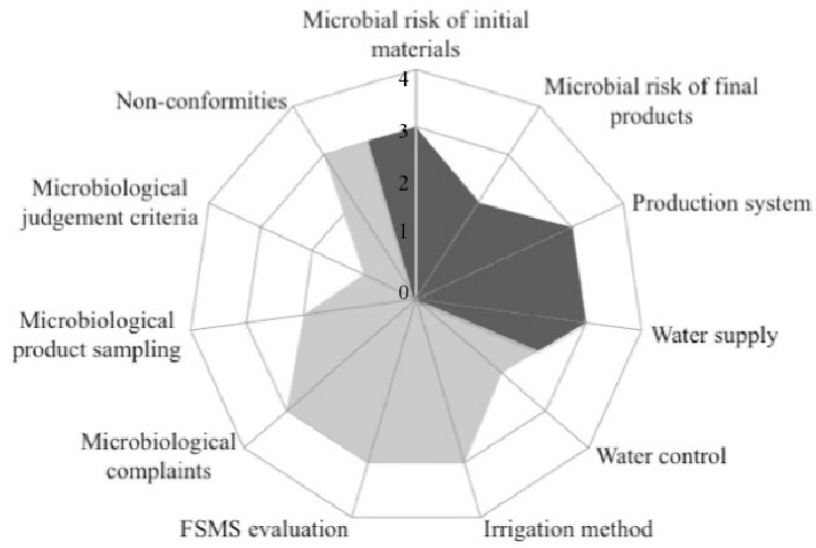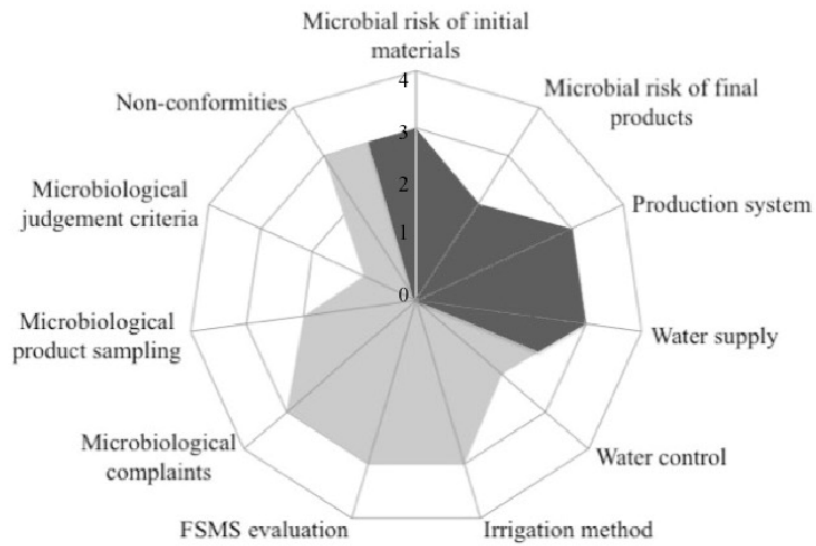
Figure 6. Figure indicating dominant coliforms isolated from tomatoes, soil and water and identified using MALDI-TOF MS

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CHAPTER 3

Enrichment cultivation of *Escherichia coli* O157:H7 using enzyme controlled glucose release

ABSTRACT

Foodborne outbreaks caused by *Escherichia coli* O157:H7 is no longer only associated with animal derived products but is increasingly implicated in the consumption of contaminated water and fresh produce. Cells often occur in physiologically stressed states frequently encountered when dealing with environmental sources. Methods aimed at the detection of viable *E. coli* O157:H7 cells are limited by the poor physiological condition of target cells and the high number of background microflora in samples. Since rapid detection methods still rely on culture-based enrichment methods to resuscitate cells and increase numbers to detectable levels, improved methods for the enrichment of injured target cells are needed. This study therefore aimed at improving the recovery of heat-injured *E. coli* O157:H7 from water. The supplementation of sorbitol MacConkey agar (SMAC) and buffered peptone water with enzyme-controlled substrate delivery EnPresso® B to improve the recovery of injured cells in solid and liquid media, respectively, were investigated. Buffered peptone water supplemented with EnPresso® B and Reagent A proved effective for the resuscitation and growth of *E. coli* O157:H7 under pristine axenic conditions, but performed poorly against the golden standard of pure buffered peptone water under non-pristine conditions. A combination of EnPresso® B and SMAC was effective for selective resuscitation of injured cells on solid media, but due to high buffering capacity did not provide adequate differentiation between target *E. coli* O157:H7 cells and other coliforms.

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1   INTRODUCTION

Since the first confirmed outbreak of *Escherichia coli* O157:H7 in the United States in 1982, this foodborne pathogen has become a global public health concern (Hiramatsu 2002) and one of the most significant human pathogens (Park *et al.* 2011). *Escherichia coli* O157:H7 produces Shiga-like toxins and is able to cause haemorrhagic colitis and haemolytic uremic syndrome (HUS), which can be life threatening (Weagant *et al.* 2011). This pathogen has also caused outbreaks linked to contaminated water and fresh produce (Pachepsky *et al.* 2011; Castro-Ibáñez *et al.* 2015). Furthermore, *E. coli* O157:H7 can remain viable in water for extended periods of time (Ngwa *et al.* 2013) and lead to the contamination of fresh produce (Castro-Rosas *et al.* 2012; Estrada-Acosta *et al.* 2014). These pathogens attach and colonise various growing crops and remain viable and be vectored back to the warm-blooded host (Jablasone *et al.* 2005; Berger *et al.* 2010; Teplitski *et al.* 2012).

Good detection systems within the production and processing environments contribute greatly to fresh produce safety assurance in the absence of a kill-step (Geng *et al.* 2011; Park *et al.* 2013). Rapid detection of contaminated produce and water therefore reduces the risk of outbreaks and causing economic losses such as the cost of recalls and healthcare (Ngwa *et al.* 2013; Hirneisen 2015). Furthermore, detection of potential contaminants within the environment where produce is grown can serve as an early warning system.

Traditional culturing methods are time consuming, laborious, and may take up to seven days for confirmed results (Chen *et al.* 2010; Cheung and Kam 2012; Khan *et al.* 2014; Lee *et al.*

2015). Fresh produce has a short shelf-life and the time between harvest and consumption is short and therefore require rapid and reliable methods of pathogen detection (Lee *et al.* 2015). Traditional culturing methods also often fail to detect pathogens if they are in a viable but non-culturable (VBNC) state (Dinu *et al.* 2009; Gilmartin and O'Kennedy 2012). High levels of background microflora in relation to pathogenic cells make cultural methods difficult (Vimont *et al.* 2006) due to overgrowth of target cells (de Boer and Heuvelink 2000) and target cell growth suppression by background microflora during enrichment (Duffy *et al.* 1999; Vold *et al.* 2000; Vimont *et al.* 2006). The high pathogenicity, low infective dose, and lack of methodology to isolate *E. coli* O157:H7 from environmental samples make this study important.

The shortcomings of traditional culturing techniques can be overcome by molecular methods such as the polymerase chain reaction (PCR) (Almeida *et al.* 2014; Delbeke *et al.* 2015). But although sensitive and rapid, effective PCR diagnostic implementation faces technical challenges when applied to detection of foodborne pathogens in a complex food matrix (Taskila *et al.* 2012) due to the presence of inhibitory substances and non-specific amplification (Bottero *et al.* 2004). Furthermore, conventional PCR methods fail to (1) detect the low numbers of cells usually contaminating fresh produce, and (2) differentiate between viable and dead cells (Cheung and Kam 2012; Dinu and Bach 2013).

The detection limit of even highly sensitive techniques is still in the range of 10^2 to 10^3 cells per millilitre (Zhang *et al.* 2012). This detection limit is far higher than the contamination usually occurring in food and environmental samples (Gehring *et al.* 2012). Therefore, an enrichment step remains necessary (Taskila *et al.* 2012) and if used successfully, can allow

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the detection of viable cells at a concentration less than one cell per sample unit (Zhang *et al.* 2014). The goals of an enrichment step is to resuscitate sub-lethally injured cells, increase the target cell concentration to above the detection limit of rapid methods, and select against competing background microflora (Asperger *et al.* 1999; Kim and Bhunia 2008; Xiao *et al.* 2010; Fedio *et al.* 2011; Gehring *et al.* 2012; Tsai *et al.* 2012). Detection of injured cells is of utmost importance, since even when injured, infections may occur at a low doses (Park *et al.* 2011). Optimal enrichment depends on the enrichment media, choice of selective agents, incubation temperature, and enrichment duration (Weagant *et al.* 2011).

The enzyme based substrate delivery technique (Enbase®) (Panula-Perälä *et al.* 2008) utilizes enzymatic glucose release by a glucoamylase from a glucose polymer in combination with a highly nutritional mixture of mineral salts and complex nutrients (Krause *et al.* 2010). A controlled glucose release platform limits overflow metabolism, restricting harmful metabolite build-up and oxygen limitation (Krause *et al.* 2010). Continuous glucose release, complex nutrients, and a high buffering capacity allow high-density growth of cells (Krause *et al.* 2010). This study aims to evaluate the performance of EnPresso® B as a media to support the detection and recovery of injured *E. coli* O157:H7 from water.

2   MATERIALS AND METHODS

2.1   Materials

Escherichia coli O157:H7 ATCC 35150 was obtained from Anatech Instruments (Sloane Park, South Africa). Working cultures were maintained on tryptone soy agar (Merck Biolab, Wadeville, South Africa), supplemented with 0.6% yeast extract (TSAYE) (Merck). A single colony was inoculated into tryptone soy broth (Merck) with 0.6% yeast extract (TSBYE) (Merck Biolab) and grown for 8 h at 37 °C with shaking rotation (160 rpm).

Liquid culture media for these experiments include the EnPresso® B (BioSilta, Oy, Finland) cultivation system with glucoamylase Reagent A (BioSilta), tryptone soy broth (TSB) (Merck) buffered peptone water (BPW) (Merck), and brain heart infusion broth (BHI) (Merck). To quantify bacteria throughout the experiment, serial 10-fold dilutions were made in half-strength BPW and 0.1 ml volumes spread-plated on Sorbitol MacConkey Agar (Oxoid, Hampshire, England) supplemented with cefixime and potassium tellurite (CT-SMAC) (Oxoid).

The ingredients for Sorbitol Peptone EnPresso B (SPEB) agar is as follows: 10 g BPW (Merck), half-strength EnPresso B (BioSilta, Oy, Finland), 15 g agar (Merck Biolab), 0.5 g bile salts (Sigma-Aldrich Fluka, Steinheim am Albuch, Germany), 0.03 g neutral red (Sigma-Aldrich), 10 g D-Sorbitol (Saarchem UniLab, Krugersdorp, South Africa), and 5 g sodium chloride (ACE Chemicals, Johannesburg, South Africa) per litre.

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2.2   Heat-injury of Cells

Exponentially growing cells in TSBYE were heat-injured by incubating 1ml aliquots at 55 °C (Precisterm waterbath, J.P. Selecta, Spain) for 15 min.   A 10-fold serial dilution was conducted for inoculation.   Sublethal injury of *E. coli* O157:H7 was determined by plating serial dilutions on CT-SMAC and TSAYE in parallel and duplicate.   To calculate cell die-off and sublethal stress (% injury) after heat exposure, the following formula was used (Aruscavage *et al.* 2010):

$$\%Injury = \left(\frac{\text{Counts on non-selective media } - \text{ Counts on Selective Medium}}{\text{Counts on non-selective media}}\right) \times 100$$

1.1   Axenic growth of *Escherichia coli* O157:H7 in different enrichment broths

To test growth under axenic conditions, EnPresso® B, TSB, BPW, BPW-EnPresso B (1:1) (PEB) and BHI were inoculated with *E. coli* O157:H7 at a concentration of 50 cells/mL in 1.5 ml broth in a 24-well deep well plate (BioSilta, Oy, Finland).   Reagent A was added to EnPressoB at various concentration (0.1-100 U/L) after inoculation. The deep well plates were sealed with airporous seals (Biosilta).   Cell concentrations were determined from 100 µl samples at 600nm with the Multiskan GO 96-Multiwell plate reader (Thermo Scientific, Dreieich, Germany). Measurements were taken at 24 and 48 h post inoculation. The experiment was performed three times, each in triplicate.

2.3 Solid media for concurrent resuscitation and enumeration of *Escherichia coli* O157:H7

Growth of heat-injured *E. coli* O157:H7 on the selective and differential SPEB agar was compared to SMAC and TSAYE. Exponentially growing cells were heat-injured, 10-fold serially diluted and plated in parallel and in triplicate on TSAYE, SPEB, and SMAC. 15ml were poured per plate. For the SPEB, 0.5 U/L Reagent A was spread plated with the cells onto the plate. Colonies were counted after incubation for 18-24 h at 37 °C. The experiment was repeated three times.

2.4 Isolation of *Escherichia coli* O157:H7 from spiked surface water samples

The potential for resuscitation and selective and differential isolation of injured *E. coli* O157:H7 from surface water was evaluated. 100ml water samples were collected in triplicate from Walkerspruit in Pretoria, Gauteng Province. Samples were then vacuum-filtered through 0.45 µm pore-sized nitrocellulose membrane disks with a 47 mm diameter (Sartorius Stedim Biotech, Goettingen, Germany). The membranes were transferred to 10 ml BPW then spiked with 10, 100, and a 1000 heat-injured *E. coli* O157:H7 cells. From BPW cells were serially diluted and plated on SPEB and SMAC in triplicate and in parallel. Plates were incubated for 24 h at 37 °C and colourless colonies counted to obtain total *E. coli* O157:H7 counts.

2.5 Growth of *Escherichia coli* O157:H7-spiked water samples in different broths

The performance of different non-selective growth mediums were compared for the enrichment of *E. coli* O157:H7 from spiked natural surface water samples. Similar to the previous experiment, 100 ml water samples were collected in triplicate from Walkerspruit,

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Pretoria, vacuum-filtered, and the membranes transferred to 10 ml BPW, TSB, EnPresso B (with 0.5 U/L reagent A), and BPW-EnPresso B (with 0,5 U/L reagent A) then spiked with 10 heat-injured *E. coli* O157:H7 cells. The samples were enriched for 24 h at 37°C with shaking (160 rpm). Following enrichment, serial dilutions were performed in half-strength BPW and plated in triplicate on SMAC without supplements. After incubation, plate counts were performed and colourless colonies counted as *E. coli* O157:H7, while pink colonies were counted as coliforms.

3 RESULTS

3.1 Axenic growth of *Escherichia coli* O157:H7 in different enrichment broths

The resuscitation and growth of heat-injured *E. coli* O157:H7 in different broths were compared in 1.5 ml cultures. The cultures were inoculated with approximately 50 cfu and cell growth was determined by OD_{600} measurements. The PEB medium containing 0.5 U/L Reagent A achieved significantly higher ($P < 0,0001$) cell densities over a 24 h growth period than all other broths tested. The combination of BPW and EnPresso® B resulted in far higher cell concentrations than the two media on their own. Therefore the PEB medium was used as base for the development of the solid plate medium.

3.2 Growth of *Escherichia coli* O157:H7 on SPEB

One loopful of *E. coli* O157:H7 and *E. coli* were streaked on SPEB after 24 h incubation in TSBYE and incubated at 37 °C for 18-24 h. *Escherichia coli* O157:H7 is colourless on SPEB

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before 24 h, whereas sorbitol positive bacteria produce dark pink colonies on SPEB before 24 h. The *E. coli* O157:H7 colonies turned pink on SPEB after 48 h incubation.

3.3 Resuscitation and growth of injured *Escherichia coli* O157:H7 on SPEB

Sorbitol Peptone EnPresso® B (SPEB) agar was compared to SMAC and TSAYE for resuscitation and growth following heat injury. Serial dilutions were performed and plates incubated at 37°C for 18-24 h. SPEB recovered significantly higher numbers of *E. coli* O157:H7 ($P < 0.0001$) than SMAC, but still significantly less on SPEB compared to TSAYE.

3.4 Isolation of *Escherichia coli* O157:H7 from spiked surface water samples

No *E. coli* O157:H7 was detected in any of the samples prior to inoculation. SPEB agar failed to differentiate sufficiently between *E. coli* O157:H7 and other coliforms. The high buffering capacity of EnPresso® B combined with that of peptone water produced colonies that were all colourless, light pink, or cream-coloured, and therefore sorbitol-fermenting and non-fermenting bacteria were hard to distinguish. The same colonies produced on SMAC were clearly colourless or pink. SMAC, unlike SPEB, can therefore successfully and clearly differentiate between *E. coli* O157:H7 and other sorbitol-fermenting coliforms.

3.5 Growth of *Escherichia coli* O157:H7-spiked water samples in different broths

Following 24 h of enrichment, BPW, BHI, and TSB yielded significantly higher ($P < 0.0001$) *E. coli* O157:H7 numbers, compared to EnPresso® B and BEB. All other broths tested

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outperformed BEB. BPW, BHI, and TSB were not significantly different yielding 8.4, 8.2, and 8.4 log cfu/ml *E. coli* O157:H7, respectively.

4   DISCUSSION AND CONCLUSIONS

Water can be an important vector for the transmission of *E. coli* O157:H7 and other pathogens to fresh produce (Pachepsky *et al.* 2011). It is therefore important to monitor irrigation water quality to mitigate the risk of pathogen transmission to crops and humans (Campbell *et al.* 2001). Water quality is usually determined by monitoring indicator coliforms and *E. coli*, instead of actual pathogens (Pachepsky *et al.* 2011). Presence and levels of indicator organisms, however, do not predict potential for causing disease (Shelton *et al.* 2011). *Escherichia coli* O157:H7 is traditionally detected by culturing on SMAC, however, use is limited due to high incidence of false positive colonies. Additionally, there is no recognized standard for the detection of *E. coli* O157:H7 from water. Therefore a media with higher specificity and sensitivity for higher detection and recovery is needed (Ngwa *et al.* 2013).

The growth of heat-injured *E. coli* O157:H7 was significantly higher on SPEB than SMAC, which made it promising for the recovery of injured cells from water samples. The highest number of cells were recovered on TSA, similar to results of Rocelle *et al.* (1995). The success of SPEB may in part be as a result of the high buffering capacity of the medium allowing the recovery of injured cells (Krause *et al.* 2010), as well as the absence of crystal violet that may impair recovery of injured cells (Rocelle *et al.* 1995). Unfortunately, the medium's high buffering capacity prevented the chromogenic reaction of neutral red to the

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reduction of pH as a result of sorbitol fermentation. Additionally, the fermentation of enzymatically-released glucose from EnPresso® B lowers the pH sufficiently to change colony colour of *E. coli* O157:H7. Therefore, all colonies on the media appear pink after 48 h, regardless of sorbitol fermenting properties. Supplying glucose gradually helps recovery and growth of heat-injured cells, but also interferes with the chromogenic properties of the medium. EnPresso® B with Reagent A may be useful as part of a solid-repair method, specifically the thin agar layer (TAL) method developed by Kang and Fung (1999) and reviewed by Wu and Fung (2006). Coupled with selective differential media for *E. coli* O157:H7, solidified EnPresso® B may create favourable conditions for the resuscitation and growth of injured cells, before selection and differentiation on the underlying chromogenic media (Wu 2008).

Enrichment cultivation of *Salmonella enterica* in BPW supplemented with EnBase-Flo® was successful in a study conducted by Taskila *et al.* (2011). Similar results were obtained for *E. coli* O157:H7 when grown under axenic conditions in BPW supplemented with EnPresso® B with Reagent A. Unlike the study conducted by Taskila *et al.* (2011), when grown in non-pristine conditions, the medium failed to perform. For the detection of *E. coli* O157:H7 from the pre-enriched sample, a target cell concentration of 1×10^5 CFU/ml should be obtained (Gehring *et al.* 2012). Water samples were spiked with low levels (<10 cfu/ml) injured cells to simulate low levels of contamination in environmental samples. Cells grew to a concentration exceeding 1×10^5 cfu/ml in all broths tested, which should allow detection when coupled to other detection methods. Buffered peptone water performed best of all the broths tested for the enrichment of spiked water samples, whereas it performed the worst under axenic growth conditions. Plating on CT-SMAC after immunomagnetic separation (IMS)

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with Dynabeads anti-*E. coli* O157:H7 should increase chances of recovery after enrichment (Weagant and Bound 2001). The lower performance of EnPresso® B against other enrichment broths in a non-pristine environment indicates the potential influence of competing bacteria in the water samples that have an inhibitory effect on the growth of *E. coli* O157:H7 as opposed to pristine conditions (Al-Zeyara *et al.* 2011; Zhang *et al.* 2012). Weagant *et al.* (2011) found *E. coli* O157:H7 was recovered most effectively from inoculated sprouts by increasing selectivity by the addition of antibiotics, higher incubation temperature (42 °C) and shaking. Optimising growth and selectivity into EnPresso® B may drastically increase the recovery of *E. coli* O157:H7 from water samples (Liamkaew *et al.* 2012), however, it cannot be said whether this broth will ever outperform buffered peptone water under non-pristine conditions. Increased selectivity may also lead to diminished recovery of *E. coli* O157:H7 from water samples since selective conditions may inhibit growth and recovery of injured target cells (Jasson *et al.* 2007; Kim and Bhunia 2008). Prior to selective enrichment, incorporating a non-selective resuscitation step may increase recovery success (Nakagawa *et al.* 2000).

This study was the first to investigate the use of enzyme-controlled glucose release technology for the resuscitation and growth of *E. coli* O157:H7 in liquid and solid media. Although the performance of EnPresso® B was not comparable to BPW, further studies may still prove this technology's worth for enrichment purposes. Cells were injured with heat due to the reproducibility the injury method provides for initial EnPresso® B evaluations. Different injury methods such as hypochlorite and UV treatment of *E. coli* O157:H7 cells might obtain different results due to differences in the repair mechanisms involved and should be investigated in future studies. The addition of growth factors and selective agents and

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changes to incubation conditions may improve performance. Furthermore, EnPresso® B may be valuable to use in designing a multipathogen detection platform. For example, pre-enrichment coupled to direct multiplex PCR for simultaneous detection of *Salmonella enterica, E. coli* O157:H7, and *Listeria monocytogenes* may yield superior results compared to BPW.

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Figure 1. Cell densities obtained with different enrichment broths after 24 and 48 hours of cultivation. Different concentrations of Reagent A were tested in EnPresso-B. Cultures were inoculated with 50 cfu. Capital and lower case letters indicate significant differences at the 95% confidence level ($P < 0,001$) at 24 and 48 hours post inoculation, respectively. Error bars indicate standard error from the mean.
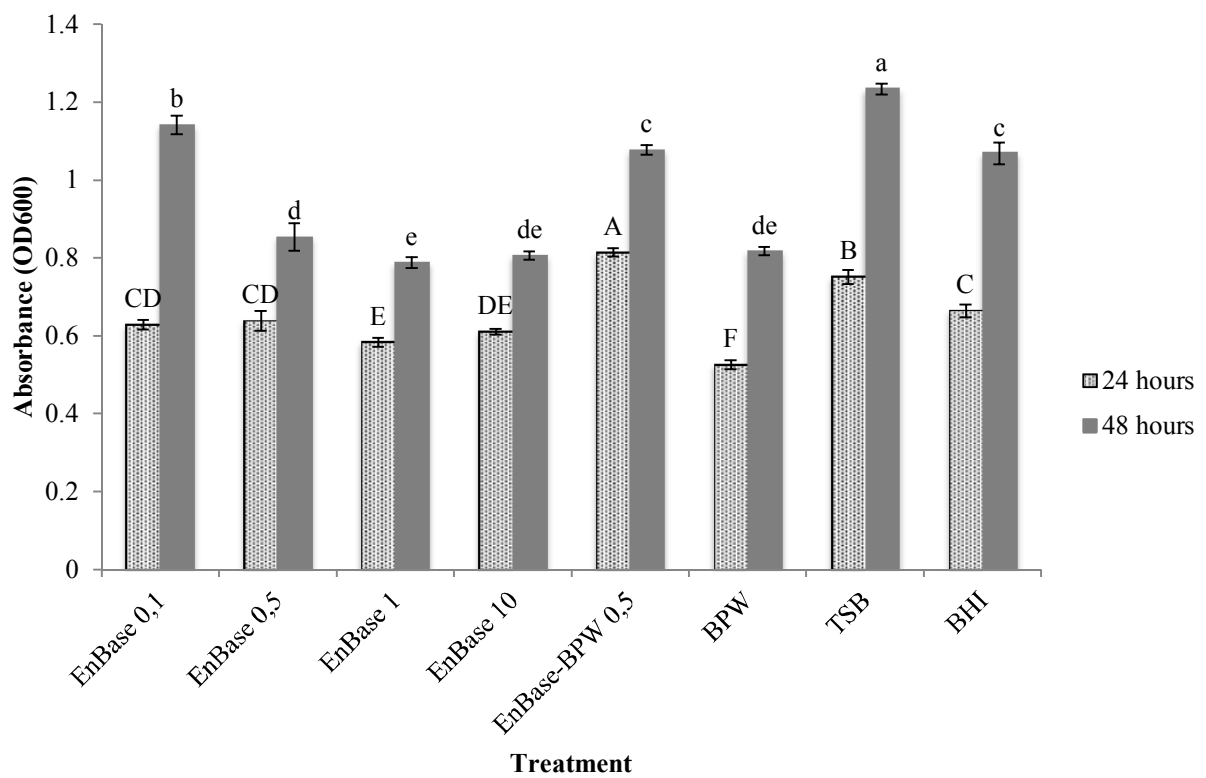
Figure 2. Recovery of heat-injured *Escherichia coli* O157:H7 on selective media (SPEB, SMAC, and TSAYE). The graph shows the mean values obtained after repeating the experiment three times. Capital letters indicate significant differences at the 95% confidence level ($P < 0,001$). Error bars indicate standard error from the mean.
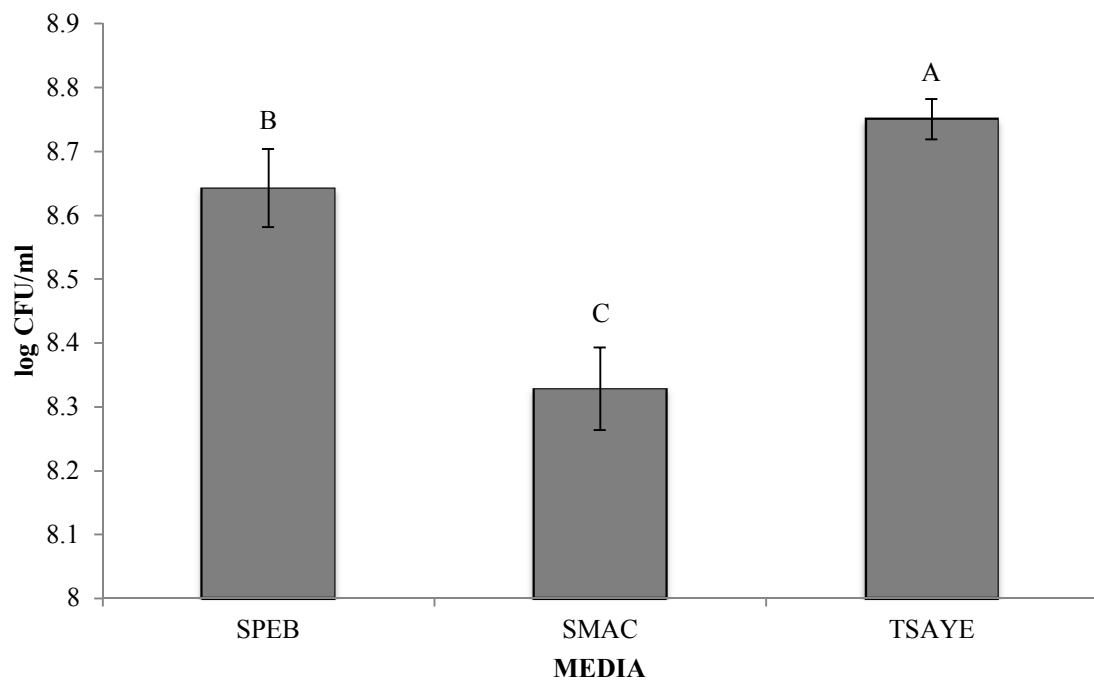
Figure 3. Growth of *Escherichia coli* O157:H7 and coliforms isolated from spiked water samples enriched for 24hours in different broths and recovered on SMAC. Capital and small letters indicate significant differences at the 95% confidence level ($P < 0,001$) between *Escherichia coli* O157:H7 and coliforms, respectively. Error bars indicate Standard Error from the mean.
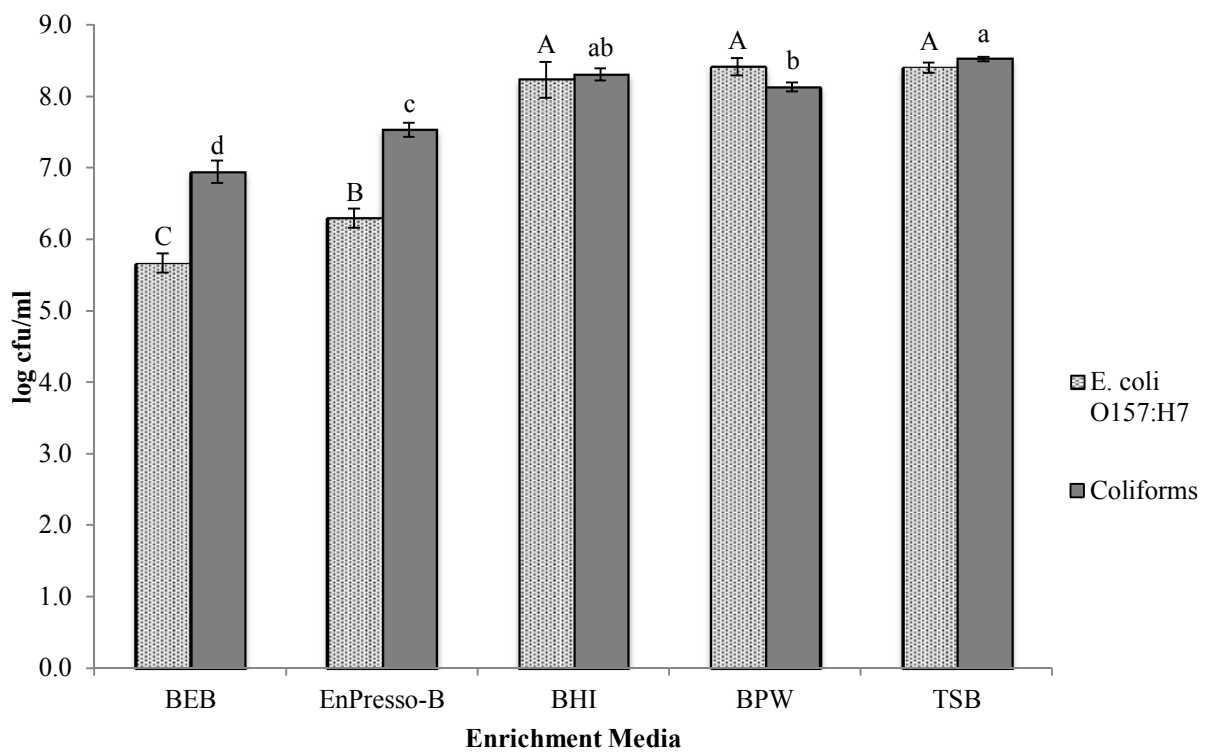
SUMMARY

Since the discovery of *Escherichia coli* O157:H7 in the early 1980s and the increased association of foodborne outbreaks linked to fresh produce, a growing number of research articles relating to this subject have been published. Although leaps in our understanding of foodborne pathogen association with fresh produce have been made, our knowledge regarding this subject remains limited, with many research findings being contradictory. The microbial safety of commercially produced tomatoes in South Africa was investigated during 2012-2014. Although no tomatoes tested were contaminated with either *Salmonella enterica* or *Escherichia coli* O157:H7, the presence of *Salmonella enterica* in the packinghouse of the first farm investigated in 2012 does indicate a potential risk associated with fresh tomatoes.

Rapid, sensitive, and accurate detection of foodborne pathogens is vital to ensure produce safety. Conventional culturing techniques should be improved to reduce the analysis time, increase the efficiency of recovery, improve the resurrection and growth of injured cells, and to exclude other microorganisms present in the samples. The part of this dissertation therefore aimed to investigate the application of EnBase® technology for the enhanced recovery of sub-lethally injured *E. coli* O157:H7 from the environment and particularly surface water. Limiting fermentable carbohydrate availability through enzymatic glucose release would hypothetically prevent metabolic overload during enrichment cultivation, allowing enhanced resuscitation and growth of target organisms. This was found to be the case when *E. coli* O157:H7 was grown axenically under pristine conditions. EnPresso® B and variations thereof performed sub-optimally compared to buffered peptone water when used for the enrichment of *E. coli* O157:H7-spiked surface water in the presence of naturally occurring coliforms. The

utilization of EnBase® technology for the selective and differential isolation and enumeration of sub-lethally injured *E. coli* O157:H7 from spiked surface water on solid media were not effective due to interference of the buffering capacity of EnPresso® B with the pH indicator neutral red.

Understanding the biological and ecological factors enabling survival and persistence of human enteric bacteria in fresh produce, as well as better detection and monitoring techniques, are crucial in developing pre- and postharvest strategies to ensure safe produce in the future.