THE OCCURRENCE OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* IN SOUTH AFRICAN GAME SPECIES

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

The consumption of game meat on a global scale is increasing every year and South Africa is no exception. Consumers have started incorporating low kilojoule and low cholesterol products into their lifestyles, of which game meat is one such product. These increases give rise to a range of challenges for the game meat industry in terms of the microbial safety of the meat.

The aims of this study were to optimise a DNA extraction protocol for the isolation of the Shiga-toxin producing *Escherichia coli* (STEC) positive control and PCR conditions for the amplification of the *stx*$_1$, *stx*$_2$ and *eaeA* virulence genes; to determine the prevalence of STEC contamination in South African game species; to determine the microbial population present on South African game carcasses after dressing; and to develop and implement an organic acid spray in order to reduce microbial growth on game carcasses.

Optimisation of a DNA extraction protocol for STEC positive control included an ethanol wash and re-suspension in sterile distilled water to purify the DNA from any PCR inhibitors. Primer concentrations were also optimised to prevent non-specific binding, which causes primer-dimer formation. PCR amplification conditions were compared to determine the optimum annealing temperature, as a too low or too high temperature led to unsatisfactory amplification results.

The optimised protocol and conditions were used to determine the prevalence of STEC in South African game species. Animals from two different farms were used in this study. Farm 1 consisted of four species, of which two species (Zebra and Black Wildebeest) had STEC present in the faecal matter. The meat of all four species (Zebra, Black Wildebeest, Impala and Eland) contained STEC suggesting possible cross contamination from the hide to the carcass. Faecal samples from Farm 2 (Blesbok and Springbok) tested negative for STEC.

During the microbial population study it was found that aerobic bacteria prevalence on the meat ranged from 1.60 to 4.97 log cfu·cm^{-2} whereas total coliforms varied from 5.04 to 5.59 log cfu·cm^{-2}. *E. coli* prevalence ranged from 0.00 to 1.71 log cfu·cm^{-2} while *Staphylococcus aureus* varied from 0.00 to 2.97 log cfu·cm^{-2}. The results from the faecal matter displayed aerobic bacteria prevalence in the range of 5.78 to 6.44 log cfu·g^{-1} while total coliforms ranged from 6.53 to 7.04 log cfu·g^{-1}. *E. coli* prevalence varied from 3.00 – 4.54 log cfu·g^{-1} while *Staphylococcus aureus* ranged from 3.63 to 4.40 log cfu·g^{-1}. None of the faecal samples tested positive for *Salmonella* or *Listeria*.

Throughout the organic acid spray development, different organic acids (lactic acid, citric acid, acetic acid and octanoic acid) and sodium benzoate were tested singly and in combination. Development started with enrichment mediums and progressed to meat samples. Based on the results of the meat samples a final combination of 5% lactic acid, 0.5% octanoic acid and 0.1% sodium benzoate was chosen to use on game carcasses. Although the combination provided

promising results in laboratory trials, its efficacy on the carcasses was unsatisfactory with low overall log reductions achieved.

To conclude, the high prevalence of STEC (26.9%) detected in the game species was alarming and requires further investigation.

OPSOMMING

Die verbruik van wildsvleis op ‘n globale skaal neem jaarliks toe en Suid-Afrika is geen uitsondering nie. Verbruikers het begin om lae kilojoule en lae cholesterol produkte in hul lewenswyse te inkorporeer, waarvan wildsvleis een so 'n produk is. Hierdie verbruiksverhoging gee aanleiding tot 'n reeks uitdagings vir die vleis industrie in terme van die mikrobiologiese veiligheid van die vleis.

Die doelwitte van hierdie studie was om 'n DNA ekstraksie protokol vir die isolasie van die Shiga-toksien produserende *Escherichia coli* (STEC) positiewe kontrole asook die Polimerase Ketting Reaksie (PKR) kondisies vir die amplifisering van die stx_1, stx_2 en *eaeA* virulensie gene te optimiseer; om die voorkoms van STEC kontaminasie in Suid-Afrikaanse wildspesies te bepaal; om die mikrobiologiese populasie teenwoordig op Suid-Afrikaanse wildsvleis karkasse na afslag te bepaal; en om 'n organiese suur spuitmiddel te ontwikkel en te implementeer om mikrobiese groei op wildsvleis karkasse te verminder.

Optimisering van 'n DNA ekstraksie protokol vir die STEC positiewe kontrole het 'n etanolwas en heroplossing in steriele gedistilleerde water ingesluit om te verseker dat die DNA vry is van PKR inhibeerders. Inleier konsentrasies is ook geoptimiseer om nie-spesifieke binding wat inleier dimere tot gevolg het, te verhoed. PKR amplifiseringskondisies is met mekaar vergelyk om die optimale inleier aanhegtings temperatuur te bepaal, aangesien ‘n te hoë of te lae temperatuur tot onbevredigende amplifikasie resultate sou lei.

Die geoptimiseerde protokol en kondisies is gebruik om die voorkoms van STEC in Suid-Afrikaanse wildspesies te bepaal. Diere vanaf twee verskillende plase is tydens hierdie studie gebruik. Plaas 1 het bestaan uit vier spesies, waarvan twee spesies (Zebra en Swart Wildebees) STEC in die ontlasting teenwoordig gehad het. Die vleis van al vier spesies (Zebra, Swart Wildebees, Impala en Eland) het STEC bevat wat moontlike kruis-kontaminasie vanaf die vel na die karkas aandui. Fekale monsters vanaf Plaas 2 (Blesbok en Springbok) het negatief getoets vir STEC.

Gedurende die mikrobiologiese populasie studie is gevind dat die voorkoms van aerobiese bakterieë op die vleis gewissel het tussen 1.60 en 4.97 log kve·sm^{-2} terwyl totale kolivorme gewissel het tussen 5.04 – 5.59 log kve·sm^{-2}. Die voorkoms van *E. coli* het gewissel vanaf 0.00 tot 1.71 log kve·sm^{-2} terwyl *Staphylococcus aureus* gewissel het tussen 0.00 en 2.97 log kve·sm^{-2}. Die resultate van die fekale monsters het getoon dat die voorkoms van aerobiese bakterieë gevarieer het tussen 5.78 en 6.44 log kve·g^{-1}, terwyl totale kolivorme gewissel het tussen 6.53 en 7.04 log kve·g^{-1}. Die voorkoms van *E. coli* het gevarieer tussen 3.00 en 4.54 log kve·g^{-1}, terwyl *Staphylococcus aureus* gewissel het tussen 3.63 en 4.40 log kve·g^{-1}. Geen fekale monsters het positief getoets vir *Salmonella* of *Listeria* nie.

Gedurende die organiese suur spuitstof ontwikkeling, is verskillende organiese sure (melksuur, sitroensuur, asynsuur en oktanoësuur) en natriumbensoaat afsonderlik asook in kombinasie getoets. Ontwikkeling het begin met verrykingsmediums en daarna voortgegaan met vleismonsters. Op grond van die resultate behaal vir die vleismonsters was 'n finale kombinasie van 5% melksuur, 0.5% oktanoësuur en 0.1% natriumbensoaat gekies om op die wildsvleis karkasses te gebruik. Alhoewel dié kombinasie belowende resultate in laboratoriumtoetse getoon het, was die doeltreffendheid daarvan op die karkasse onbevredigend in terme van die algehele log reduksies wat bereik is.

Om af te sluit, die hoë voorkoms van STEC (26.9%) wat waargeneem is in die wildspesies, is kommerwekkend en vereis verdere ondersoek.

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RESEARCH CONTRIBUTIONS

Conference Contributions

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Research Outputs

Gouws, P.A., Germishuys, Z. & Hoffman, L.C. (2017). Determination of the microbial population of Blesbok (*Damaliscus pygargus phillipsi*) meat in South Africa. In: *Game meat hygiene – Food safety and security* (edited by P. Paulsen, A. Bauer & F. J. M. Smulders). Pp. 235-240. Netherlands: Wageningen Academic Publishers.

CONTENTS

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion, recommendations and conclusions. Language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ABBREVIATIONS

A/E	Attaching and Effacing
AA	Acetic Acid
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BB	Blesbok
bp	Base pair
CA	Citric Acid
CAP	Capsular Polysaccharides
CDC	Centres for Disease Control and Prevention
cfu	Colony Forming Units
DAFF	Department of Agriculture, Forestry and Fisheries
dNTP	Deoxyribonucleioside Triphosphate
dsDNA	Double Stranded Deoxyribonucleic Acid
EAEC	Enteroaggregative *Echerichia coli*
EHEC	Enterohemorrhagic *Echerichia coli*
EIEC	Enteroinvasive *Echerichia coli*
EPEC	Enteropathogenic *Echerichia coli*
ETEC	Enterotoxigenic *Echerichia coli*
Gb_3	Globotriaosyl Ceramide
GIT	Gastrointestinal Tract
GMP	Guanosine Monophosphate
GRAS	Generally Regarded as Safe
HC	Haemorrhagic Colitis
HUS	Haemolytic Uremic Syndrome
LA	Lactic Acid
LB-broth	Luria Bertani broth

LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharides
LT	Heat-liable
MAC	MacConkey Agar
MCFA	Medium Chain Fatty Acid
mdh	Malate dehydrogenase
MUG	4-methylumbelliferyl-β-D-glucuronide
OA	Octanoic Acid
PCR	Polymerase Chain Reaction
PMF	Proton Motive Force
Saa	STEC autoagglutinating adhesion
SB	Springbok
SDS	Sodium Dodecyl Sulphate
ST	Heat-stable
STEC	Shiga-Toxin producing *Echerichia coli*
T_a	Annealing Temperature
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TCA	Tricarboxylic Acid
T_m	Melting Temperature
TSA	Tryptone Soy Agar
TTP	Thrombotic Thrombocytopaenic Purpura
UN DESA	United Nations Department of Economic and Social Affairs
VRBA	Violet Red Bile Agar
VTEC	Verocytotoxigenic *Escherichia coli*

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CHAPTER 1
INTRODUCTION

Currently the world global population is sitting at approximately 7.4 billion people of which South Africa constitutes around 54 million (Anonymous, 2016a). The United Nations Department of Economic and Social Affairs (UN DESA) estimates that the global population could rise to around 9.7 billion people by 2050 (UN DESA, 2015). This rapid increase in global population poses a great challenge to food security, as the increasing population has to be fed while still ensuring that the world's natural resources do not become depleted (Cooper, 1995). The production of game meat as an alternative and or additional protein source to that of livestock may lighten the demand load on the meat industry (Hoffman & Cawthorn, 2013).

The consumption of game meat on a global scale is increasing with every year that passes. In this regard South Africa is no exception (Reilly *et al.*, 2003; Taylor *et al.*, 2015). This increase in game meat production and consumption can mainly be attributed to consumers becoming more health conscious about the products they consume. Consumers have started adapting their lifestyles to incorporate low kilojoule and low cholesterol products of which game meat is one such product (Hoffman & Wiklund, 2006; Klein, 2005). South Africa produced around 23 700 tonnes of game meat during 2014 (Taylor *et al.*, 2015).

The increases in game meat consumption further gives rise to a range of difficulties for the game meat industry in terms of the microbial safety of the meat. As game meat originates from animals that live in the wild, careful attention has to be given to comply with the strict health and safety regulations to ensure that the meat is safe for human consumption (Atanassova *et al.*, 2008).

The deep tissue of meat is generally accepted as being sterile, provided the animal is in a healthy condition (Mackey & Derrick, 1979). The harvesting process of animals can introduce microbial contaminations onto this once sterile meat. This is particularly relevant in cases where the animal is shot in the field and internal organs become damaged. This open wound then provides the perfect environment for microbial contamination in the field setting to occur (Mackey & Derrick, 1979; Gill, 2007). Further contamination of the meat can occur during dressing of the carcass as the hides of wild animals naturally contain microorganisms (van Schalkwyk & Hoffman, 2016). This can be attributed to their surroundings as well as contamination of the hide with faecal matter present in the field (Nørrung & Buncic, 2008; Blagojevic *et al.*, 2011). Blagojevic *et al.* (2011) further showed a significant carryover of microbial contamination from the hide to the carcass during the dressing process.

The microbial population present on any food product can be discussed in terms of either food spoilage or food safety. Food spoilage refers to the process whereby undesired changes

occur within a food product leading to the degradation of biochemical and sensory characteristics, making the product unacceptable to the consumers. Spoilage bacteria include aerobic bacteria, total coliforms and *Pseudomonas*. Meat specifically, is very prone to spoilage due to the presence of enzymatic and chemical activity. Fresh meat further has a high moisture content, contains nutrients and has an optimum pH range which favour the growth of a variety of microorganisms (Iulietto *et al.*, 2015). Food safety on the other hand refers to the presence/absence of pathogenic bacteria present on the foodstuff. For food products to be regarded as safe, no or limited (according to legislation) pathogenic bacteria or toxin-producing bacteria may be present on the food (D'Alessandro & Zolla, 2012). These bacteria include *Escherichia coli* (*E. coli*), *Salmonella, Listeria* and *Staphylococcus aureus* (*S. aureus*).

An important human pathogen previously linked to cattle now poses a great risk to game species. First isolated in 1982 after two haemorrhagic colitis outbreaks, Enterohemorrhagic *Escherichia coli* (EHEC) but more specifically Shiga-toxin producing *Escherichia coli* (STEC) was determined as the responsible pathogen (Erdoğan *et al.*, 2008; O'Brien & Holmes, 1987; Riley *et al.*, 1983). *E. coli* O157:H7 is the most well-known serotype of STEC (Gannon *et al.*, 1997). STEC are defined as *E. coli* that have the ability to produce at least one of the two shiga-toxins, namely stx_1 and stx_2 and are not serotype specific. In addition to the toxin genes they may also possess the *eaeA* gene that encodes for the intimin protein that is required for attaching and effacing lesions (Tarr *et al.*, 2005; Chekabab *et al.*, 2013).

Since the first outbreak much research has been done in order to detect STEC, especially the virulence genes, from an array of different sources. The most widely used method for the detection of these genes is the use of Polymerase Chain Reaction (PCR) (Kulp, 2014; Elnifro *et al.*, 2000). During a standard PCR amplification only one gene is amplified but in the case where three different genes must be amplified, a multiplex PCR is performed. There are many components that can hinder the PCR amplification process, thus optimisation of the process is critical to minimise the possibility of false negative or false positive results (Henegariu *et al.*, 1997).

Ingestion of food contaminated with STEC, could lead to an array of different illnesses, especially considering its low infective dose of less than 100 colony forming units (cfu) (Welinder-Olsson & Kaijser, 2005). Diseases associated with STEC include bloody diarrhoea, haemorrhagic colitis (HC), thrombotic thrombocytopaenic purpura (TTP) as well as the life threatening disease haemolytic uremic syndrome (HUS) (McClure, 2000; Gannon *et al.*, 1992).

STEC related outbreaks associated with the meat industry, especially in the United States, are becoming more prominent each year. The Centres for Disease Control and Prevention (CDC) estimated that around 390 *E. coli* O157 outbreaks occurred in the United States between 2003 and 2012. These included 4 928 illnesses, 1 272 hospitalization, 299 cases of HUS and 33 deaths (Zuraw, 2015). A slaughterhouse in Massachusetts had to recall 27 lots of beef, veal and bison due to *E. coli* O157:H7 contamination during June to September 2016 (Anonymous, 2016b). One

of the most significant STEC outbreaks in recent years occurred in Germany in 2011 where sprouts were the vehicles of transmission. This outbreak resulted in 3 842 illnesses of which 855 developed HUS and more than 50 deaths were reported (Muniesa *et al.*, 2012).

Decreasing the microbial contamination on carcasses after dressing is an integral part in not only ensuring the meat is safe for consumption but also to decrease the possibility of meat spoilage. The use of microbial interventions in the meat industry is readily used to decontaminate carcasses (Loretz *et al.*, 2011; Sofos & Smith, 1998). The most widely used microbial interventions used in the meat industry, especially in the United States, include hot water washes, steam pasteurisation of carcasses as well as organic acid carcass washes (Bosilevac *et al.*, 2006; Berry & Cutter, 2000).

Hot water washes are used to remove environmental contaminants such as soil and faecal matter from carcasses but are only effective if the water is heated to at least 75°C (Loretz *et al.*, 2011; Pipek *et al.*, 2005). Hot water treatments can be applied either before or after the removal of the hide. If done before, it will inherently decrease the amount of cross contamination from the hide to the carcass. If done after skinning, it can remove any contaminants that could have been carried over during the skinning process. The use of a combination of the two will lead to maximum reduction of contaminants (Small *et al.*, 2005; Sofos & Smith, 1998).

Another effective method of reducing microbial contamination on carcasses is the use of steam pasteurisation. This involves exposing the carcasses to steam at 82 – 97°C inside a chamber for approximately 6 – 12 s (Aymerich *et al.*, 2008). The advantage of steam pasteurisation is that there is a large amount of heat that is rapidly transferred to the carcass, increasing the surface temperature (James *et al.*, 2000). However, a disadvantage is that it is energy and water intensive and thus not readily applicable to use in smaller abattoirs or in field situations. It is also important to note that the in field practicality of the two above mentioned techniques could pose a challenge. The slaughtering infrastructure on many game farms is inferior compared to that of a functional abattoir. Lack of hot water, proper facilities and electricity combined with a non-sterile environment all contribute to the challenges faced.

The use of organic acid washes in an abattoir setting has proven to be a useful method to decontaminate carcasses. Organic acids are generally regarded as safe (GRAS) and when used in low concentrations (0.1 – 5%) pose little risk to the consumer. The most widely used organic acids include lactic acid, acetic acid and citric acid (Barco *et al.*, 2015; Mani-López *et al.*, 2012; Brul & Coote, 1999; Hamby *et al.*, 1987). The use of medium chained fatty acids (MCFA) in combination with organic acids has been proven to increase its effectiveness (Kim & Rhee, 2013). MCFA's have the ability to diffuse through the bacterial cell membrane creating pores, which then results in altered membrane permeability and ultimately cell death (Bergsson *et al.*, 2001). As organic acids do not require any specialised equipment, it makes it a viable carcass decontamination choice for use in field setting, unlike the other techniques mentioned.

The greater aim of the current research was to determine the prevalence of STEC in South African game species. Several studies were performed focussing on: optimisation of a DNA extraction protocol for the isolation of the STEC positive control; optimisation of the PCR conditions and reaction composition for the amplification of the *stx*$_1$, *stx*$_2$ and *eaeA* virulence genes; determining the prevalence of STEC contamination in South African game species; determining the microbial population present on South African game carcasses after dressing to determine whether the meat would meet the microbiological criteria in terms of food spoilage and food safety aspects; developing an organic acid spray in laboratory trials to determine the decontamination efficacy of different organic acids and mixtures thereof; and determining the efficacy of the developed organic acid spray in a field study on game carcasses.

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CHAPTER 2
LITERATURE REVIEW

2.1 GAME SPECIES

2.1.1 Background

The game meat industry of South Africa is an ever-increasing industry growing as each year passes. Already in 2003 the wildlife ranching sector of South Africa was recognized as a fast growing sector in the agricultural industry. It was estimated that South Africa had 9 000 commercial game ranches in 2003 covering more than 17 million hectares of land (Reilly *et al.*, 2003). Ultimately, all the game species and animals ranched end up producing meat. The game meat industry is not only growing in South Africa, as increases in game meat production can be seen worldwide. The North American Elk Breeders Association, in 2003, estimated that there were approximately 110 000 elk distributed across 2 300 U.S. farms valued at more than 150 million US Dollars. This was an increase of about 20 000 elk since 1997 (Klein, 2005). In 2007 it was estimated that the world farmed deer population was around five million, with half of this population situated in New Zealand (Gill, 2007).

This increase in game meat production and thus consumption is mainly attributed to consumers becoming more concerned about their health and the products they consume. Due to these health concerns, consumers have started adapting their lifestyles to incorporate low kilojoule and low cholesterol products (Hoffman & Wiklund, 2006). One such product is game meat, known for its health benefits (Klein, 2005). During 2014, South Africa produced 23 700 tonnes of game meat (Taylor *et al.*, 2015).

Some of the most common animals harvested in South Africa for the production of game meat include feral Fellow Deer (*Dama dama)*, Springbok (*Antidorcas marsupialis*), Blesbok (*Damaliscus pygargus phillipsi*), Black Wildebeest (*Connochaetes gnou*), Zebra (*Equus quagga*), Impala (*Aepyceros melampus*), Eland (*Taurotragus oryx*), Kudu (*Tragelaphus*) as well as Gemsbok (*Oryx gazella*) (van Schalkwyk *et al.*, 2010). Game meat refers to meat obtained from non-domesticated, farm raised and/or free ranging animals. Historically, only poor and rural communities in Middle East, Asia, Africa and South America ate game meat as a cheap source of protein (Klein, 2005).

As game meat comes from animals that live in the wild, health and safety regulations have to be followed to ensure the meat is safe for human consumption.

2.1.2 Harvesting process

The following section will describe the harvesting process of game animals. Throughout the process possible sources of meat cross-contamination will be highlighted.

The harvesting of game animals can occur both during the day and at night but it is believed that harvesting at night is less stressful to the animal (Hoffman & Laubscher, 2009). Night harvesting is done from an open vehicle where the animals are spotted using a spotlight. Once the animal has been spotted the shooter/marksman can take the shot. The most common shot placement is the head. Headshots are preferred as these cause immediate death and also minimises the chance of meat contamination. Abdominal shots on the other hand could lead to the rupturing of the stomach and intestines causing the body cavity to be contaminated with the intestinal contents and are not preferred (van Schalkwyk & Hoffman, 2010). Nonetheless, shoulder shots with the objective of damaging vital organs (heart, liver, lungs) are common amongst biltong and trophy hunters, whilst headshots are a prerequisite for the commercial harvesting of game animals.

When the animal has been shot, exsanguination has to take place as soon as possible to stop circulation of blood through the body. A clean sterilised knife should be used. The hide could be heavily contaminated with environmental contaminants that can be transferred to the knife during exsanguination, which could in turn be transferred to the next animal if the knife is not cleaned. It was suggested by van Schalkwyk & Hoffman (2010) that knifes have to been cleaned in 82°C water for at least 10 seconds. Abdalla *et al.* (2009) showed that this recommended submersion time is hardly ever reached. After exsanguination the carcasses are transported to the field abattoir where they are hung to bleed out. Carcasses are typically hung vertically or on a slope to facilitate bleeding

Once the carcasses have bled out they need to be eviscerated. The carcass is cut open where after the stomach, intestines and organs are removed. Evisceration has to take place as soon as possible. When evisceration is delayed for a few hours to a few days the intestines could start swelling increasing the difficulty of removing the stomach without puncturing it (Gill, 2007; Klein, 2005).

The next step is skinning of the carcass. Skinning of the animal could lead to further contamination of the carcass meat. The hide of the animal naturally contains microbial loads. This is due to their surrounding as well as contamination from faecal matter present in the field (Blagojevic *et al.*, 2011; Bell, 1997). If skinning of the carcass is thus not done properly, cross contamination can occur where microbial organisms are transferred from the hide of the animal to the meat (Nørrung & Buncic, 2008). Blagojevic *et al.* (2011) showed a significant carryover of microbial load from the hide to the carcass during dressing of the carcass. After dressing carcass chilling occurs at ± 4°C till further processing takes place.

2.2 MICROBIAL POPULATION

2.2.1 Background

Coliforms

Coliforms are a group of bacteria characterised on the basis of biochemical reactions instead of genetic relationships. They are aerobic or facultative anaerobic, Gram-negative, motile or non-motile, non spore-forming bacteria that have the ability to ferment lactose. When they ferment lactose, acid and gas are produced (Kornacki & Johnson, 2001). Coliforms are present in the intestines of both humans as well as warm-blooded animals and can thus be found in the faecal matter. Due to the fact that coliforms are present in faecal matter it is used as an indicator organisms to test whether food has been in contact with or contaminated with faecal matter (Rompré *et al.*, 2002). Coliforms are also responsible for the reduced shelf-life of food products due to food spoilage (Baylis, 2006).

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a common cause of foodborne intoxication, especially in warm and humid regions such as India and Africa. *S. aureus* is a Gram-positive, non-motile, facultative anaerobic coccus. Most humans carry this bacterium within their throat and nasal passages. *S. aureus* has the ability to grow at temperatures ranging from 7 – 48°C with its optimum temperatures being between 30 – 37°C. *S. aureus* also grows at a pH range of 4 – 9 with its optimum being between 7 – 7.5 (Bhatia & Zahoor, 2007).

S. aureus are generally present on meats, dairy products and bakery products (Willey *et al.*, 2011f). They produce enterotoxins that are responsible for food poisoning (Schmitt *et al.*, 1990). The incubation period of *S. aureus* is between 2 – 8 hours and symptoms last for around 24 hours. Symptoms of *S. aureus* food poisoning include severe vomiting, diarrhoea, abdominal cramps, fever and dehydration (Willey *et al.*, 2011f).

Salmonella

Salmonella is considered to be one of the most important pathogenic bacteria related to foodborne diseases (Govaris *et al.*, 2010). In 2011 it was estimated that around 170 000 cases of salmonellosis were reported on an annual basis in the European Union alone (Vieira-Pinto *et al.*, 2011). *Salmonella* forms part of the family *Enterobacteriaceae* that also includes *Escherichia coli*. They are Gram-negative, facultative anaerobic, rod-shaped, motile and non spore-forming bacilli (Baylis, 2006). *Salmonella* can grow at temperatures ranging from 8 – 45°C with its optimum temperatures being between 35 – 37°C. It also grows at a pH range of 4 – 9 and a water activity (a_w) of above 0.94. *Salmonella* are heat sensitive and are usually killed off at temperatures above 70°C (Mani-López *et al.*, 2012).

Salmonella are generally associated with food products such as eggs, chicken, sprouts and meat where contamination usually occurs via contact with faecal matter from the animal itself or from the environment. When food contaminated with *Salmonella* is ingested, symptoms can start occurring anywhere from 6 – 72 hours after ingestion. Typical symptoms include gastroenteritis, low-grade fever, nausea and headaches. Symptoms typically last around 48 hours but in more complicated cases it can last up until 14 days (Mani-López *et al.*, 2012; Willey *et al.*, 2011f).

Listeria monocytogenes

Listeria monocytogenes (L. monocytogenes) is a highly pathogenic bacterium that is dangerous to those with compromised immune systems, pregnant woman, newborns as well the elderly (Yde *et al.*, 2010). *L. monocytogenes* is a Gram-positive, facultative anaerobic, non spore-forming rod. *L. monocytogenes* can grow at temperatures ranging from -0.4 – 50°C with its optimum temperatures being between 30 – 37°C (Farber & Peterkin, 1991).

L. monocytogenes is associated with contaminated vegetables, fish, meat products, milk and ready-to-eat products (Kérouanton *et al.*, 2010). The incubation time of *L. monocytogenes* can range anywhere from a few days up until three months making it difficult to pin point the exact source of contamination (Yde & Genicot, 2004). Symptoms of listeriosis include fever, headache, vomiting, muscle aches and diarrhoea. *L. monocytogenes* can also lead to septicaemia, meningoencephalitis, meningitis, abortion and death (Cox *et al.*, 1998).

2.2.2 Sampling Methods for microbiological evaluation

The two most commonly used sampling techniques are described below. The first technique involves taking an actual meat sample from the carcass. This is also known as excision sampling. A steel borer, 2.52 cm in diameter, is sterilised with a flame and firmly pressed onto the surface of the carcass. The borer is then twisted to make an initial cut. Pre-sterilised disposable scalpels can then be used to further cut the piece of meat away from the carcass. This borer size will remove a piece of meat with a 5 cm^2 surface area (van der Merwe *et al.*, 2011; Hutchison *et al.*, 2005).

The second technique involves taking swabs of the carcass. This can be done in two ways depending on the size of the surface area that has to be sampled. If the surface area to be swabbed is small such as 100 cm^2 or less, sterile cotton swabs are used. A sterilised template is placed on the carcass and the swab is rolled between the thumb and the index finger ensuring to cover the entire area (Ghafir & Daube, 2008). If the area to be swabbed is much larger such as in the study done by Wang *et al.* (2013) where a 4 000 cm^2 surface was swabbed, a sponge (Speci-Sponges) can be used. The sponge is rubbed over the surface where after it is sealed in a bag and stored on ice (Brichta-Harhay *et al.*, 2007).

2.2.3 Prevalence of microorganisms on carcasses

Several studies have been performed over the years to determine the microbial population present on cattle, pork and other livestock. The prevalence of these types of studies on game animals has been done to a much lesser extent. The following section will explore the prevalence of the most commonly tested bacteria on game animals.

Atanassova *et al.* (2008) investigated the prevalence of aerobic plate count (APC), *Enterobacteriaceae, Salmonella* and *Listeria* in wild boars, red deer and roe deer. The mean APC was 3.2 log cfu·cm^{-2}, 2.9 log cfu·cm^{-2} and 2.6 log cfu·cm^{-2} respectively. The mean *Enterobacteriaceae* counts for all three species were 2.1 log cfu·cm^{-2}. A total of 289 samples were analysed of which 14 samples gave positive results for *Listeria*. No *Salmonella* was detected in any of the samples.

Avagnina *et al.* (2012) also used these species in a study to determine the prevalence of APC and *Enterobacteriaceae*. The mean APC counts for wild boars, red deer and roe deer were 4.61 log cfu·cm^{-2}, 3.31 log cfu·cm^{-2} and 3.46 log cfu·cm^{-2} respectively. The *Enterobacteriaceae* counts for the three species in the order stated above were 3.00 log cfu·cm^{-2}, 1.70 log cfu·cm^{-2} and 2.47 log cfu·cm^{-2} respectively.

Springbok carcasses that were tested over a two-year period (2009 – 2010) reported a mean APC count of 2.58 cfu·cm^{-2} for 2009 and 3.49 cfu·cm^{-2} for 2010. The study further reported a mean *Enterobacteriaceae* count of 1.33 cfu·cm^{-2} for 2009 and 2.93 cfu·cm^{-2} for 2010 (Magwedere *et al.*, 2013).

2.3 ESCHERICHIA COLI

2.3.1 Background

Escherichia coli (*E. coli*) was first discovered by Theodor Escherich in 1887 when it was isolated from human stool samples (Kornacki & Johnson, 2001). *E. coli* forms part of the family *Enterobacteriaceae* that also includes other pathogens such as *Salmonella* and *Shigella* (Baylis, 2006). Most commensal *E. coli* strains are harmless but some of them are pathogenic and could cause an array of different diseases.

E. coli is a facultative anaerobic, Gram-negative, non-sporulating bacterium. Their cells are rod-shaped with a length of approximately 2.0 µm and a diameter ranging from 0.25 – 1.0 µm (Willey *et al.*, 2011c). *E. coli* can be either motile, with peritrichous flagella, or non-motile. Those that are motile typically have between 5 and 10 flagella per cell that are situated around the cell surface in a random fashion. Each flagellar filament has a diameter of around 20 nm and could be as long as 20 µm. Each flagellum is made up of subunits of one single protein known as flagellin. In addition to the flagella, most *E. coli* strains also possess fimbriae, otherwise known as pili (Scheutz & Strockbine, 2005).

E. coli isolates have three major surface antigens which can be used to differentiate between them on a serological and genetic level. These include the O, H and K antigens (Meng *et al.*, 2013). The O antigen refers to a repetitive glycan polymer that is contained within a lipopolysaccharide (LPS). The O antigen is used to determine the serogroup of a strain. The H antigen refers to the flagella proteins. The K antigen is the acidic capsular polysaccharide (CAP) and refers to the capsule. When the O and H antigens are combined, the serotype of the strain can be determined (Scheutz & Strockbine, 2005; Meng *et al.*, 2001).

The primary habitat of *E. coli* is within the gastrointestinal tract (GIT), of both humans as well as warm-blooded animals (Dixit *et al.*, 2004). The secondary habitat is water, soil and sediment. These two habitats greatly differ from each other and so will the strains that are present there. The optimum growth temperature of *E. coli* is ± 37°C as this is the temperature of its primary host. *E. coli* living in the secondary habitat can grow at temperatures between 10 – 14°C but can survive temperatures below freezing point (Savageau, 1983; Willey *et al.*, 2011c).

Since the primary habitat of *E. coli* is within the GIT of humans and animals, it is used as an indicator organism for faecal contamination of water and food sources. There are also reference strains of *E. coli* that are routinely used in laboratory studies and trials. One such reference strain is *E. coli* K-12. It has furthermore been estimated that *E. coli's* total population size is approximately 10^{20} when taking into consideration all *E. coli* found in the wild (Tenaillon *et al.*, 2010; Scheutz & Strockbine, 2005).

2.3.2 Conventional methods for the detection of *Escherichia coli*

Microbiological tests

The most widely used microbiological technique for the enumeration of microorganisms is the plate count method. These include both the spread and pour plate techniques. When using this method the assumption is that every microbial cell present in a sample will form a separate colony when mixed with a growth medium such as bacteriological agar (Swanson *et al.*, 2001). To ensure that colonies form on the growth media, the media should meet the microorganism's physiological and nutritional needs. The media should thus have sufficient minerals, proteins and carbohydrates which the microorganisms can utilize (Forsythe, 2010b). It should further be noted that the requirements for each microorganism differs, making it imperative to choose the correct culture media.

The two most commonly used culture media for the detection of *E. coli* and coliforms are Violet Red Bial Agar (VRBA) and MacConkey Agar (MAC). Both these media contain bile salts that inhibit the growth of Gram-positive bacteria. Lactose is also added to the media, as *E. coli* is a lactose-fermenting bacterium. The indicator, neutral red, causes these lactose-fermenting bacteria to produce pink-red colonies in the medium (Forsythe, 2010b). As VRBA is mostly used for the detection of coliforms, which include *E. coli* among others, 4-methylumbelliferyl-β-D-glucuronide

(MUG) is added to the media. As *E. coli* has been shown to have β-glucuronidase activity, they hydrolyse the MUG into its glucuronides. The red/purple fluorescent *E. coli* colonies can then be visualized by illuminating them with a UV lamp at 366 nm due to the presence of 4-methylumbelliferone (Shah *et al.*, 2010; Forsythe, 2010b).

After enumeration of *E. coli* on a media plate it can further be confirmed by determining the morphology of the enumerated bacterium. The simplest way of determining the morphology is to perform a simple Gram stain. The main purpose of a Gram stain is to distinguish between Gram-positive and Gram-negative bacteria. When a Gram stain is performed on *E. coli,* red/pink rod shaped cells can be observed under a microscope (Willey *et al.*, 2011i).

Biochemical tests

After microbiological tests have been done, identification can be taken a step further by performing biochemical tests. One of these biochemical tests includes the API20E system from bioMérieux. This system is used for the identification of *Enterobacteriaceae* as well as other Gram-negative bacteria. It is composed of 20 microtubes on a plastic strip all containing different dehydrated biochemical compounds (Willey *et al.*, 2011e). The tests that form part of the API20E system include: β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, acetoin production, gelatinase as well fermentation of glucose, inositol, mannitol, sorbitol, saccharose, rhamnose, melibiose, amygdalin and arabinose (Swanson & Collins, 1980).

Each of the 20 microtubes is inoculated with a pure culture of the bacteria in question where after the strip is incubated at 37°C according to the manufactures instructions. During incubation the bacteria metabolise the reagents in the tube causing colour changes. These colour changes are either spontaneous or are revealed after incubation by the addition of reagents to selected tubes. Upon reading the results of the 20 tests, a seven-digit profile code is generated (Holmes *et al.*, 1978; Swanson & Collins, 1980; Willey *et al.*, 2011e). Using this code on the API database, the species of the bacterium can be determined.

2.3.3 Molecular based detection of *Escherichia coli*

Polymerase Chain Reaction

Probably the most widely used molecular method for the detection of *Escherichia coli* (*E. coli*) as well as many other pathogenic and non-pathogenic microorganisms is the Polymerase Chain Reaction (PCR). Kary B. Mullis first developed this technique in 1983 while working at the Cetus Corporation in Emeryville, CA. Since then PCR has revolutionized the field of molecular biology (Gibbs, 1990; Kulp, 2014). Before the development of PCR, molecular biologists had to make use of methods that were not only very labour intensive but also very time consuming when they had to identify or clone specific DNA sequences (Muhlrad, 2003). PCR is a technique that is based on the

natural process of DNA replication. This natural process was used to manipulate DNA or fragments thereof to be amplified for the purpose of easily studying them. The polymerase enzyme is involved in the DNA replication process which under normal circumstances requires a chain of events to occur and then only produces DNA on a small scale (Kulp, 2014).

PCR is used for the amplification of either single or double stranded DNA (dsDNA) sequences or fragments (Gorski & Csordas, 2010). The technique is based on a three-step process of first denaturing the DNA followed by the attachment of oligonucleotides, also known as primers, and lastly DNA polymerase extension. This three-step process is then repeated for a number of cycles depending on the application it is used for (Gibbs, 1990; Gorski & Csordas, 2010). It is a chain reaction that occurs hence the name polymerase chain reaction.

PCR facilitators

PCR starts by formulating the optimum reaction mixture for the amplification of the target DNA fragments or specific genes. The reaction mixture consists of a variety of different components each with their own unique purpose. The first important component of the reaction mixture is the oligonucleotide primers (Gibbs, 1990). The primers have to be carefully designed, as they need to perfectly fit onto the part of the DNA that needs to be amplified by providing the 3'-OH required for DNA synthesis (Willey *et al.*, 2011h). The second component is a pH buffer containing $MgCl_2$. The third component is each of the four deoxyribonucleoside triphosphates (dNTPs), dATP, dCTP, dGTP and dTTP. The fourth component is the target DNA template that will be used for the amplification of the fragments (Gorski & Csordas, 2010). The last component is the DNA polymerase. DNA polymerase is used to extend the primers, synthesising new copies of the target DNA using the free dNTP's (Chien *et al.*, 1976; Willey *et al.*, 2011h). The most common polymerase used in PCR is *Taq* polymerase as it is a thermostable enzyme extracted from the thermophilic bacterium *Thermus aquaticus (Taq)* (Gibbs, 1990).

PCR Stages

The PCR amplification process consists of three steps. The first step of PCR is denaturation. During denaturation the DNA that contain the sequence to be amplified is heated to approximately 95°C. This causes the double-stranded DNA to separate into two single DNA strands that are complementary to one another (Alberts, 2003). The second step is annealing. During annealing the temperature of the reaction mixture is cooled down to ± 50°C. By lowering the temperature the primers can form hydrogen bonds with both of the DNA single strands. Due to the small size of the primers (usually between 20 – 30 base pairs) as well as the fact that they are in excess, the DNA strands would rather anneal to the primers than themselves (Willey *et al.*, 2011h). The last step, and also the end of one cycle, is polymerase extension. During extension the temperature is raised again, usually to 68 – 72°C. The *Taq* polymerase can now rebuild the DNA strand using the free dNTPs in the reaction mixture (Forsythe, 2010b; Gibbs, 1990).

As this process of denaturing the DNA, annealing the primers and polymerase extension continues, the primers will repeatedly bind with both the target DNA as well as all the newly synthesized DNA strands to produce more copies with every cycle that passes. As the number of DNA copies double with each cycle, it can be noted that there is an exponential increase in the number of copies after each cycle (Gibbs, 1990). After only 30 cycles the initial double stranded DNA has been amplified to produce over one billion copies (Willey *et al.*, 2011h). This process can be seen in Figure 2.1.

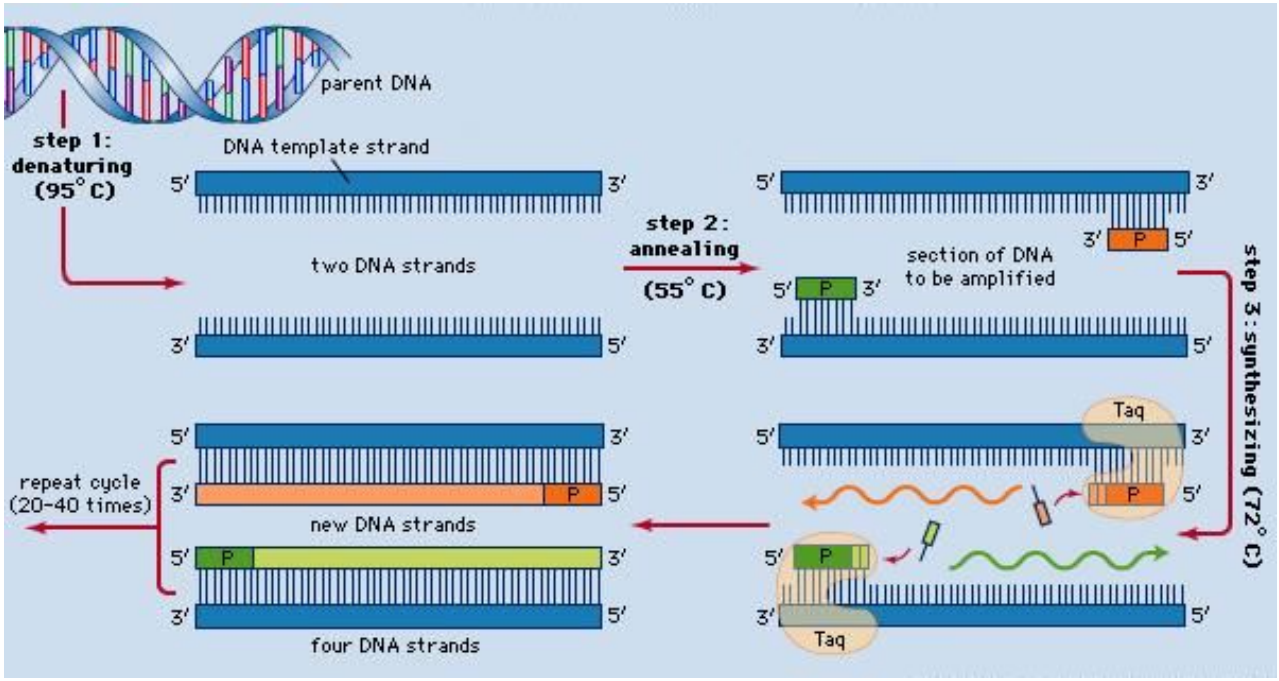

Figure 2.1 Polymerase chain reaction process (adapted from Anonymous, 2014).

DNA visualisation by Gel Electrophoresis

Once the DNA fragments or genes of concern have been amplified they will be separated in order to visualize them. One of the most effective ways of separating DNA fragments with sizes varying from 100 bp to 2 500 bp is agarose gel electrophoresis (Fajardo *et al.*, 2010). Agarose is isolated from two genera of seaweed, namely *Gracilaria* and *Gelidium*. These consist of repeated agarobiose (L- and D-galactose) subunits (Lee *et al.*, 2012). Agarose is prepared by the addition of an appropriate gel running buffer such as Tris-Acetate-EDTA (TAE) or Tris-Borate-EDTA (TBE) buffer. This same buffer should be used to run the gel. If the same buffer is not used, the current running through the gel will differ from that running through the buffer the gel is submerged in. This will in turn hinder the PCR products from migrating through the gel. During the gelation step the agarose polymers non-covalently associate forming a network of bundles. The pore sizes of the bundles determine the molecular sieving properties of the gel (Lee *et al.*, 2012). Agarose percentages used in gel electrophoresis range from 0.5 – 2.0%. The percentage used depends on the size of the DNA fragments that have to be separated. If the fragments are small a higher percentage will be used to better separate them. If the fragments on the other hand are large, a

lower percentage agarose gel will be prepared. The PCR product containing the amplified DNA is then loaded into the pre-cast wells of the gel and a tracking dye, such as bromophenol blue is added. The tracking dye is used to firstly sink the DNA into the wells due to its high density and secondly to visualize DNA migration progress through the gel as the solution containing the DNA is colourless (Lee *et al.*, 2012).

The phosphate backbone of the DNA molecule is negatively charged and when it is loaded into a gel that has a current, usually between 70 – 100 V, running through it the DNA will migrate through the gel towards the positive pole (Willey *et al.*, 2011h). Depending on the density of the gel, the different DNA fragments will be separated based on their size. The smaller fragments will move faster through the gel and will be found further along the gel. The bigger fragments will move a lot slower through the gel and will usually be found more to the top of the gel. To determine the size of the DNA fragments a marker is also included in the gel (Lee *et al.*, 2012).

Gels are generally stained with ethidium bromide. The reason for this is due to the fact that they are effective and relatively inexpensive. The biggest disadvantage is that ethidium bromide is considered to be carcinogenic and mutagenic making them harmful to work with (Sonmezoglu & Ozkay, 2015). Safer options such as SYBR Green are thus used to overcome this problem. Gels are stained to allow DNA bands on the gel to be visualized by UV transillumination. Due to SYBR Greens large fluorescence enhancement and its high quantum yield, when it is bound to double-stranded DNA, it can be used to detect dsDNA in the pico gram regions (Konschak & Tinhofer, 2011).

PCR inhibitors

PCR inhibitors are the most common cause of PCR failure even if there was enough DNA present. These substances could not only affect the sensitivity of the reaction but could also lead to false-negative results. The inhibitory effects of these substances could alter any component of the reaction from the DNA to the *Taq* polymerase (Alaeddini, 2012). Most PCR inhibitors are organic compounds such as ethanol, phenol, polysaccharides, bile salts, urea, sodium dodecyl sulphate (SDS) and humic acids. Proteins such as haemoglobin, myoglobin and collagen are also known PCR inhibitors. One mechanism of inhibiting PCR is during annealing where the inhibitor competes with the primers to bind to the DNA template (Schrader *et al.*, 2012). To prevent inhibitors in the reaction, the DNA extraction method must be adequate to remove them. Some of the most effective methods of removing inhibitors is the use of phenol-chloroform extractions to remove inhibitors such as lipids, activated carbon to remove inhibitory salts as well as DNA clean-up kits (Hu *et al.*, 2015; Schrader *et al.*, 2012).

Genes amplified for the detection of Escherichia coli

Conventional methods are not the only way to test for *Escherichia coli* in a sample. The use of molecular techniques such as PCR can reduce the analyses time and is also much more accurate,

provided it is done correctly. When using PCR to test for *E. coli,* the genes to be amplified are genes that are present in all *E. coli*. These genes are referred to as housekeeping genes (Wose Kinge *et al.*, 2012).

The first housekeeping gene that can be tested for is the malate dehydrogenase (*mdh)* gene. The malate dehydrogenase enzyme is required for the tricarboxylic acid (TCA) cycle. This enzyme is also involved in the noncyclic anaplerotic pathway of *E. coli* (Park *et al.*, 1995). The main purpose of this pathway is to replenish TCA cycle intermediates that have been depleted (Willey *et al.*, 2011a). During this pathway malate and oxaloacetate are interconverted leading to the oxidation of NADH or reduction of NAD (Park *et al.*, 1995).

As mentioned in the traditional methods section, it has been shown that *E. coli* display β-glucuronidase activity. The next housekeeping gene that can be tested for is the *uidA* gene that encodes for β-glucuronidase (Ud-Din & Wahid, 2014). Due to the fact that β-glucuronidase activity can be found in bacteria other than *E. coli* and that there have been reports of β-glucuronidase-negative *E. coli* strains, Molina *et al.* (2015) developed an alternative primer set for the *yaiO* gene.

The *yaiO* gene is an orphan gene belonging to *E. coli* and was used in the before mentioned study to determine whether it would select more specifically for *E. coli* than the *uidA* gene. The *uidA* gene primer set used for this study was designed by Bej *et al.* (1991). During a trial with 87 samples, 95% of the samples were correctly identified as *E.* coli with the *yaiO* gene while 87.5% of the samples were correctly identified using the *uidA* gene. Molina *et al.* (2015) thus proved that the orphan gene provides better specificity in detecting *E. coli* in the samples. The primer sets for the three genes mentioned above is displayed in Table 2.1.

Table 2.1 PCR primer sets for the detection of *E. coli* housekeeping genes

Primer Set	Sequence (5' – 3')	Product size (bp)	Source
mdh	F: GGTATGGATCGTTCCGACCT R: GGCAGAATGGTAACACCAGAGT	304	Tarr *et al.*, (2002)
uidA	F: TGGTAATTACCGACGAAAACGGC R: ACGCGTGGTTACAGTCTTGCG	162	Bej *et al.*, (1991)
yaiO	F: TGATTTCCGTGCGTCTGAATG R: ATGCTGCCGTAGCGTGTTTC	115	Molina *et al.*, (2015)

2.3.4 Pathogenic *Escherichia coli*

Pathogenic *E. coli* strains are classified into five groups based on the virulence determinants they possess. These include those that control invasion, adhesion, toxins, motility, genetic

characteristics as well as antiphagocytic surface structure (Meng *et al.*, 2013). The different groups of gastrointestinal pathogenic *E. coli* are discussed below.

Enterotoxigenic E. coli (ETEC)

Enterotoxigenic *E. coli* is a major cause of diarrhoea in infants in developing countries. They are also responsible for traveller's diarrhoea (Forsythe, 2010a). These strains resemble the bacterium *Vibrio cholerae* as they adhere to the proximal small intestine via surface fimbriae. ETEC do not produce symptoms by invading the mucosa but rather by producing one or both of the two different enterotoxins, namely heat-liable (LT) and heat-stable (ST) (Isidean *et al.*, 2011). These enterotoxins then induce fluid accumulation, which is responsible for the diarrhoea (Meng *et al.*, 2001). The structure and mode of action of the LT enterotoxin is similar to that of the cholera toxin whereas the ST enterotoxin causes increased levels of cyclic GMP in the host cell cytoplasm (Fleckenstein, 2013; Meng *et al.*, 2013).

Enteropathogenic E. coli (EPEC)

Enteropathogenic *E. coli* is mostly responsible for infantile diarrhoea that can also be watery (Hartl & Dykhuizen, 1984). Although EPEC causes diarrhoea, there is no blood present (Forsythe, 2010a). EPEC transmission is mostly via the faecal-oral route induced by contaminated foods and hands. During 1995 there were two EPEC outbreaks in Northern France associated with lettuce and prawn mayonnaise. The EPEC O111 strain was isolated from infected patients (Meng *et al.*, 2001). EPEC do not produce enterotoxins like ETEC but rather instigate attaching and effacing (A/E) lesions in cells resulting in adherence and invasion of the epithelial cells (Donnenberg *et al.*, 1997). Some of the major O serogroups that are associated with illnesses caused by EPEC include O86, O119, O126 as well as O128ab (Campos *et al.*, 2004).

Enteroinvasive E. coli (EIEC)

Enteroinvasive *E. coli* is also a cause of diarrhoea and dysentery such as the other pathogenic *E. coli* (Forsythe, 2010a). Although these strains are very closely related to *Shigella* spp. in terms of their biochemistry, pathogenicity and genetics, they differ in the sense that they do not produce Shiga-toxins. Haemolytic uremic syndrome (HUS) is not one of the complications associated with EIEC (Meng *et al.*, 2001; Ud-Din & Wahid, 2014). EIEC obtains its invasiveness by the presence of a large 140 MDa plasmid that encodes for numerous outer membrane proteins. The O antigen of EIEC and the antigenicity of the outer membrane proteins are closely related. Bacterial localization takes place in the colon where the epithelial cells are invaded and proliferated by EIEC. This then causes cell death on a large scale. The most common EIEC serotype is O124 (Meng *et al.*, 2013).

Enteroaggregative E. coli (EAEC)

Enteroaggregative *E. coli* differ from other types of pathogenic *E. coli* as they have the ability to produce a distinct pattern of aggregative adherence on Hep-2 cells. Their adherence to the Hep-2 cells appears as stacked bricks. These strains furthermore possess a toxin known as the enteroaggregative ST (EAST) toxin (Meng *et al.*, 2013; Hebbelstrup Jensen *et al.*, 2014). EAEC infections have symptoms including vomiting as well as diarrhoea that in some cases persist for up to 14 days (Forsythe, 2010a). A large outbreak of EAEC was reported in 2011 in Germany with sprouts being the vehicle of transmission. The total number of cases reported from this outbreak was 3 842 of which 855 developed HUS and more than 50 deaths were reported (Muniesa *et al.*, 2012). The strain that was associated with this outbreak was *E. coli* O104:H4, which produced the Stx2a toxin. Due to the fact that this strain produced a Shiga toxin, is was classified as a Shiga-toxin producing *E. coli* (STEC). However, when sequencing of the genome of the pathogen was done, it was found that it had a 93% homology with the 55589 EAEC strain. The strain also possessed the *aggR* gene that is found on the virulence plasmid of EAEC. Further genetic analyses also revealed that this O104:H4 strain had obtained its Stx producing ability via phage conversion (Meng *et al.*, 2013; Muniesa *et al.*, 2012). The mechanism used in this case was horizontal gene transfer, but more specifically the transduction process.

Transduction refers to the process by which DNA is transferred from one bacterium to another via a bacteriophage (Hartl & Jones, 1998). Bacteriophages are not able to multiply autonomously and therefore require a host cell that they can infect and take control of. This in effect forces the host cell to make copies of the bacteriophage instead of the bacterium (Willey *et al.*, 2011g). During the transduction process two types of bacteriophages can occur. These include virulent and temperate bacteriophages. After virulent bacteriophages enter the host cell they rapidly start to multiply until they reach a certain number of progeny phages. The progeny phages then cause the host cell to lyse; releasing them from the cell so they can infect more cells. This is known as the lytic cycle (Penades *et al.*, 2015). Temperate bacteriophages follow a different approach than that of the virulent bacteriophages. When they enter the host cell they do not kill it but rather insert their genome into the chromosome of the bacterial host. As the host bacterium is not killed, it replicates the viruses' genome as the bacteria replicates. This is known as the lysogenic cycle. These phages have the ability to stay inactive for many generations until they are exposed to stresses such as UV light. Upon exposure to stress they change from the lysogenic cycle to the lytic cycle and lyse the host cell (Penades *et al.*, 2015; Willey *et al.*, 2011g).

Enterohemorrhagic E. coli (EHEC)

In 1982, after the two outbreaks of haemorrhagic colitis, Enterohemorrhagic *E. coli* (EHEC) was recognised as the pathogen responsible for the incidences (Erdoğan *et al.*, 2008; O'Brien & Holmes, 1987; Riley *et al.*, 1983). It was during these outbreaks that EHEC was classified as a human pathogen. EHEC is also responsible for diarrhoea, bloody diarrhoea as well as haemolytic

uremic syndrome (HUS). This study will focus mainly on EHEC but more specifically a group of pathogenic *E. coli* known as Shiga-toxin producing *E. coli* that fall under the group EHEC. This will be further discussed in the section to follow.

2.3.5 Shiga-toxin producing *Escherichia coli* (STEC)

Background

Shiga-toxin producing *Escherichia coli* (STEC) also known as Verocytotoxigenic *E. coli* (VTEC) falls under the EHEC group of pathogenic *E. coli.* The most well know serotype of STEC is O157:H7. STEC causes an array of different diseases including bloody diarrhoea, haemorrhagic colitis, thrombotic thrombocytopaenic purpura (TTP) as well as the life threatening disease HUS (McClure, 2000; Gannon *et al.*, 1992).

STEC has the ability to produce two shiga toxins that are very similar to those produced by *Shigella dysenteriae*. The stx_1 and stx_2 genes encode for the two toxins respectively. Shiga toxin 1 is almost indistinguishable from the toxin that is produced by *Shigella dysenteriae* whereas shiga toxin 2 only has a 56% amino acid homology to shiga toxin 1 (O'Brien & Holmes, 1987; Mead & Griffin, 1998; Tarr *et al.*, 2005). In addition to the toxins that STEC produce, it also has the *eaeA* gene that encodes for the intimin protein. This protein is responsible for attaching and effacing lesions. The gene is encoded in the chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (Chekabab *et al.*, 2013; Fagan *et al.*, 1999).

One of the main reservoirs of STEC is cattle where the bacteria are transmitted to humans via undercooked meat or inadequately pasteurized dairy products (Chekabab *et al.*, 2013). STEC has also been isolated from other animals including goats, sheep, deer, dogs, birds, chickens, turkeys, horses and even flies (Mead & Griffin, 1998; Chekabab *et al.*, 2013). STEC is not limited to animals but can also occur on other products such as lettuce, unpasteurized apple juice, sprouts and water. This is usually due to cross-contamination from animal derived products or faeces (Acheson & Jaeger, 1999).

Due to the severity of STEC infections, much research has gone into detection methods over the years. The most widely used method for the detection of STEC is the use of PCR. Almost any product suspected of being contaminated with STEC can be tested provided the DNA extraction method is adequate in removing PCR inhibitors. This will be discussed further in a following section.

Different serotypes of STEC and related outbreaks

As previously mentioned, the most well known serotype of STEC is O157:H7, but this is not the only serotype. There are also a variety of non-O157 serotypes that are classified as STEC. Some of the non-O157 serotypes include O26, O45, O103, O111, O121 and O145 (Meng *et al.*, 2013). In Walkerton, Canada in May 2000, an outbreak related to drinking water contaminated with faeces

caused 2 300 people to become sick. *E. coli* O157:H7 was identified as being one of the main pathogens in this outbreak leading to seven deaths (Chekabab *et al.*, 2013). An outbreak in South Australia in 1995 linked to fermented meat caused 23 cases of HUS and lead to one death. The serotype responsible for this outbreak was determined to be a non-motile *E. coli* O111:NM (McClure, 2000). In a study by the Centres for Disease Control and Prevention (CDC), it was estimated that around 390 *E. coli* O157 outbreaks occurred in the United States between 2003 and 2012. These outbreaks included 4 928 illnesses, 1 272 hospitalization and a total of 33 deaths. It was also estimated that there were 299 cases of HUS (Zuraw, 2015).

Mechanism of infection

STEC has an incubation period of 2 – 5 days (Acheson & Jaeger, 1999; Karmali, 2004). The infective dose required to cause illness may be as low as a 100 cfu (Welinder-Olsson & Kaijser, 2005). After ingestion of food that had been contaminated with the STEC organism they start to colonize in parts of the lower intestine. Here they produce attaching/effacing (A/E) lesions whereby the bacteria can adhere to epithelial cells and induce destruction of brush border villi (Mele *et al.*, 2014). The development of these A/E lesions requires the *eaeA* gene that is located on the locus of enterocyte effacement (LEE) pathogenicity island. The *eaeA* gene encodes for the outer membrane adhesion protein intimin which further encodes for the *tir* (translocated intimin receptor) gene (Acheson & Jaeger, 1999; Karmali, 2004). Once the bacteria have adhered to the intestinal epithelial cells they start producing and releasing the shiga toxins into the gut lumen. This usually occurs within a few days of colonization. Although the exact mechanism of how the toxins reaches the target organs are not clearly understood, it is assumed that blood cells such as erythrocytes, monocytes and platelets serve as Stx carriers. These carriers then transport the toxins to the target organs via the blood stream (Mele *et al.*, 2014). The toxins can then bind to the target cells.

This binding process is mediated by B-subunits via interaction with the globotriaosylceramide (Gb_3) located on the endothelium (Mele *et al.*, 2014; Meng *et al.*, 2013). Inside the cells these toxins have the ability to cleave a single adenine base from the 28S rRNA within the ribosomal subunit. During this irreversible process the ribosome is rendered defective, inhibiting protein synthesis causing cell death (Obrig, 2010; Welinder-Olsson & Kaijser, 2005). Without the Gb_3 receptor the toxins cannot bind to the target cells to cause cell death. This makes cattle the perfect vehicle of transmission, as they do not have this receptor (Connolly *et al.*, 2015).

In recent years a new subtilase cytotoxin encoded by the *subAB* gene has been detected (Tozzoli *et al.*, 2010). LEE-negative STEC usually produce these toxins. This means they do not encode for the *eaeA* gene required for attaching/effacing lesions. Instead of the *eaeA* gene, they possess the STEC autoagglutinating adhesion (Saa) plasmid encoding for the *saa* gene. The *saa* gene is the LEE-negative alternative to the *eaeA* gene, functioning as the attaching/effacing mechanism (Sánchez *et al.*, 2012; Sánchez *et al.*, 2013; Hoang Minh *et al.*, 2015; Nüesch-Inderbinen *et al.*, 2015).

2.3.6 Molecular detection of STEC

DNA extraction methods

There are several different DNA extraction protocols that can be used to extract DNA from faecal and meat samples. The two most widely used techniques will be briefly explained in this section.

The first method is the boiling method using a lysis buffer. During this method DNA can be extracted from either a pure culture on a plate or from a culture that was grown in an enrichment medium. If DNA is extracted from a plate, a 1 cm sweep of growth is suspended in 300 µl of lysis buffer (Reischl *et al.*, 2002). If DNA is extracted from an enrichment medium, 200 µl of the medium is transferred to a sterile 1.5 ml centrifuge tube and centrifuged for 3 min at 14 000 x g where after the supernatant is discarded and the pellet re-suspended in 300 µl lysis buffer (Gilmour *et al.*, 2009). The lysis buffer is composed of 1 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl (pH 8.0) and 0.5% Tween 20. The buffer suspension is then heated to ± 100°C for 10 min. After boiling, the suspension it is centrifuged at 1 000 x g for 2 min to allow all the bacterial cell walls and other PCR inhibitors to collect at the bottom. The supernatant containing the DNA can then be collected (Reischl *et al.*, 2002).

The second method is phenol/chloroform extraction. One millilitre of the enrichment that had been incubated overnight (37°C) is centrifuged for 2 min at 13 000 rpm where after the pellet is re-suspended in an equal volume of Tris-EDTA buffer. In addition to the buffer, 100 µl of lysozyme solution, 100 µl of proteinase K enzyme and 1% sodium dodecyl sulphate (SDS) is added (Shawish *et al.*, 2014). This mixture is then incubated at 37°C for 30 min where after the DNA lysate is extracted with a mixture of chloroform and isoamyl alcohol in a 24:1 ratio (Brian *et al.*, 1992). Further extraction with a mixture of phenol, chloroform and isoamyl alcohol in a 25:24:1 ratio is then performed (Shawish *et al.*, 2014). The precipitated DNA is then washed with 70% ethanol and dissolved in Tris-EDTA buffer (Bej *et al.*, 1991).

Genes associated with STEC, PCR primers and conditions

As previously mentioned the three most important genes used to test for the presence of STEC are the *stx*$_1$, *stx*$_2$ and *eaeA* genes. In addition to these genes, the *subAB* and *saa* genes were also included. The primer sequences of these five genes can be seen in Table 2.2.

Table 2.2 PCR primer sets for the detection of STEC

Primer Set	Sequence (5' – 3')	Product size (bp)	Source
stx1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATG	614	Gannon *et al.*, (1992)
stx2	F: CCATGACAACGGACAGCAGTT R: CCTGTCAACTGAGCACTTTG	779	Gannon *et al.*, (1992)
eaeA	F: GTGGCGAATACTGGCGAGACT R: CCCCATTCTTTTTCACCGTCG	890	Gannon *et al.*, (1997)
saa	F: CGTGATGAACAGGCTATTGC R: ATGGACATGCCTGTGGCAAC	119	Paton & Paton, (2002)
subAB	F: CTT CCC TCA TTG CCT CAC G R: GGC TGG CCT GTT GTG TAA A	1066	Funk *et al.*, (2013)

When determining the conditions at which a PCR has to be performed, one of the most important variables that have to be taken into account is the annealing temperature (T_a) of the primers. If the annealing temperature is too low it will lead to the amplification of non-specific DNA fragments. This will in turn lead to multiple bands on the agarose gel. If the annealing temperature on the other hand is too high, the purity and the yield of the desired products are reduced. Also, the annealing temperature should always be lower than the melting temperature (T_m) of the primers. When performing a multiplex PCR such as the one for STEC, the least stable primer set's melting temperature must be used to calculate the annealing temperature for the reaction (Rychlik *et al.*, 1990).

A study by Gannon *et al.* (1992) where a PCR reaction was done to amplify the stx_1 and stx_2 genes, the following conditions were used. The reaction consisted of 35 cycles of 94°C for 1 min followed by 60°C for 1 min and finally 72°C for 2 min. A final extension of 72°C for 5 min ended the reaction. The annealing temperature used in this study was thus 60°C. When Gannon *et al.* (1997) performed a similar study a few years later, the *eaeA* gene was added to the PCR reaction. Due to the additional primer set that was added, the reaction conditions had to be adapted. The reaction consisted of 35 cycles of 94°C for 15 s followed by 65°C for 15 s and finally 72°C for 75 s. A final extension of 72°C for 5 min ended the reaction. It can thus be noted that the annealing temperature changed from 60°C to 65°C to accommodate the additional primer set.

2.4 MICROBIAL INTERVENTIONS

As discussed earlier, the harvesting process of game animals can introduce microbial contamination onto the meat. Decreasing the microbial load on carcasses is thus an integral part of ensuring that the meat is safe for consumption as well as to decrease the possibility of spoilage.

Two of the most commonly used interventions include hot water washes and steaming of the carcasses.

The use of hot water is readily used in meat processing plants to remove contaminants such as soil, faecal matter, hairs and any other environmental contaminants (Loretz *et al.*, 2011). For the hot water washes to be effective the water has to be heated to at least 75°C (Pipek *et al.*, 2005). Hot water treatments can be applied to the hide to remove as much contaminants before skinning, which will inherently decrease the amount of cross contamination from the hide to the carcass. Hot water treatments can also be applied to the carcass after skinning to remove any contaminants that could have been carried over during the skinning process. Another possibility is the use of a combination of the two as this will lead to maximum reduction of contaminants (Small *et al.*, 2005; Sofos & Smith, 1998). Gill *et al.* (1995) showed a 2 log reduction of spoilage bacteria on pig carcasses that had been treated with 85°C water for 20 s. One thing to keep in mind is that hot water washes could also cause heat damage to the meat if the treatment time is too long (Pipek *et al.*, 2005).

Steam pasteurisation of carcasses is another effective way of reducing the microbial load that may be present on carcasses. Steam pasteurisation involves exposing carcasses to steam at 82 – 97°C inside a chamber for approximately 6 – 12 s (Aymerich *et al.*, 2008). The advantage steam pasteurisation has over hot water washes is that there is a large amount of heat that is being transferred to the carcass, rapidly increasing the surface temperature (James *et al.*, 2000). In a study on beef carcasses where steam at 93.3°C was applied for just over 6 s, a reduction of 1 log cfu·cm^{-2} was reported for *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria innocua* (Aymerich *et al.*, 2008).

The two interventions discussed above have been shown to work in an abattoir setting but will not be practically viable to use in the field on game carcasses on an open wound and/or on the exsanguination and evisceration cuts. An intervention is required that is inexpensive to produce, requires little to no equipment, must be easy to use as well as be generally regarded as safe (GRAS). The use of chemical agents such as organic acids or mixtures thereof possess great potential to decontaminate carcasses and reduce microbial growth. The following section will explore the use of organic acids as microbial intervention for in-field use.

2.5 ORGANIC ACID SPRAY

2.5.1 Cell wall structure background

The cell wall structure of Gram-positive and Gram-negative bacteria greatly differ from one another and might thus respond differently to environmental stresses such as exposure to organic acids. The following section will discuss these differences in cell wall structure.

Gram-positive bacteria

Some of the most common examples of Gram-positive bacteria include *Staphylococcus, Bacillus, Streptococcus* and *Listeria*. Gram-positive bacteria have thick cell walls composed mainly of peptidoglycan (Malanovic & Lohner, 2016). In addition to the peptidoglycan, the cell walls also contains high amounts of teichoic acid, which is a secondary cell wall polymer. Teichoic acid is a polymer made up of ribitol or glycerol bound by phosphate groups. These acids are bound to either peptidoglycan or plasma membrane lipids via covalent bonds (Willey *et al.*, 2011b). These acids also give Gram-positive bacteria their negative charge as teichoic acid is negatively charged (Malanovic & Lohner, 2016). Teichoic acid is only found in Gram-positive bacteria and not in Gram-negative bacteria. One of the main functions of teichoic acid is to create and maintain the cell envelope structure and to protect the cell against substances in the environment that may be harmful to the cell (Lambert *et al.*, 1977). Further functions include the regulation of cell division, invasion of the host's tissue as well as autolysis regulation (Hanson & Neely, 2012). Between the cell wall and the plasma membrane lies the periplasmic space. Interactions of the cell with its environment are mediated by proteins that are located on the surface of the peptidoglycan of most Gram-positive bacteria (Willey *et al.*, 2011b).

Gram-negative bacteria

Some of the most common examples of Gram-negative bacteria include *Salmonella, Escherichia coli,* Coliforms, *Pseudomonas* and *Shigella*. Unlike Gram-positive bacteria, the cell walls of Gram-negative bacteria are much more complex. Gram-negative cell walls have a thin peptidoglycan layer which is situated next to the plasma membrane and is bound by the periplasmic space on both sides (Neyen & Lemaitre, 2016). The periplasmic space of these cells is also much larger than that of Gram-positive cell walls. The second difference between Gram-positive and Gram-negative cell walls is the presence of an outer membrane in Gram-negative cell walls (Willey *et al.*, 2011b). The thin peptidoglycan layer is bound to the outer membrane via Braun's lipoproteins. Braun's lipoproteins are embedded in the outer membrane and covalently bound to the peptidoglycan layer beneath it (Nakayama *et al.*, 2012). Another component of the outer membrane is the lipopolysaccharides (LPSs). These complex molecules are made up of a lipid as well as a polysaccharide. Lipopolysaccharides are an important constituent of the cell wall as they have many functions. They stabilize the outer membrane structure and also contribute to the negative charge on the cell surface (Willey *et al.*, 2011b). LPS further creates a permeability barrier restricting the uptake of any toxic substances that could harm the cell. The LPS also protect pathogenic bacteria from host defences (Beveridge, 1999).

2.5.2 Organic acids

The use of organic acids for decontamination of meat and carcasses is a widely used practice in abattoirs and the meat industry (Mani-López *et al.*, 2012). Although research on the use of these

acids on game species is lacking, there is abundant research on other farm-raised animals such as cattle, pigs and chickens. Table 2.3 depicts some of the most common organic acids used on meat surfaces and their efficacy on some of the most common foodborne pathogens. One important aspect to take into consideration is the temperature at which the organic acid mixture is applied to the carcass. The higher the temperature, the greater the log reduction achieved (Mani-López *et al.*, 2012).

Table 2.3 Different organic acids used against common foodborne pathogens and their respective log reductions

Organic acid	Microorganism	Reduction (Log CFU)	Concentration	Temperature (°C)	Reference
Acetic acid	Aerobic bacteria	1.8 – 4.3/cm^2	1%	NA	Hamby *et al.*, (1987)
		2.5/cm^2	2%	40	Cutter, (1999)
	E. coli O157:H7	1.28/g	2%	NA	Raftari *et al.*, (2009)
		3.2/cm^2	2%	40	Cutter, (1999)
	Staphylococcus aureus	1.58/g	2%	NA	Raftari *et al.*, (2009)
	Coliforms	0.8/cm^2	2.5%	NA	Algino *et al.*, (2007)
	Salmonella typhimurium	0.5 – 0.8/cm^2	2%	NA	Dickson, (1992)
Lactic acid	Aerobic bacteria	3.0 – 3.3/100 cm^2	4%	55	Castillo *et al.*, (2001a)
	E. coli O157:H7	1.35/g	2%	NA	Raftari *et al.*, (2009)
		4.0 – 4.8/cm^2	4%	55 – 65	Castillo *et al.*, (2001b)
	Staphylococcus aureus	1.69/g	2%	NA	Raftari *et al.*, (2009)
	Coliforms	1.8/cm^2	1.5%	25	Barboza de Martinez *et al.*, (2002)
	Salmonella typhimurium	3.4/cm^2	4%	55	King *et al.*, (2005)
Citric acid	*E. coli* O157:H7	1.2 – 1.8/cm^2	1 – 5%	24	Cutter & Siragusa, (1994)
	Salmonella typhimurium	1.9	4%	NA	Mani-López *et al.*, (2012)
Propionic acid	*E. coli* O157:H7	1.17/g	2%	NA	Raftari *et al.*, (2009)
	Staphylococcus aureus	1.45/g	2%	NA	Raftari *et al.*, (2009)

2.5.3 Effect of organic acids on microorganisms

When organic acids are introduced into the environment surrounding bacterial cells, their main action against the bacterial cell is to disrupt the proton motive force (PMF). The PMF plays an integral part in the survival of the cell (Willey *et al.*, 2011d). When looking at the electron transport chain, the PMF is generated at the final electron acceptor (Simon *et al.*, 2008). The potential energy generated here is then used to synthesize ATP from ADP and P_i. The PMF also functions as a transport mechanism transporting molecules directly into the cell without the use of ATP (Montville & Bruno, 1994). Another important function of the PMF is to rotate the bacterial flagellar to allow the cell to move (Mitsui & Ohshima, 2005). It can thus be noted that the PMF plays very important roles in the cell's physiology. The following section will look into how organic acids disrupt the PMF.

There are two primary mechanisms by which organic acids can influence the microbial activity of the bacterial cell. The first mechanism is via acidification of the cytoplasm thus leading to the interference of the energy production system and secondly, via the accumulation of dissociated acid anions till it reaches a toxic level (Mani-López *et al.*, 2012). A transmembrane gradient can be established when the pH of the environment is lower than that of the cellular cytoplasm. This occurs when undissociated acids diffuse through the microbial membrane. When these protonated acids diffuse through the microbial membrane it creates an alkaline environment that in turn leads to the dissociation of the acid into an acid anion and a free proton. The cell then tries to efflux these protons by exchanging them for other cations such as sodium or potassium (Mani-López *et al.*, 2012). It is further believed that the cell has to make use of active transport to efflux these protons, as the microbial membrane is impermeable to protons (Harold, 1972). This action is important to ensure the cellular interior maintains pH homeostasis (Brul & Coote, 1999).

The accumulation of the dissociated acid anions leads to the shifting of the internal pH to a range where it is no longer optimal for enzymatic activity. It also negatively affects protein synthesis as well as DNA/RNA synthesis. Further accumulation of the acid anion creates the driving force for cell inhibition as they ultimately hinder the PMF. This in turn inhibits the bacteria's ability to re-alkalinize its cytoplasm leading to the death of the cell (Mani-López *et al.*, 2012; Willey *et al.*, 2011d).

Gram-positive bacteria, such as *Staphylococcus aureus,* have the ability to combat the effects of the organic acids by increasing their internal pH. Rode *et al.* (2010) found that when lactic acid treatments were administered to *S. aureus* cells, they were able to increase the pH of their external environment and in doing so also increase their own internal pH. It is believed that they metabolize the acids, producing diacetyl (2,3-butanedione), ammonia and pyrazine. When headspace GC-MS analysis of the lactic acid stressed *S. aureus* was performed, a 10-fold increase in the amount of pyrazine was detected after a 20 h period.

2.6 OUTLINE OF RESEARCH CHAPTERS

From the literature review it is clear that information on the bacteria found on game carcases hunted in South Africa is scarce and warrants further research. On a more practical level, the discussion above highlighted the challenges faced by hunters in the field to attempt to minimise bacterial contamination on the carcasses and meat. It was therefore decided to initiate research into these factors. Therefore the following section will briefly discuss the outline of each of the four research chapters for this study.

The first research chapter will be based on the methods and techniques used in the optimisation of the rapid molecular method for the detection of Shiga-toxin producing *Escherichia coli.* These include the optimisation of a DNA extraction technique to extract DNA from a positive control; optimizing the PCR conditions, PCR master mix and gel electrophoresis conditions.

The second research chapter will explore the incidences of STEC in Game Meat. Meat and faecal samples will be taken from game species and tested with the methods optimised in research chapter one to determine whether Shiga-toxin producing *Escherichia coli* are present in the intestines as well as on the meat of South African game species.

The third research chapter will entitle the identification and quantification of the microbes found in and on game species. Analyses for *Escherichia coli, Staphylococcus* aureus and total coliforms will be performed using the Tempo system from bioMérieux. Testing for *Listeria* and *Salmonella* will be performed using PCR and aerobic count will be analysed using Petrifilm.

The first part of the fourth research chapter will focus on the methods used in the development of an organic acid spray. Different organic acids will be researched to determine their efficacy on decontamination of carcasses. All trials for this part of the chapter will be performed on laboratory scale.

The second part of this chapter will look into the efficacy of the organic acid mixture. The spray will be applied to carcasses after they have been dressed. All analyses will be performed using the Tempo system except for aerobic count that will be done on Petrifilm.

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CHAPTER 3
OPTIMISATION OF A RAPID MOLECULAR METHOD FOR THE DETECTION OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI*

3.1 ABSTRACT

DNA extraction optimisation as well as PCR optimisation for the detection of the three virulence genes (*stx*$_1$, *stx*$_2$ and *eaeA*) associated with Shiga-toxin producing *Escherichia coli* (STEC) were performed. The most common factor that can hinder the PCR amplification process is the presence of PCR inhibitors, which is directly related to the purity of the template DNA. Four DNA extraction trials were performed, each one improving on the previous. The first DNA extraction trial led to DNA contaminated with inhibitors. The implementation of an ethanol wash rectified this problem. The final DNA extraction method proved to be adequate in extracting pure DNA from the STEC positive control but inadequate in extracting PCR inhibitor free DNA from faecal samples. The ZR Fecal DNA MiniPrep kit was thus chosen as an alternative. Different primer concentrations and PCR conditions were also evaluated to determine the optimum conditions for the amplification process. The final DNA extraction method chosen for the positive control was the DNA extraction with an ethanol wash as well as a re-suspension in sterile distilled water.

3.2 INTRODUCTION

Shiga-toxin producing *Escherichia coli* (STEC) are a group of pathogenic *E. coli* that form part of the Enterohemorrhagic *E. coli* (EHEC) group. *E. coli* O157:H7 is the most well-known serotype of STEC. STEC are defined as *E. coli* that produce at least one of the two shiga-toxins and are not serotype specific. They can include both O157 and non-O157 serotypes (Meng *et al.*, 2013). STEC have the ability to produce two shiga-toxins, namely *stx*$_1$ and *stx*$_2$. In addition to the toxin genes they may also possess the *eaeA* gene that encodes for the intimin protein that is required for attaching and effacing lesions (Tarr *et al.*, 2005; Chekabab *et al.*, 2013). After two outbreaks of haemorrhagic colitis in 1982, EHEC was recognized as the responsible pathogen (Viazis & Diez-Gonzalez, 2011). Since the first outbreak much research was done in order to detect STEC, especially the virulence genes, from an array of different sources. The most widely used method for the detection of these genes is the use of Polymerase Chain Reaction (PCR) (Kulp, 2014; Elnifro *et al.*, 2000). During a normal PCR only one gene is tested for but in the case where three different genes need to be tested, a multiplex PCR is performed. As there are many components that can hinder the PCR reaction, some optimisation is required for each individual sample type (Henegariu *et al.*, 1997).

The most important component of the PCR reaction, namely the template DNA containing the genes to be amplified, can in many cases be the downfall of the reaction. This happens when the DNA is not pure enough; meaning it still contains some PCR inhibitors. Therefore, the DNA extraction process first has to be optimised to ensure that no inhibitors are present in the final DNA intended for PCR (Alaeddini, 2012). This optimisation in itself can be quite difficult depending on the source from where the DNA is to be extracted. In this study faecal samples will be the main source from where DNA will be extracted. Faecal matter contains many PCR inhibitors such as heparin, polysaccharides, bile salts as well as bilirubins (Trochimchuk *et al.*, 2003). Another factor that complicates the matter is the presence of non-specific DNA from other faecal microbial flora present in samples (Plain *et al.*, 2014). If these PCR inhibitors are not effectively removed during the DNA extraction process they could compete with the primers during the annealing phase of the PCR and reduce the efficiency of the amplification process (Schrader *et al.*, 2012).

After optimisation of the DNA extraction process, the PCR reaction composition and conditions have to be optimised to ensure effective amplification of the target genes. Optimizing a multiplex PCR can prove difficult due to the fact that there is more than one primer set present in the reaction (Elnifro *et al.*, 2000). This can be problematic for two reasons. The first is that the total primer concentration in the reaction will be relatively high which could lead to primers annealing to one another instead of the DNA template leading to the formation of primer-dimers (Brownie *et al.*, 1997). When primers anneal to one another they consume some of the reaction components, such as the *Taq* polymerase, in effect decreasing the amount remaining for amplification of the target genes (Elnifro *et al.*, 2000). Secondly, every primer set has its own optimum annealing temperature. When combining more than one primer set, or three in the case of this study, a combined optimum annealing temperature must be determined. If the chosen annealing temperature is higher than the melting temperature (T_m) of the lowest primer set, those primers will not anneal to the template DNA leading to false negative results (Ran *et al.*, 2008). On the other hand, if the annealing temperature is too low, it could lead to non-specific binding causing the formation of primer-dimers (Anonymous, 2016).

The aims of this study was to firstly optimise a DNA extraction protocol for the isolation of the STEC positive control as well as to determine whether this protocol will be able to extract inhibitor free DNA from faecal samples. The second aim of this study was to optimise the PCR conditions and reaction composition for the amplification of the *stx*$_1$, *stx*$_2$ and *eaeA* virulence genes.

3.3 MATERIALS AND METHODS

3.3.1 Culture Preparation

A pure *E. coli* O157:H7 culture was obtained from the Department of Microbiology, Stellenbosch University and used to prepare stock cultures. Stock cultures were suspended in Luria Bertani Broth (LB-broth) (Biolab, Merck, South Africa) for enrichment DNA extractions. Stock cultures were

also streaked onto Tryptone Soy Agar (TSA) (Biolab, Merck, South Africa) for pure culture DNA extractions. Both LB enrichments and TSA plates were incubated at 37°C for 24 h prior to DNA extraction.

3.3.2 DNA Extraction

All DNA extraction trials were done from pure cultures on TSA as well as from enrichments in LB-broth.

DNA Extraction Trial 1 (DNA-T1)

One millilitre of LB-broth enriched culture was transferred to a sterile 1.5 mL Eppendorf tube. Samples were centrifuged at 13 000 x g for 2 min where after the supernatant was discarded. The pellet was suspended in 300 µL lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5% TWEEN 20, 1 mM EDTA). For DNA extracted from pure cultures, 300 µL lysis buffer was added to a sterile 1.5 mL Eppendorf tube and one loop full of culture from the TSA plate was suspended in the lysis buffer. The samples were boiled for 10 min at 100°C. After boiling, samples were centrifuged at 1 000 x g for 2 min and 250 µL of the supernatant containing the DNA was transferred to a sterile 1.5 mL Eppendorf tube. DNA samples were held on ice until use or frozen at -20°C for later use.

DNA Extraction Trial 2 (DNA-T2)

One millilitre of LB-broth enriched culture was transferred to a sterile 1.5 mL Eppendorf tube. Samples were centrifuged at 13 000 x g for 2 min where after the supernatant was discarded. The pellet was suspended in 300 µL lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5 % TWEEN 20, 1 mM EDTA). For DNA extracted from pure cultures, 300 µL lysis buffer was added to a sterile 1.5 mL Eppendorf tube and one loop full of culture from the TSA plate was suspended in the lysis buffer. The samples were boiled for 10 min at 100°C and centrifuged at 1 000 x g for 2 min. After centrifuging, 250 µL of the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and 250 µL of ice cold 99.9% ethanol was added to the tube. Samples were then centrifuged at 13 000 x g for 1 min and the supernatant was discarded. The tubes were allowed to dry for 45 – 60 min where after the dried pellet was suspended in either 100 µL sterile distilled H_2O or 100 µL 10 mM Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA). DNA samples were held on ice until use or frozen at -20°C for later use.

DNA Extraction Trial 3 (DNA-T3)

One millilitre of LB-broth enriched culture was transferred to a sterile 1.5 mL Eppendorf tube. Samples were centrifuged at 13 000 x g for 2 min where after the supernatant was discarded. The pellet was suspended in 300 µL lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5% TWEEN 20, 1 mM EDTA). For DNA extracted from pure cultures, 300 µL lysis buffer was added to a sterile 1.5 mL Eppendorf tube and one loop full of culture from the TSA plate was suspended in the lysis

buffer. The samples were boiled for 10 min at 100°C and centrifuged at 1 000 x g for 2 min. After centrifuging, 250 µL of the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and 250 µL of ice cold 99.9% ethanol was added to the tube. Samples were then centrifuged at 13 000 x g for 1 min and the supernatant was discarded. The tubes were allowed to dry for 45 – 60 min where after the dried pellet was suspended in 100 µL sterile distilled H_2O. DNA samples were held on ice until use or frozen at -20°C for later use.

DNA Extraction Trial 4 (DNA-T4)

An additional DNA extraction method developed by Altalhi & Hassan (2009) was included to compare with the method described in DNA-T3. DNA-T1 and DNA-T2 did not form part of this comparative study. One colony from a TSA plate was transferred to a sterile 1.5 mL Eppendorf tube containing 100 µL of sterile distilled H_2O. The sample was boiled for 13 min and centrifuged at 14 000 x g for 15 min. After centrifuging, the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and held on ice until use or frozen at -20°C for later use.

3.3.3 PCR Primers

Table 3.1 represents the three primer sets that were used in the multiplex PCR for the detection of the three virulence genes.

Table 3.1 STEC PCR Primers sequences used for the multiplex PCR

Primer	Primer Sequence (5'-3')	Fragment size (Bases)	Reference
***stx*$_1$**	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	614	Gannon *et al.*, (1992)
***stx*$_2$**	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA GCA CTT TG	779	Gannon *et al.*, (1992)
eaeA	F: GTG GCG AAT ACT GGC GAG ACT R: CCC CAT TCT TTT TCA CCG TCG	890	Gannon *et al.*, (1993)

3.3.4 Reaction mixture composition

Reaction Composition Trial 1 (RC-T1)

PCR amplification of extracted DNA was performed in 50 µL volumes consisting of 1X One *Taq* standard reaction buffer (New England BioLabs Inc), 2 µL template DNA, 1 µM of each primer (Inqaba Biotec), 200 µM of each dATP, dCTP, dGTP and dTTP (New England BioLabs Inc) and 1 U One *Taq* DNA polymerase (New England BioLabs Inc).

Reaction Composition Trial 2 (RC-T2)

PCR amplification of extracted DNA was performed in 50 µL volumes consisting of 1X One *Taq* standard reaction buffer (New England BioLabs Inc), 2 µL template DNA, 0.2 µM of each primer (Inqaba Biotec), 200 µM of each dATP, dCTP, dGTP and dTTP (New England BioLabs Inc) and 1 U One *Taq* DNA polymerase (New England BioLabs Inc).

3.3.5 PCR Conditions

PCR Conditions Trial 1 (PCR-T1)

The PCR conditions followed was an initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s and 72°C for 90 s and ending with a final extension step at 72°C for 5 min (Fagan *et al.*, 1999). PCR was performed with a Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa).

PCR Conditions Trial 2 (PCR-T2)

The PCR conditions followed was an initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 20 s, 48°C for 40 s and 72°C for 90 s and ending with a final extension step at 72°C for 5 min. PCR was performed with a Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa).

PCR Conditions Trial 3 (PCR-T3)

The PCR conditions followed was an initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 15 s, 55°C for 30 s and 68°C for 30 s and ending with a final extension step at 72°C for 5 min (Lamprecht *et al.*, 2014). PCR was performed with a Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa).

3.3.6 Gel Electrophoresis

Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain (Lab Supply Mall, InnoVita Inc). Gels were run for 100 min at 70 V. A 100 bp DNA Ladder (New England BioLabs Inc) was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) in combination with the Image Lab Software (version 5.2.1).

3.3.7 Comparison of DNA extraction methods and PCR conditions

The purpose of this experiment was to determine which DNA extraction method and PCR condition combinations produced the optimum results.

Sample Preparation

E. coli O157:H7 stock culture was streaked onto TSA. The stock culture was further suspended in LB-broth. The plates and enrichments were incubated at 37°C for 24 h.

DNA Extraction

DNA was extracted from the pure culture TSA plate as well as the LB-broth enrichment using the DNA extraction method described in DNA-T3. DNA was further extracted from the pure culture TSA plate using the DNA extraction method described in DNA-T4.

PCR Conditions

The PCR conditions of PCR-T1, PCR-T2 as well as PCR-T3 were used in this experiment. All DNA extraction samples were exposed to the three PCR conditions. The PCR reaction mixture composition used was as described in RC-T2. Table 3.2 depicts the different DNA extraction and PCR conditions combinations.

Table 3.2 Description of DNA extraction methods and PCR conditions combinations used for each sample

Sample Number	DNA extraction method	PCR Conditions
1	DNA-T3 – Pure culture	
2	DNA-T3 – Enrichment	PCR-T1
3	DNA-T4 – Pure Culture	
4	DNA-T3 – Pure culture	
5	DNA-T3 – Enrichment	PCR-T2
6	DNA-T4 – Pure Culture	
7	DNA-T3 – Pure culture	
8	DNA-T3 – Enrichment	PCR-T3
9	DNA-T4 – Pure Culture	

Gel Electrophoresis

Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain. Gels were run for 100 min at 70 V. A 100 bp DNA Ladder was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System in combination with the Image Lab Software.

3.3.8 Finalizing Method

Sample Preparation

E. coli O157:H7 stock culture was streaked onto TSA. After incubation at 37°C for 24 h, two loops full of culture was suspended in 200 µL sterilised distilled water. Five 22.5 mL LB-broths were prepared in 50 mL grinder tubes of which the second to fifth tubes received 2.5 g of Fellow Deer

(*Dama dama)* faecal sample before sterilisation. These were artificially contaminated with different concentrations of *E. coli* O157:H7 prepared earlier (Table 3.3). The first grinder only received the faecal sample after sterilisation and received no artificial contamination. All five samples were incubated at 37°C for 24 h.

Table 3.3 Amount of artificial contamination of each sample

Sample Number	Amount of artificial contamination (µL)
FD1	0.00
FD2	12.50
FD3	6.30
FD4	3.20
FD5	1.60

DNA Extraction

DNA was extracted from the pure culture TSA plate using the method described in DNA-T3. This served as the positive control. DNA was extracted from all five samples (FD1 – FD5) using the ZR Fecal DNA MiniPrep kit (Zymo Research, Inqaba Biotec, South Africa). DNA was further extracted from FD2 and FD5 using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc, Biocom, South Africa).

PCR Conditions

The PCR conditions of PCR-T3 were used in this experiment. The PCR reaction mixture composition used was as described in RC-T2. PCR was performed on the positive control, all samples as well as a negative water control.

Gel Electrophoresis

Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain. Gels were run for 100 min at 70 V. A 100 bp DNA Ladder was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System in combination with the Image Lab Software.

3.4 RESULTS AND DISCUSSION

3.4.1 DNA Extraction

Four different DNA extraction trials were performed to determine which extraction produced the cleanest DNA to be used for PCR. DNA-T1 produced DNA that resulted in DNA smears on the gel.

During DNA-T2 an ethanol wash was incorporated to precipitate the DNA in the mixture and to suspend all other components that could possibly act as PCR inhibitors (Bej *et al.*, 1991). The ethanol wash was sufficient in removing PCR inhibitors from the DNA. During this trial, the DNA that was suspended in TE-Buffer produced no bands on the gel. The possible reason for this could be that the TE-Buffer contained EDTA, which is known for its inhibitory effects on PCR amplification, as EDTA causes chelation of magnesium ions (Schrader *et al.*, 2012). Magnesium is an integral part of the reaction as it helps with the annealing of the primers to the DNA strands. When the EDTA chelates with the magnesium ions, they are unavailable to be used by the primers, in turn leading to poor amplification of the DNA (Rossen *et al.*, 1992; Khosravinia & Ramesha, 2007). The DNA was secondly suspended in sterilised distilled water (DNA-T3). This suspension medium produced acceptable results in terms of PCR products. Distinct bands appeared on the gels that were run with these samples. This was chosen as the final extraction method as described in DNA-T3. The results of the method described in DNA-T4 will be discussed in a later section.

3.4.2 Reaction mixture composition

During this study two different reaction mixture compositions were tested to determine which produced the best PCR products. The composition used in RC-T1 resulted in the formation of primer-dimers suggesting the primer concentration used was too high. Brownie *et al.* (1997) noted that high primer concentrations, especially in multiplex PCR where there are more than two primers, will likely cause primer-dimers. During the second trial (RC-T2), the primer concentration was lowered from 1 µM to 0.2 µM. The lowered concentration produced better bands on the gel with little to no primer-dimers. The reaction composition of RC-T2 was therefore chosen as the final composition for all further experiments.

3.4.3 Comparison of extraction methods and PCR conditions

Optimum PCR amplification was tested using different DNA extraction methods (DNA-T3 or DNA-T4) in combination with different PCR conditions (PCR-T1, PCR-T2 or PCR-T3). When observing lanes 2 – 4 (Figure 3.1) depicting the PCR products obtained from PCR-T1 it is clear that DNA-T3 from a pure culture (lane 2) had the highest intensity bands followed by DNA-T3 from enrichment (lane 3) with lower intensity bands. Lane 4, representing DNA-T4, produced no visible bands suggesting that the DNA extraction method was either inadequate in extracting DNA from the sample or that the DNA could possibly have still contained PCR inhibitors that influenced the PCR amplification process (Alaeddini, 2012). As this extraction method is somewhat of a crude extraction method without an additional washing step, the possibility of the DNA still containing PCR inhibitors is quite high. The overall results for PCR-T1 was unsatisfactory and this could be due to the annealing temperature (58°C) being too high, which in turn lowers the efficacy of the PCR amplification process (Anonymous, 2016).

Lanes 5 – 7 depict the results of PCR-T2. No noticeable differences were observed between lanes 5 and 6 representing DNA-T3 from a pure culture and from an enrichment respectively. Both lanes showed bands of similar intensities. Once again DNA-T4 produced no visible bands (lane 7). The overall results for PCR-T2 were unacceptable as the band intensities were even lower than that of PCR-T1. The possible reason for these results could be that the annealing temperature (48°C) was too low for the primers to effectively bind to the single stranded DNA (Anonymous, 2016).

The last three lanes (8 – 10) represent the results of PCR-T3. Lanes 8 and 9 representing DNA-T3 from a pure culture and enrichment respectively displayed high intensity bands. The DNA extraction method was adequate for the respective samples. Although both samples showed promising results, the final extraction method chosen was DNA-T3 from a pure culture in combination with PCR-T3 conditions. The reason for this is that some external factors could come into play when the enrichment is used. These include other microorganisms that could contaminate the enrichment leading to the extraction of DNA from not only the pure culture but also other microorganism. Tebbe & Vahjen (1993) reported that high concentrations of non-target DNA could lead to PCR inhibition. The components that make up the enrichment media could also possibly act as PCR inhibitors if they are not efficiently removed during the DNA extraction process (Knutsson *et al.*, 2002). PCR-T3 showed better amplification results for DNA-T4 (lane 10), but the results were still not satisfactory in terms of band intensity.

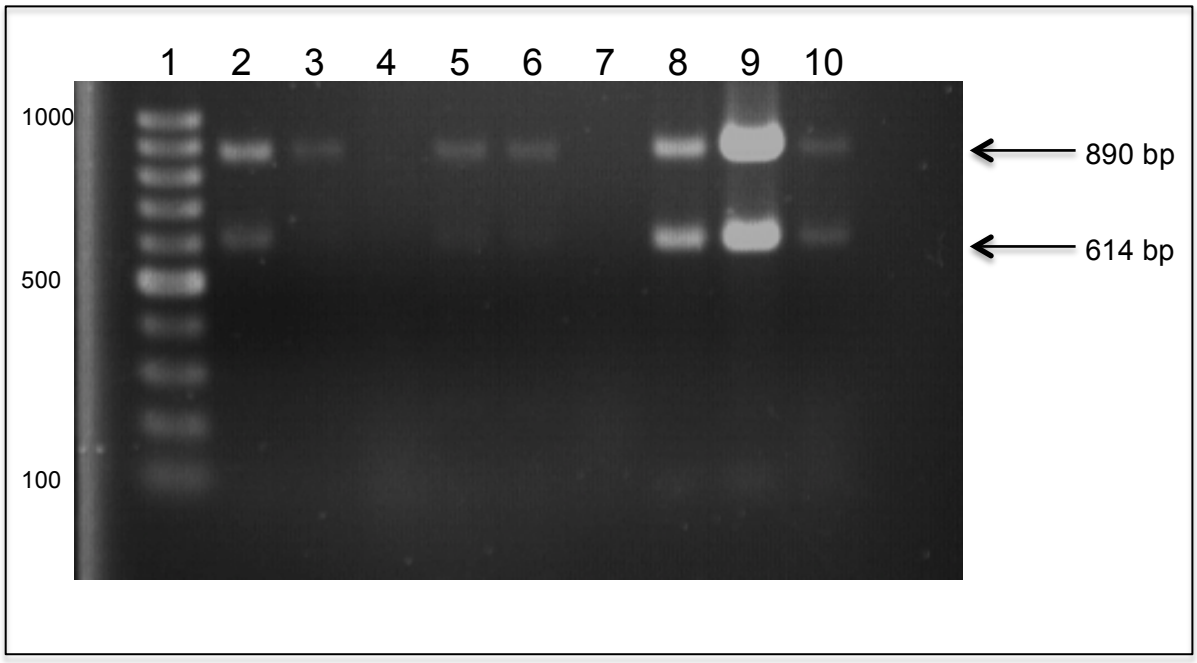

Figure 3.1 Agarose gel (1.2%) electrophoresis of PCR products obtained from the DNA extraction and PCR conditions comparison experiment. Primer sequences for target genes (5'-3'): *stx*$_1$ (F – ACACTGGATGATCTCAGTGG, R – CTGAATCCCCCTCCATTATG); *stx*$_2$ (F – CCATGACAACGGACAGCAGTT, R – CCT GTCAACTGAGCAGCACTTTG) & *eaeA* (F – GTGGCGAATACTGGCGAGACT, R – CCCCATTCTTTTTCACCGTCG). **Lane 1**: 100 bp ladder; **lane 2**: DNA-T3 (pure culture) & PCR-T1; **lane 3**: DNA-T3 (enrichment) & PCR-T1; **lane 4**: DNA-T4 (pure culture) & PCR-T1; **lane 5**: DNA-T3 (pure culture) & PCR-T2; **lane 6**: DNA-T3 (enrichment) & PCR-T2; **lane 7**: DNA-T4 (pure culture) & PCR-T2; **lane 8**: DNA-T3 (pure culture) & PCR-T3; **lane 9**: DNA-T3 (enrichment) & PCR-T2; **lane 10**: DNA-T4 (pure culture) & PCR-T3.

3.4.4 Finalizing Method

During the previous experiments it had been established that the extraction method described in DNA-T3 would be used for the positive control. It was also established that the conditions of PCR-T3 in combination with the reaction compositions of RC-T2 would be used for the amplification of the PCR products. The aim of this final experiment was to determine which DNA extraction kit would be used to extract DNA from the faecal samples. The reason for using an extraction kit is due to the fact that the DNA-T3 extraction method was inadequate in extracting inhibitor free DNA from the samples. This could be due to the fact that faecal matter contains numerous inhibitors such as bile salts and polysaccharides, which this extraction method might not be able to eliminate (Trochimchuk *et al.*, 2003; Monteiro *et al.*, 1997).

The results of the electrophoresis gel of the faecal contaminated samples are shown in Figure 3.2. Judging by the intensity of the bands in lane 2 (positive control) in Figure 3.2, it can be concluded that the optimum DNA extraction method, PCR conditions and reaction composition were chosen. Lanes 3 – 7 represent DNA extracted with the ZR Fecal DNA MiniPrep kit. When observing lane 3 no visible band can be seen. This could be due to the fact that the original faecal sample had no STEC present, as this sample received no artificial contamination. Lanes 4 (FD2) and 5 (FD3) that received the highest concentration of artificial contamination showed high intensity bands meaning there was more than enough DNA extracted from the faecal sample. Lanes 6 (FD4) and 7 (FD5) that had the least amount of artificial contamination still displayed visible bands although they were of a lower intensity. Lanes 8 (FD2) and 9 (FD5) represent the DNA extracted with the PowerSoil DNA Isolation kit. It can be noted that the results were not as satisfactory as those of the ZR Fecal DNA MiniPrep kit. When comparing lanes 4 and 8 that represent the same sample (FD2) with the different extraction kits, it can be seen that lane 8 had low intensity bands whereas those in lane 4 were of a much higher intensity. This shows that the PowerSoil DNA Isolation kit was not effective enough in removing inhibitors from the sample and extracting inhibitor free DNA. It was therefor decided that the ZR Fecal DNA MiniPrep kit would be used for all DNA extractions from faecal samples as this produced the best results. Lane 10, which is the negative water control, had no visible bands, confirming that the PCR amplification process worked perfectly.

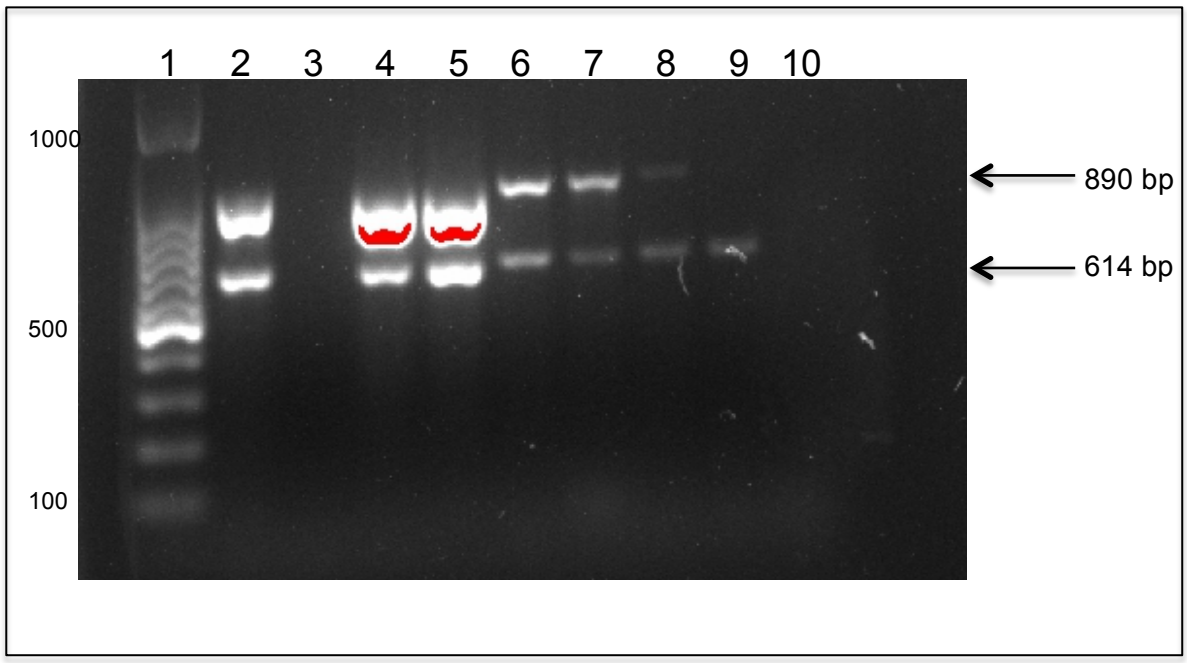

Figure 3.2 Agarose gel (1.2%) electrophoresis of the PCR products obtained from samples FD1 – FD5 using the ZR Fecal DNA MiniPrep kit as well as the PowerSoil DNA Isolation kit. Primer sequences for target genes (5'-3'): *stx*$_1$ (F – ACACTGGATGATCTCAGTGG, R – CTGAATCCCCCTCCATTATG); *stx*$_2$ (F – CCATGACAACGGACAGCAGTT, R – CCT GTCAACTGAGCAGCACTTTG) & *eaeA* (F – GTGGCGAATACTGGCGAGACT, R – CCCCATTCTTTTTCACCGTCG). **Lane 1**: 100 bp ladder; **lane 2**: *E. coli* O157:H7 positive control; **lane 3**: FD1 extracted with ZR Fecal DNA MiniPrep kit; **lane 4**: FD2 extracted with ZR Fecal DNA MiniPrep kit; **lane 5**: FD3 extracted with ZR Fecal DNA MiniPrep kit; **lane 6**: FD4 extracted with ZR Fecal DNA MiniPrep kit; **lane 7**: FD5 extracted with ZR Fecal DNA MiniPrep kit; **lane 8**: FD2 extracted with PowerSoil DNA Isolation kit; **lane 9**: FD5 extracted with PowerSoil DNA Isolation kit; **lane 10**: negative control (water).

3.5 CONCLUSIONS

During this study, DNA extraction optimisation was investigated as well as optimisation of the PCR amplification process conditions. The optimisation of the DNA extraction protocol consisted of four trials, each improving on the last. DNA-T1 displayed poor amplification results due to PCR inhibitors still being present which led to DNA-T2 receiving an additional ethanol wash to remove the PCR inhibitors. In DNA-T3 the inhibitor free DNA was then suspended in sterilised distilled water as the TE-Buffer acted as a PCR inhibitor. Optimisation of the PCR reaction composition required experimenting with the primer concentrations. Initially the primer concentration was set at 1 µM but was later lowered to 0.2 µM as the higher concentration led to non-specific binding causing the formation of primer-dimers. The lowered concentration produced satisfactory amplification results.

DNA-T3 & 4 was subjected to comparison tests in combination with PCR-T1, 2 & 3. PCR-T1 produced the highest intensity bands when combined with DNA-T3 from a pure culture although the results were not satisfactory. PCR-T2 produced bands of equal intensity for DNA-T3 from both a pure culture as well as the enrichment. Both PCR-T1 and 2 showed no visible bands when combined with DNA-T4. PCR-T3 showed the most promising results of all the PCR trials when combined with DNA-T3 from a pure culture. DNA-T3 from a pure culture was chosen as the final extraction method for the STEC positive control. The previously mentioned extraction method was used to extract DNA from faecal samples but due to the large amount of PCR inhibitors present in faecal matter the method was inadequate in removing all these inhibitors.

Two commercially available DNA extraction kits, namely the ZR Fecal DNA MiniPrep kit as well as the PowerSoil DNA Isolation Kit was tested to determine which extracted the purest DNA. Although the DNA extracted from the PowerSoil DNA Isolation Kit showed visible bands on the agarose gel, the ZR Fecal DNA MiniPrep kit was chosen as the final kit as these bands were of a much higher intensity.

To conclude it was determined that DNA-T3 from a pure culture would be used for the STEC positive control and the ZR Fecal DNA MiniPrep kit would be used to extract DNA from faecal samples in the following study. It was also determined that PCR RC-T2 in combination with PCR-T3 conditions produced the optimum results and was chosen as the final conditions for the experimental work to be conducted in the following study.

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CHAPTER 4
DETERMINING THE INCIDENCES OF SOUTH AFRICAN GAME SPECIES CONTAMINATED WITH SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI*

4.1 ABSTRACT

The game meat industry is growing globally as each year passes. Microbial safety of these meat products is thus essential. Incidences of red meat contaminated with Shiga-toxin producing *Escherichia coli* (STEC) are prominent, especially in the USA. Limited literature is available on the incidences of game meat contaminated with STEC in southern Africa. The aim of this study was therefore to determine the prevalence of STEC in South African game species. Faecal and meat samples were obtained from six game species (Zebra, Impala, Eland, Black Wildebeest, Blesbok and Springbok) from two different farms in the Western Cape, South Africa. These were analysed via the Polymerase Chain Reaction (PCR) for the *stx*$_1$, *stx*$_2$ and *eaeA* genes. Additional faecal samples from cattle were taken from both farms and analysed for the *stx*$_1$ *or stx*$_2$ and *eaeA* genes by means of the BAX® System. It was found that two of the four (Zebra and Black Wildebeest) species from the first farm tested positive for STEC in the faecal matter but all four species tested positive with regards to the meat samples, suggesting possible cross contamination. It was also found that none of the faecal samples from the second farm tested positive for STEC. Cattle samples from the two farms displayed the same trend as that of the game samples and could thus explain why the game animals on Farm 1 tested positive for STEC and those on Farm 2 negative. It was concluded that 19.2% of samples tested positive for STEC and that 26.9% of game animals had STEC present in either the faecal matter or on the meat. The high prevalence of positive animals may be alarming in terms of financial losses that can occur during export as well as posing a health risk to consumers.

4.2 INTRODUCTION

The game meat industry is growing globally as each year passes and the South African game meat industry is no exception. In 2003, South Africa had approximately 9 000 commercial game ranches covering approximately 17 million hectares of land (Reilly *et al.*, 2003). In 2014, South Africa produced around 23 700 tonnes of game meat (Taylor *et al.*, 2015). The most common animals harvested in South Africa for the production of game meat include feral Fellow Deer (*Dama dama)*, Black Wildebeest (*Connochaetes gnou*), Zebra (*Equus quagga*), Springbok (*Antidorcas marsupialis*), Blesbok (*Damaliscus pygargus phillipsi*), Impala (*Aepyceros melampus*),

Eland (*Taurotragus oryx*), Kudu (*Tragelaphus*) as well as Gemsbok (*Oryx gazella*) (van Schalkwyk *et al.*, 2010).

The increase in game meat production and consumption is mainly attributed to consumers becoming more concerned about their health and the products they consume. Due to these health concerns, consumers have started adapting their lifestyles to incorporate low kilojoule and low cholesterol products (Hoffman & Wiklund, 2006). One such product is game meat, known for its health benefits (Klein, 2005). As game meat comes from animals that life in the wild, strict health and safety protocols have to be followed during the slaughter and handling process to minimise possible contamination of the meat by faecal matter (van Schalkwyk & Hoffman, 2016).

Escherichia coli are common inhabitants of the gastrointestinal tracts of warm-blooded animals and are generally commensal or non-pathogenic. Although most strains are commensal, there are a number of strains that are pathogenic (Ahmed *et al.*, 2015; Altalhi & Hassan, 2009). One of the most well known classes of pathogenic *E. coli* is Shiga-toxin producing *Escherichia coli* (STEC). The most recognized serotype of STEC is O157:H7. STEC causes an array of different diseases including bloody diarrhoea, haemorrhagic colitis (HC), thrombotic thrombocytopaenic purpura (TTP) as well as the life threatening disease haemolytic uremic syndrome (HUS) (McClure, 2000; Gannon *et al.*, 1992; Brandal *et al.*, 2015).

STEC has the ability to produce two shiga toxins that are very similar to those produced by *Shigella dysenteriae*. The stx_1 and stx_2 genes encode for the two toxins respectively. Shiga toxin 1 is almost indistinguishable from the toxin that is produced by *Shigella dysenteriae* whereas shiga toxin 2 only has a 56% amino acid homology to shiga toxin 1 (Tarr *et al.*, 2005; Mead & Griffin, 1998'O'Brien & Holmes, 1987). In addition to the toxin genes, STEC also have the *eaeA* gene that encodes for the intimin protein. This protein is responsible for attaching and effacing lesions. The gene is encoded in the chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (Chekabab *et al.*, 2013; Fagan *et al.*, 1999).

One of the main reservoirs of STEC is cattle, where the bacteria are transmitted to humans via undercooked meat or inadequately pasteurized dairy products (Chekabab *et al.*, 2013). STEC has also been isolated from other animals including goats, sheep, deer, chickens, turkeys and horses (Chekabab *et al.*, 2013; Mead & Griffin, 1998). The main source of STEC contamination on meat is usually due to contamination by faecal matter. This usually happens during dressing of the carcass.

The hide of the animal naturally contains microbial loads. This is due to their surrounding as well as contamination from faecal matter present in the field (Blagojevic *et al.*, 2011; Bell, 1997). If skinning of the carcass is not done properly, cross contamination can occur where microbial organisms are transferred from the hide of the animal to the meat (Nørrung & Buncic, 2008). In this same manner STEC can also be transferred from the hide to the meat if the hide had been

contaminated with STEC via an external source. Blagojevic *et al.* (2011) showed a significant carryover of microbial load from the hide to the carcass during dressing of the carcass.

STEC outbreaks in the meat industry, especially in the United States, are becoming more prominent each year. The Centres for Disease Control and Prevention (CDC) estimated that around 390 *E. coli* O157 outbreaks occurred in the United States between 2003 and 2012. These outbreaks included 4 928 illnesses, 1 272 hospitalization and a total of 33 deaths. It was also estimated that there were 299 cases of HUS (Zuraw, 2015). During the months of June to September 2016, a slaughterhouse in Massachusetts had to recall 27 lots of beef, veal and bison due to *E. coli* O157:H7 contamination. Seven individuals from four different states became ill after consumption of the contaminated meat and five of these individuals required hospitalization (Anonymous, 2016).

Literature regarding STEC outbreaks and STEC research on both livestock and game animals in the United States are available (Fischer *et al.*, 2001; Renter *et al.*, 2001; Kistler *et al.*, 2011), but little research has been done to determine the prevalence of STEC in game species in southern Africa. The aim of this study was to determine the prevalence of STEC contamination in South African game species.

4.3 MATERIALS AND METHODS

4.3.1 Harvesting and Sample Collection

During this study, 26 game animals were harvested from two different farms in the Western Cape, South Africa. Harvest 1 (Farm 1) included four Zebra (*Equus quagga*), four Impala (*Aepyceros melampus*), four Eland (*Taurotragus oryx*) and four Black Wildebeest (*Connochaetes gnou*). Harvest 2 (Farm 2) included eight Blesbok (*Damaliscus pygargus phillipsi*) and two Springbok (*Antidorcas marsupialis*). The two farms were approximately 160 km apart. Both farms had cattle that were in close proximity to the wildlife. All animals were harvested according to the standard operating procedure (SU-ACUM14-001SOP) approved by the Stellenbosch University Animal Care and Use Committee.

Animals were shot and exsanguinated, within a few minutes, where after they were transported to the on-farm slaughtering facility. Carcasses were dressed (removal of head, legs and skin) and eviscerated according to the procedures described by van Schalkwyk & Hoffman (2010) and van Schalkwyk & Hoffman (2016). The slaughtering infrastructure on Farm 1 was not ideal, compared to that of an abattoir, but carcass dressing was performed in the most hygienic way possible. Farm 2 had a considerably better abattoir setup where management of the strict hygiene protocols were easier to control, compared to that of Farm 1. All skinned carcasses were transported back to the University of Stellenbosch by means of a cool truck (4°C). Upon arrival two pieces of meat (± 100 g each) were aseptically removed from the flank region of each carcass, vacuum packed and frozen (-18°C). Faecal matter was gathered directly from the small intestines

immediately after evisceration of the carcass and frozen (-18°C) for further analysis. Additionally, faecal samples were also gathered (removed aseptically from the anus) from five Aberdeen Angus (Farm 1) and five Hereford (Farm 2) cows on each of the two farms and frozen (-18°C) for further analysis.

4.3.2 Sample Preparation

Game samples

E. coli O157:H7 stock cultures (prepared from a pure culture received from the Department of Microbiology, Stellenbosch University) were streaked onto tryptone soy agar (TSA) (Biolab, Merck, South Africa) for positive control DNA extraction. Plates were incubated at 37°C for 24 h prior to DNA extraction. All meat samples from Harvest 1 and faecal samples from both Harvest 1 and 2 were defrosted overnight at 4°C. A 25 cm^2 swab was taken from each piece of meat and transferred to 10 mL of sterilised buffered peptone water (Biolab, Merck, South Africa). For the faecal samples, 10 g of faecal matter was transferred to 90 mL of buffered peptone water. All the samples were then incubated at 37°C for 24 h.

Cattle samples

E. coli O157:H7 stock cultures were used to prepare the positive control by suspending 100 µL of stock culture in 10 mL of sterilised buffered peptone water. One gram of faecal matter from each cow was suspended in 9 mL of sterilised buffered peptone water. All samples were then incubated at 37°C for 24 h before testing on the BAX® System (DuPont, South Africa). Cow samples were not tested using the optimised method that was used for the game samples.

4.3.3 DNA Extraction

Positive control

For DNA extracted from pure cultures (positive control), 300 µL lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 0.5% TWEEN 20, 1 mM EDTA) was added to a sterile 1.5 mL Eppendorf tube and one loop full of culture from the TSA plate was suspended in the lysis buffer. The samples were boiled for 10 min at 100°C and centrifuged at 1 000 x g for 2 min. After centrifuging, 250 µL of the supernatant was transferred to a sterile 1.5 mL Eppendorf tube and 250 µL of ice cold 99.9% ethanol was added to the tube. Samples were then centrifuged at 13 000 x g for 1 min and the supernatant was discarded. The tubes were allowed to dry for 45 – 60 min where after the dried pellet was suspended in 100 µL sterile distilled H_2O. DNA samples were held on ice until use or frozen at -20°C for later use.

Meat and faecal samples

DNA was extracted from all samples using the ZR Fecal DNA MiniPrep kit (Zymo Research, Inqaba Biotec, South Africa). For the meat samples, the volume of sample required for the kit was divided between the two samples from each carcass. The two samples from each carcass were thus pooled together to create one sample from which DNA was extracted.

4.3.4 Amplification of Multiplex PCR

Amplification of the multiplex PCR was performed using the method optimised in Chapter 3. The primer sets for the multiplex PCR amplification of STEC target genes are depicted in Table 4.1. The target genes that were selected were the *stx*$_1$, *stx*$_2$ and *eaeA* genes. PCR amplification of extracted DNA was performed in 50 µL volumes consisting of 1X One *Taq* standard reaction buffer (New England BioLabs Inc), 2 µL template DNA, 0.2 µM of each primer (Inqaba Biotec, South Africa), 200 µM of each dATP, dCTP, dGTP and dTTP (New England BioLabs Inc) and 1 U One *Taq* DNA polymerase (New England BioLabs Inc). A negative water control was also included where the DNA was replaced with water. The PCR mixtures were subjected to the following conditions: initial denaturation at 95°C for 3 min followed by 30 cycles of 95°C for 15 s, 55°C for 30 s and 68°C for 30 s and ending with a final extension step at 72°C for 5 min (Lamprecht *et al.*, 2014). PCR was performed with the Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa). Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain (Lab Supply Mall, InnoVita Inc). Gels were run for 100 min at 70 V. A 100 bp DNA Ladder (New England BioLabs Inc) was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) in combination with the Image Lab Software (version 5.2.1).

Table 4.1 Primer sets for the multiplex PCR amplification of STEC target genes

Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
stx$_1$	F: ACA CTG GAT GAT CTC AGT GG R: CTG AAT CCC CCT CCA TTA TG	614	Gannon *et al.*, (1992)
stx$_2$	F: CCA TGA CAA CGG ACA GCA GTT R: CCT GTC AAC TGA GCA GCA CTT TG	779	Gannon *et al.*, (1992)
eaeA	F: GTG GCG AAT ACT GGC GAG ACT R: CCC CAT TCT TTT TCA CCG TCG	890	Gannon *et al.*, (1993)

4.3.5 BAX System

The BAX® System real-time PCR assay for STEC-Screening (*stx*$_1$ *or stx*$_2$ and *eaeA*) was used to test the cattle faecal samples. The lysis reagent was prepared by transferring 150 µL of protease to 12 mL of lysis buffer. Aliquots of 200 µL lysis reagent was transferred to cluster tubes and 20 µL of sample was added. Cluster tubes were heated to 37°C for 20 min followed by 95°C for 10 min. The cluster tubes were then transferred to the cooling block to cool down to 4°C for 5 min. Aliquots of 30 µL of lysate were transferred to PCR tubes and left for 20 min to hydrate the tablets (Wasilenko *et al.*, 2014). The PCR tubes were loaded into the BAX® System Q7 instrument (DuPont, South Africa) and the process was run according to the BAX® System User Guide. Results were analysed using the BAX® System software version 3.2 (DuPont, South Africa).

4.4 RESULTS AND DISCUSSION

During this study the prevalence of STEC in South African game species was investigated. The faecal samples isolated from the first harvest (Table 4.2) showed that only the faecal matter of two Zebras (1 & 4) and two Black Wildebeest (1 & 3) had STEC present. None of the samples taken from the Eland and Impala had any STEC present. The meat of all the animals from Harvest 1 were thus also analysed to determine whether cross contamination from the faecal matter or hide could have contaminated the meat.

When the faecal samples from Harvest 2 were analysed it was noted that none of the Blesbok or the Springbok had any STEC present. As none of the faecal samples from Harvest 2 contained any STEC, the meat of these animals was not tested. It was interesting to note that animals from Farm 1 tested positive for STEC but the animals from Farm 2 tested negative. The initial thought was that Farm 2 might not have STEC present on the farm but this could not be said with certainty until further testing is done. It is known that cattle are one of the main reservoirs of STEC and that STEC could be transferred between animals living on the same farm (Diaz-Sanchez *et al.*, 2013; Gortázar *et al.*, 2007).

Table 4.2 Prevalence of STEC target genes isolated from Harvest 1 faecal samples

Animal	*eaeA*	*stx*$_1$	*stx*$_2$
Zebra 1	+	+	+
Zebra 2	-	-	-
Zebra 3	-	-	-
Zebra 4	-	+	+
Impala 1	-	-	-
Impala 2	-	-	-
Impala 3	-	-	-
Impala 4	-	-	-
Eland 1	-	-	-
Eland 2	-	-	-
Eland 3	-	-	-
Eland 4	-	-	-
Black Wildebeest 1	+	-	+
Black Wildebeest 2	-	-	-
Black Wildebeest 3	-	+	+
Black Wildebeest 4	-	-	-

An additional study was done where faecal samples of cattle from both farms were tested to determine whether they possess STEC and in turn could transfer it to the game animals (Table 4.3). As it was only important to know whether the potential STEC in the cattle possess the *stx* gene and not specifically the *stx*$_1$ or *stx*$_2$ genes, the BAX System STEC-Screening kit was the best choice for the analysis. Only one of the cows from Farm 1 tested positive for both the *stx* and *eaeA* genes (Table 4.3). It can further be observed that none of the cattle from Farm 2 tested positive for either of the two genes. These results follow the exact same trend that was observed with the game animals. It can thus be speculated that due to the fact that the cattle on Farm 1 tested positive for STEC, the STEC could have been transferred to the game animals whereas there were no STEC present on the cattle in Farm 2 to transfer to the wild game. It is also known that the farmer on Farm 1 sometimes moves the cattle into the game paddocks increasing the possibility of STEC transfer from livestock to wild game.

Table 4.3 Prevalence of STEC target genes isolated from Farm 1 and 2 cattle faecal samples

Farm	Sample	*stx*	*eaeA*
1	Cow 1	-	-
	Cow 2	+	+
	Cow 3	-	-
	Cow 4	-	-
	Cow 5	-	-
2	Cow 1	-	-
	Cow 2	-	-
	Cow 3	-	-
	Cow 4	-	-
	Cow 5	-	-

Observing the results for the meat samples from Harvest 1 (Table 4.4), it is interesting to note that all four species had one sample that had STEC present whereas only two species had STEC present in the faecal matter. Although strict hygiene protocols were followed during the slaughtering of the animal, some cross contamination could still have occurred during the dressing of the carcass. The hide of the animals could have been contaminated with excreted faeces present in the field. This contamination on the hide could then have been transferred to the meat when the incision in the hide was made to dress the carcass; this is particularly applicable to the harvest on Farm 1 where no suitable infrastructure were available for dressing the carcasses (compared to that on Farm 2). Also, the species from Farm 1 are all large animals (live weights displayed in Table 4.4) with thicker skins, making the actual skinning more cumbersome than when skinning the smaller blesbok and springbok from Farm 2. Blagojevic *et al.* (2011) showed that during the process of carcass dressing, a significant amount of microorganisms were transferred from the hide to the carcass. Further cross contamination could also have occurred during the transport of the carcasses in the cooling truck due to the close proximity of the carcasses to one another. It was further noted that Black Wildebeest 3 was the only animal with STEC present in both the faecal matter as well as on the meat. When comparing the target gene combination present in the faecal matter with that found on the meat, one can note that the stx_1 and stx_2 genes were present in the faecal matter but only the stx_1 gene was isolated from the meat. This suggests that cross contamination from another carcass or from faecal matter present on the hide could have occurred (Elder *et al.*, 2000).

Table 4.4 Prevalence of STEC target genes isolated from Harvest 1 meat samples as well as the live weight of these animals

Animal	Live Weight (kg)	*eaeA*	*stx*$_1$	*stx*$_2$
Zebra 1	294.9	-	-	-
Zebra 2	307.4	-	-	-
Zebra 3	340.9	-	+	+
Zebra 4	343.0	-	-	-
Impala 1	60.5	-	-	-
Impala 2	68.4	-	-	-
Impala 3	53.0	-	-	-
Impala 4	65.3	+	+	+
Eland 1	486.6	-	-	-
Eland 2	275.4	-	-	-
Eland 3	315.3	+	+	+
Eland 4	196.8	-	-	-
Black Wildebeest 1	167.3	-	-	-
Black Wildebeest 2	175.6	-	-	-
Black Wildebeest 3	171.1	-	+	-
Black Wildebeest 4	167.9	-	-	-

During the following section the target gene combinations will be discussed for the different sample sets. Table 4.5 depicts the target gene combinations detected in the 26 faecal samples (Harvest 1 & 2) analysed for STEC. At first glance it can be noted that none of the three genes were detected singly. A study on white tailed deer in Pennsylvania found that 10% of samples contained the *stx*$_1$ gene and 46% contained the *stx*$_2$ gene (Kistler *et al.*, 2011). Another study on Red deer and Roe deer found a prevalence of 8.1% and 10% respectively for the *eaeA* gene (Obwegeser *et al.*, 2012). The *stx*$_1$ gene in combination with the *stx*$_2$ gene was detected in two of the 26 samples (7.7%). Kistler *et al.* (2011) found that 10% of the white tailed deer faecal samples displayed the *stx*$_1$ and *stx*$_2$ combination. It was further noted that the *stx*$_1$ gene in combination with the *eaeA* gene was not detected in any of the faecal samples whereas the *stx*$_2$ gene in

combination with the *eaeA* gene was detected in one of the 26 samples (3.8%). It was lastly observed that only one of the samples had all three the stx_1, stx_2 and the *eaeA* genes present. Obwegeser *et al.* (2012) found that none of the STEC isolated from either Red or Roe deer had all three genes present.

STEC requires both the *eaeA* gene in combination with at least one of the *stx* genes to cause diseases such as Haemolytic Uremic Syndrome (HUS) and Haemorrhagic Colitis (HC) (Karmali, 2004). The reason being that the *eaeA* gene encodes for the intimin protein, which is responsible for attaching/effacing lesions. Without this protein the bacterium cannot attach to cells in the body and therefore its ability to inject toxins into cells is inhibited (Viazis & Diez-Gonzalez, 2011; Karmali, 2004). Only two of the 26 samples (7.7%) that tested positive for STEC could possibly cause illness if it had come into contact with food meant for consumption due to the presence of the *eaeA* gene. The overall percentage of faecal samples that tested positive for STEC was 15.3%. This prevalence is a slightly lower than the 19.4% reported by Gilbreath *et al.* (2009) for mule deer and elk faecal samples.

Table 4.6 depicts the target gene combinations detected in the 16 meat samples (Harvest 1) analysed for STEC. Literature on the prevalence of game meat contaminated with STEC, especially in South Africa, is very limited. The prevalence of STEC on cattle carcasses will thus be used to compare to the findings of this study. Unlike the results of the faecal matter, the stx_1 gene was isolated on its own in one of the meat samples (6.3%) but once again the stx_2 and *eaeA* genes were not isolated singly. Etcheverria *et al.* (2010) found a prevalence of 2.5% and 6.2% for the stx_1 and stx_2 genes respectively, on cattle carcasses directly after slaughter. The occurrence of the stx_1 and stx_2 combination was detected in one meat sample (6.3%) whereas it was detected in two faecal samples. Neither the stx_1 gene nor the stx_2 gene in combination with the *eaeA* gene was detected in any of the meat samples. Lastly, two of the meat samples (12.5%) had the stx_1, stx_2 and the *eaeA* genes present, which is double that of the faecal samples. As with the faecal samples, there were two of the 16 meat samples (12.5%) that had the potential to cause diseases if it had been ingested. The overall percentage of meat samples that tested positive for STEC was 25.0%. This prevalence is much higher than the 17.8% found by Elder *et al.* (2000) and the 12.3% found by Etcheverria *et al.* (2010) on cattle carcasses.

Table 4.7 depicts all the results from both the faecal and meat samples. Four out of the 42 samples has the ability to cause illness due to the *eaeA* gene being present with one of the *stx* genes. The overall percentage of positive samples was 19.2%. Although eight of the 42 samples tested positive for STEC, only seven of the 26 animals tested positive as Black Wildebeest 3 had two samples that tested positive. The percentage of animals that tested positive for STEC was thus 26.9%.

Table 4.5 Fraction of faecal samples from Harvest 1 and 2 containing the different target gene combinations

Gene Prevalence	Fraction (%) Positives samples
stx$_1$	0/26 (0.0)
stx$_2$	0/26 (0.0)
eaeA	0/26 (0.0)
stx$_1$ + *stx*$_2$	2/26 (7.7)
stx$_1$ + *eaeA*	0/26 (0.0)
stx$_2$ + *eaeA*	1/26 (3.8)
stx$_1$ + *stx*$_2$ + *eaeA*	1/26 (3.8)
TOTAL	**4/26 (15.3)**

Table 4.6 Fraction of meat samples from Harvest 1 containing the different target gene combinations

Gene Prevalence	Fraction (%) Positives samples
stx$_1$	1/16 (6.3)
stx$_2$	0/16 (0.0)
eaeA	0/16 (0.0)
stx$_1$ + *stx*$_2$	1/16 (6.3)
stx$_1$ + *eaeA*	0/16 (0.0)
stx$_2$ + *eaeA*	0/16 (0.0)
stx$_1$ + *stx*$_2$ + *eaeA*	2/16 (12.5)
TOTAL	**4/16 (25)**

Table 4.7 Fraction of all samples (meat and faecal) from Harvest 1 and 2 containing the different target gene combinations

Gene Prevalence	Fraction (%) Positives samples
stx$_1$	1/42 (2.4)
stx$_2$	0/42 (0.0)
eaeA	0/42 (0.0)
stx$_1$ + *stx*$_2$	3/42 (7.2)
stx$_1$ + *eaeA*	0/42 (0.0)
stx$_2$ + *eaeA*	1/42 (2.4)
stx$_1$ + *stx*$_2$ + *eaeA*	3/42 (7.2)
TOTAL	**8/42 (19.2)**

4.5 CONCLUSIONS

The aim of this study was to determine the prevalence of STEC in game species from two different farms in the Western Cape, South Africa. Faecal samples of game species from Farm 1 showed that two Zebras and two Black Wildebeest harboured STEC. The meat samples from Farm 1 showed that all four species (Zebra, Impala, Black Wildebeest and Eland) contained STEC. Possible cross contamination could have occurred during the slaughter and dressing process. Further cross contamination could also have occurred during the transporting of the carcasses in the cooling truck.

It was interesting to note that none of the game species from Farm 2 tested positive for STEC in their faecal matter. As cattle are one of the main reservoirs of STEC, and it is known that STEC can be transferred between animals, an additional study was conducted on cattle from both farms to determine whether they possess the organism in their faeces. The results from the cattle showed the same trend as that of the game animals. This could possibly explain why the game animals from the second farm tested negative. As the cattle did not harbour the organism, they could not have transferred it to the game animals.

The high prevalence of STEC positive animals (26.9%) may have dire financial implications on the game meat export market, especially meat exported to the European Union. The health risk to consumers must also be considered, taking into account this high prevalence. Further research could focus on expanding the geographic area tested, including more farms and also increasing the sampling size. A further recommendation would be to put interventions in place on farms where cattle are known to harbour the STEC organism to prevent transfer of the organism to the wild game.

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CHAPTER 5
MICROBIAL POPULATION PRESENT ON THE MEAT AND IN THE FAECAL MATTER OF SOUTH AFRICAN GAME SPECIES

5.1 ABSTRACT

As the global, and South African, game meat industry increases each year, the pressure on the meat industry in terms of producing microbiologically safe meat also increases. This exploratory study aimed at determining the microbial population present on South African game carcasses after dressing to determine whether the meat would meet the microbiological safety criteria. Ten game animals (eight blesbok and two springbok) were harvested from a farm in the Western Cape, South Africa. Meat and faecal samples taken from the animals were tested for aerobic microbial count, total coliforms, *Escherichia coli, Staphylococcus aureus, Salmonella* and *Listeria*. Aerobic count prevalence on the meat ranged from 1.60 – 4.97 log cfu·cm^{-2} while total coliforms prevalence varied from 5.04 – 5.59 log cfu·cm^{-2}. *E. coli* prevalence ranged from 0.00 – 1.71 log cfu·cm^{-2} while *S. aureus* varied from 0.00 – 2.97 log cfu·cm^{-2}. Aerobic count prevalence in the faecal matter of the animals ranged from 5.78 – 6.44 log cfu·g^{-1} and total coliforms ranged from 6.53 – 7.04 log cfu·g^{-1}. *E. coli* prevalence varied from 3.00 – 4.54 log cfu·g^{-1} while *S. aureus* ranged from 3.63 – 4.40 log cfu·g^{-1}. All faecal samples tested negative for both *Salmonella* and *Listeria*. Using the meat export legislation set out by the Department of Agriculture, Forestry and Fisheries it was determined that all counts were below the upper limit except for total coliforms. The shelf-life stability of the meat may be compromised by the total coliforms count but in terms of food safety the meat was determined to be safe for consumption.

5.2 INTRODUCTION

The global game meat industry is an ever-increasing industry growing each year and South Africa is no exception. The South African wildlife ranching sector is recognized as a fast growing sector in the agricultural industry. In 2003, it was estimated that South Africa had 9 000 commercial game ranches covering around 17 million hectares of land (Reilly *et al.*, 2003). Ultimately, all the game species and animals ranched end up producing meat.

This increase in game meat production and consumption is mainly attributed to consumers becoming more health conscious about the products they consume. Consumers have started adapting their lifestyles to incorporate low kilojoule and low cholesterol products (Hoffman & Wiklund, 2006). Game meat is one of these products, known for its health benefits (Klein, 2005). South Africa produced 23 700 tonnes of game meat during 2014 (Taylor *et al.*, 2015).

Common game animals harvested in South Africa for the production of game meat include feral Fellow Deer (Dama dama), Kudu (Tragelaphus strepsiceros), Blue Wildebeest (Connochaetes taurinus), Black Wildebeest (Connochaetes gnou), Zebra (Equus quagga), Springbok (*Antidorcas marsupialis*) and Blesbok (*Damaliscus pygargus phillipsi*) (van Schalkwyk *et al.*, 2010) of which the latter two will be the focus of this study. Game meat refers to meat obtained from non-domesticated, farm raised and/or free ranging animals (Hoffman & Cawthorn, 2012). As these animals live in the wild, health and safety regulations have to be followed to ensure the meat is safe for human consumption.

During the harvesting process, contamination of the carcass can occur, potentially compromising the sterility of the meat. Although headshots are preferred, as these cause immediate death and minimal meat contamination, it is not always possible. Sometimes shots in the abdominal region occur. This could lead to the rupturing of the stomach and intestines causing the body cavity to be contaminated with the intestinal contents and microorganisms (van Schalkwyk & Hoffman, 2010). Although these animals are not allowed to enter the export value chain, they regularly enter the local market (van Schalkwyk & Hoffman, 2016).

Skinning of the animal could lead to the microbiological contamination of the carcass meat. The hide of an animal naturally contains microorganisms due to their surrounding environment as well as microorganisms from faecal matter present in the field (Blagojevic *et al.*, 2011; Bell, 1997). If skinning of the carcass is not done properly, cross contamination can occur where microorganisms are transferred from the hide of the animal to the meat (Nørrung & Buncic, 2008; Blagojevic *et al.*, 2011).

When determining the microbial population present on any food product, it can be evaluated/discussed from two perspectives, food spoilage and food safety. Food spoilage refers to the process whereby undesired changes occur within a food product leading to the degradation of biochemical and sensory characteristics and making the product unacceptable to the consumer. Bacteria responsible for spoilage include aerobic bacteria, total coliforms and *Pseudomonas*. Meat specifically is very prone to spoilage due to the presence of enzymatic and chemical activity. Fresh meat further has a high moisture content, contains nutrients and has an optimum pH range which favour the growth of a variety of microorganisms (Iulietto *et al.*, 2015). The second important aspect is food safety. Food safety refers to the presence/absence of pathogenic bacteria present on the foodstuff. For food products to be regarded as safe, no or limited (according to legislation) pathogenic bacteria or toxin-producing bacteria may be present on the food (D'Alessandro & Zolla, 2012). These bacteria include *Escherichia coli* (*E. coli*), *Salmonella, Listeria* and *Staphylococcus aureus* (*S. aureus*).

The aim of this study was to determine the microbial population present on Blesbok and Springbok carcasses after dressing to determine whether the meat would meet the microbiological criteria in terms of food spoilage and food safety aspects. A secondary aim was to determine the

microbiological population found in the faecal matter of these animals so as to determine whether cross contamination could have occurred.

5.3 MATERIALS AND METHODS

Harvesting

During this study, eight Blesbok (*Damaliscus pygargus phillipsi*) and two Springbok (*Antidorcas marsupialis*) were harvested from Farm 2 (refer to Chapter 4) in the Western Cape, South Africa. All animals were harvested according to the standard operating procedure (SU-ACUM14-001SOP) approved by the Stellenbosch University Animal Care and Use Committee.

Animals were shot and exsanguinated, within a few minutes, where after they were transported to the on-farm slaughtering facility. Carcasses were dressed (removal of head, legs and skin) and eviscerated according to the procedures described by van Schalkwyk & Hoffman (2010) and van Schalkwyk & Hoffman (2016). The slaughtering infrastructure on this farm was adequate to ensure that strict hygiene protocols could be followed. All skinned carcasses were transported back to the University of Stellenbosch in a refrigerated truck (4°C). Upon arrival two pieces of meat (± 100 g each) were aseptically removed from the flank region of each carcass, vacuum packed and frozen (-18°C). Faecal matter was gathered directly from the small intestines immediately after evisceration of the carcass and frozen (-18°C) for further analysis.

Sample preparation

Before analysis of the samples they were defrosted overnight at 4°C where after surface swabs (25 cm^2) of the meat samples were taken and placed in 10 ml buffered peptone water (Biolab, Merck, South Africa) (Ghafir & Daube, 2008). For the faecal samples, 10 g sample was placed in 90 ml buffered peptone water. These samples were then used for analysis on the Tempo system (BioMérieux, South Africa) and Petrifilm (3M Company, St. Paul, MN, EUA). Polymerase chain reaction (PCR) was also performed on the DNA extracted from the faecal matter in Chapter 4 for *Salmonella* and *Listeria*.

Tempo system

Aliquots of the samples were transferred to the Tempo vials containing the reconstituted media. This was prepared by adding specific volumes of autoclaved distilled water to the media powder in the vials (Owen *et al.*, 2010). *Escherichia coli* and *Staphylococcus aureus* tests were performed using one millilitre of the sample thus making a 1/40 Tempo dilution, whereas the total coliforms test was performed using 0.1 ml of sample thus making a 1/400 Tempo dilution. The contents of the vials were transferred to the Tempo cards using the Tempo Filler. Each card consists of 48 wells, 16 of each of the three volumes (225, 22.5, 2.25 µL). *E. coli* and *S. aureus* cards were incubated at 37°C while total coliform cards were incubated at 30°C. All cards were incubated for

24 h where after the results were analysed by the Tempo Reader. This system makes use of software that detects which of the wells tested positive. Positive wells would either have an increase in fluorescence or a decrease depending on the microorganism tested. The software uses the volumes of the positive wells as well as the dilution of the sample to mathematically calculate the cfu·cm^{-2} of the sample based on Most Probable Number (MPN) tables.

Petrifilm

Aerobic count (AC) enumeration was done using the Petrifilm system (Nelson *et al.*, 2013). Using the samples used for the Tempo analyses a dilution series (10^{-2} – 10^{-7}) was prepared. One-millilitre aliquots of each dilution were inoculated onto petrifilms in duplicate. Using the spreader provided, the inoculum was spread over the 20 cm^2 surface. The petrifilms were incubated at 30°C for 48 hours. All red colonies were counted as per the manufacturers instructions and deemed positive.

Salmonella PCR

The primer set for the PCR amplification of *Salmonella* target sequence is depicted in Table 5.1. PCR amplification of extracted DNA was performed in 50 µL volumes consisting of 1X One *Taq* standard reaction buffer (New England BioLabs Inc), 2 µL template DNA, 0.5 µM of each primer (Inqaba Biotec, South Africa), 400 µM of each dATP, dCTP, dGTP and dTTP (New England BioLabs Inc) and 1 U One *Taq* DNA polymerase (New England BioLabs Inc). A negative control (sterile water) was also included where the DNA was replaced with water. The PCR mixtures were subjected to the following conditions: initial denaturation at 94°C for 2 min followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and ending with a final extension step at 72°C for 10 min (adapted from Aabo *et al.*, 1993). PCR was performed with Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa). Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain (Lab Supply Mall, InnoVita Inc). Gels were run for 100 min at 70 V. A 100 bp DNA Ladder (New England BioLabs Inc) was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) in combination with the Image Lab Software (version 5.2.1).

Listeria PCR

The primer set for the PCR amplification of the *Listeria hly* target gene is depicted in Table 5.1. PCR amplification of extracted DNA was performed in 50 µL volumes consisting of 1X One *Taq* standard reaction buffer (New England BioLabs Inc), 2 µL template DNA, 0.3 µM of each primer (Inqaba Biotec, South Africa), 200 µM of each dATP, dCTP, dGTP and dTTP (New England BioLabs Inc) and 1 U One *Taq* DNA polymerase (New England BioLabs Inc). A negative water control was also included where the DNA was replaced with water. The PCR mixtures were subjected to the following conditions: initial denaturation at 95°C for 3 min followed by 30 cycles of 94°C for 40 s, 59°C for 40 s and 72°C for 1 min and ending with a final extension step at 72°C for 5

min (adapted from Gouws & Liedemann, 2005). PCR was performed with Bio-Rad T100 Thermal Cycler (Bio-Rad, South Africa). Gel electrophoresis was performed using a 1.2% agarose gel stained with GRGreen nucleic acid gel stain (Lab Supply Mall, InnoVita Inc). Gels were run for 100 min at 70 V. A 100 bp DNA Ladder (New England BioLabs Inc) was used. Gel visualization was performed using the Bio-Rad Gel Doc XR+ System (Bio-Rad, South Africa) in combination with the Image Lab Software (version 5.2.1).

Table 5.1 Primer sequences used in the PCR amplification for *Salmonella* and *Listeria*

Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
Salmonella	ST11: AGCCAACCATTGCTAAATTGGCGCA ST15: GGTAGAAATTCCCAGCGGGTACTG	429	Aabo *et al.*, (1993)
Listeria hly	F: CATTAGTGGAAAGATGGAATG R: GTATCCTCCAGAGTGATCGA	730	Gouws & Liedemann, (2005)

5.4 RESULTS AND DISCUSSION

For the purpose of comparing to literature as well as to microbial standards all log cfu·25 cm^{-2} results obtained were converted to log cfu·cm^{-2} (DAFF, 2010).

The prevalence of aerobic count (log cfu·cm^{-2}) on the meat samples from animals harvested on Farm 2 is depicted in Figure 5.1. It can be noted that the counts ranged from 1.60 log cfu·cm^{-2} (BB1, BB3 and BB5) to 3.25 log cfu·cm^{-2} (BB8) with BB1 and BB7 being the outliers with counts of 4.97 and 4.70 log cfu·cm^{-2}, respectively. It was further noted that SB1 had a high standard deviation compared to that of the other animals. This variation can be explained in terms of the microbial load present on the two meat samples from each carcass. The one sample had a substantially higher microbial load than the other one. This variation can be expected as the microbial load distribution on a carcass can vary greatly from one area to another.

Atanassova *et al.* (2008) found a mean aerobic count prevalence of 2.6 log cfu·cm^{-2} for roe deer, 2.9 log cfu·cm^{-2} for red deer and 3.2 log cfu·cm^{-2} for wild boars. Avagnina *et al.* (2012) on the other hand found a mean aerobic count of 3.6 log cfu·cm^{-2} for roe deer, 3.31 log cfu·cm^{-2} for red deer and 4.61 log cfu·cm^{-2} for wild boars. Magwedere *et al.* (2013) tested the prevalence of aerobic count on springbok carcasses during 2009 and 2010 and found a mean prevalence of 2.58 log cfu·cm^{-2} and 3.49 log cfu·cm^{-2}, respectively. Shange (2015) detected a 5.49 log cfu·cm^{-2} aerobic count for black wildebeest sampled in the flank region of the carcass. Comparing the results of the

abovementioned studies with the current one, it can be noted that their log counts all fall within the ranges found in this study.

Aerobic bacteria are one of the leading causes of meat spoilage (Iulietto *et al.*, 2015) and thus have to comply with the strict microbial standards set out by the Department of Agriculture, Forestry and Fisheries (DAFF) (DAFF, 2010). Table 5.2 depicts the acceptable aerobic count range (5.0 – 5.7 log cfu·cm^{-2}) for meat products destined for export as set out in the regulations by DAFF. When observing the results in Figure 5.1 it can be noted that the counts obtained from all the animals were well below that of the upper acceptable limit. This suggests that meat from these animals is less likely to become spoiled due to aerobic bacteria. This in effect also suggests that the meat will have improved shelf-life stability.

Table 5.2 Acceptable microbial ranges according to the microbial standards for export meat (DAFF, 2010)

	Aerobic count	***Escherichia coli***	***Enterobacteriaceae***
Acceptable range (log cfu·cm^{-2})	5.0 – 5.7	1.7 – 2.7	2.0 – 2.5

The prevalence of total coliforms (log cfu·cm^{-2}) on the meat samples from animals harvested on Farm 2 is depicted in Figure 5.2. It can be noted that the counts ranged from 5.08 log cfu·cm^{-2} (BB2 and BB3) to 5.59 log cfu·cm^{-2} (SB1). Unlike the results for aerobic count, all total coliforms counts were very close to one another and no large standard deviations were observed. Total coliforms forms part of the *Enterobacteriaceae* family that also includes bacteria such as *Salmonella, Klebsiella* and *Shigella*. Due to the fact that most studies test for the *Enterobacteriaceae* family of microorganisms and not total coliforms as per se, these results will be compared to literature on *Enterobacteriaceae* unless otherwise stated.

Obwegeser *et al.* (2012) found a mean *Enterobacteriaceae* prevalence of 2.3 log cfu·cm^{-2} for red deer, 2.6 log cfu·cm^{-2} for roe deer and 2.6 log cfu·cm^{-2} for chamois. Magwedere *et al.* (2013) found a mean *Enterobacteriaceae* prevalence of 1.33 log cfu·cm^{-2} for springbok harvested in 2009 and 2.93 log cfu·cm^{-2} for springbok harvested in 2010. Membré *et al.* (2011) found coliforms prevalence of 1.98 log cfu·cm^{-2} for red deer, 2.37 log cfu·cm^{-2} for roe deer and 1.68 log cfu·cm^{-2} for wild boar. The results obtained during these studies were substantially lower that those achieved in this study, suggesting possible contamination could have occurred during the slaughter and dressing process where microorganisms could have been transferred from the hide

to the meat (Nørrung & Buncic, 2008). Blagojevic *et al.* (2011) showed that the mean *Enterobacteriaceae* count on the hide of bovine was 1.97 log cfu·cm^{-2} and after dressing a 1.06 log cfu·cm^{-2} was detected on the carcass confirming microbial carryover during dressing.

Although there are no regulations pertaining to total coliforms as such, it can be interpreted in terms of *Enterobacteriaceae* as they form part of this family. Looking at the microbial standard for *Enterobacteriaceae* in Table 5.2 as set out by DAFF, it can be speculated that the coliform counts in this study would not fall within the specifications as they greatly exceed the upper limit for *Enterobacteriaceae*. As no tests were done for *Enterobacteriaceae,* this statement is purely based on speculation. *Enterobacteriaceae* and thus total coliforms plays an integral part in the spoilage of meat products (Baylis, 2006). As the counts in this study do not comply with the regulations it can be said with relative certainty that the shelf-life stability of the meat might be compromised leading to meat spoiling at an accelerated rate.

The *Escherichia coli* prevalence (log cfu·cm^{-2}) on the meat samples from animals harvested on Farm 2 are depicted in Figure 5.3. It can be noted that the counts ranged from 0.00 log cfu·cm^{-2} (BB1, BB2, BB5 and BB6) to 0.13 log cfu·cm^{-2} (BB8). BB3 and BB4 were outliers having the highest counts of 1.71 and 1.43 log cfu·cm^{-2}, respectively. It was also noted that BB4 had a high standard deviation compared to that of the other animals. As previously mentioned this variation can be explained in terms of the microbial load present on the two meat samples taken from that specific carcass.

Shange (2015) found a mean *E. coli* count of 2.5 log cfu·cm^{-2} on springbok carcasses after dressing. Membré *et al.* (2011) performed two studies during the 2005 – 2006 period and the 2006 – 2007 period. During the first period the prevalence of *E. coli* on red deer was 1.62 log cfu·cm^{-2}, roe deer was 2.03 log cfu·cm^{-2} and wild boar was 1.05 log cfu·cm^{-2}. During the second period much higher counts were obtained with red and roe deer having 2.78 log cfu·cm^{-2} present and wild boar 2.66 log cfu·cm^{-2}. The prevalence found in these studies is much higher than the 1.71 log cfu·cm^{-2} found for BB3 in the current study.

When it comes to food safety, *E. coli* are well known for their ability to cause illness if ingested, especially if they are of the pathogenic type (Viazis & Diez-Gonzalez, 2011). Due to this, meat products have to comply with the strict microbial standards set out by the Department of Agriculture, Forestry and Fisheries (DAFF) (DAFF, 2010). The acceptable *E. coli* range (1.7 – 2.7 log cfu·cm^{-2}) for meat products destined for export as set out in the regulations by DAFF is depicted in Table 5.2. Observing the results displayed in Figure 5.3 it can be noted that the counts obtained from all the animals were well below that of the upper acceptable limit. This would suggest that meat from these animals is safe to consume.

The prevalence of *Staphylococcus aureus* (log cfu·cm^{-2}) on the meat samples from animals harvested on Farm 2 is depicted in Figure 5.4. Almost all of the animals had 0.00 log cfu·cm^{-2}

counts except for BB4 that had a count of 0.13 log cfu·cm^{-2}, BB7 that had a count of 2.97 log cfu·cm^{-2} and SB2 that had a count of 0.36 log cfu·cm^{-2}.

Bhandare *et al.* (2007) found a *S. aureus* prevalence of 3.24 log cfu·cm^{-2} for goats directly after evisceration. This count were much higher than found in the current study except for BB7. Membré *et al.* (2011) also tested for *S. aureus* during the 2005 – 2006 and 2006 – 2007 period. During the first period the prevalence of *S. aureus* on roe deer was 0.08 log cfu·cm^{-2} and 0.00 log cfu·cm^{-2} for both red deer and wild boar. During the second period slightly higher counts were obtained with red and roe deer having 0.38 log cfu·cm^{-2} present and wild boar 0.43 log cfu·cm^{-2}. The counts obtained during these two periods are in line with what was found in the current study, not considering the BB7 outlier.

Due to *S. aureus*' ability to produce enterotoxins that cause food poisoning if ingested, it is also classified as a food safety organism (Schmitt *et al.*, 1990). The Department of Agriculture, Forestry and Fisheries have no regulations regarding the upper limit of *S. aureus* cells that may be present on meat. The infective dose of *S. aureus* required to cause food poisoning is approximately 5 log cfu·g^{-1} of food product (Bhatia & Zahoor, 2007). Comparing this with the highest count obtained for BB7 (2.97 log cfu·cm^{-2}) it can be speculated that the meat will be safe for consumption and will likely not cause food poisoning. Another factor to take into consideration is that *S. aureus* requires a temperature between 40 – 45°C to produce these enterotoxins (Smith *et al.*, 1983). As meat is held typically at a maximum of 4°C (assuming no temperature abuse occurs) *S. aureus* will not be able to produce the enterotoxins.

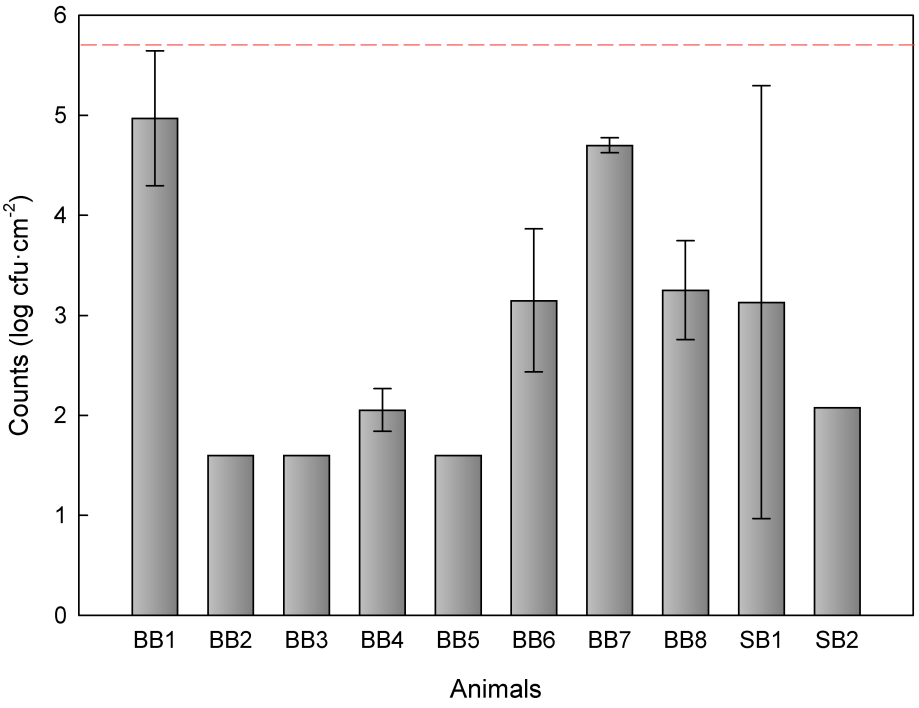

Figure 5.1 Prevalence of aerobic count (log cfu·cm^{-2}) on meat from Blesbok (BB) and Springbok (SB) harvested from Farm 2. Red line represents upper acceptable microbial limit according to DAFF regulations. Error bars were calculated based on a standard deviation at a 0.95 confidence interval.

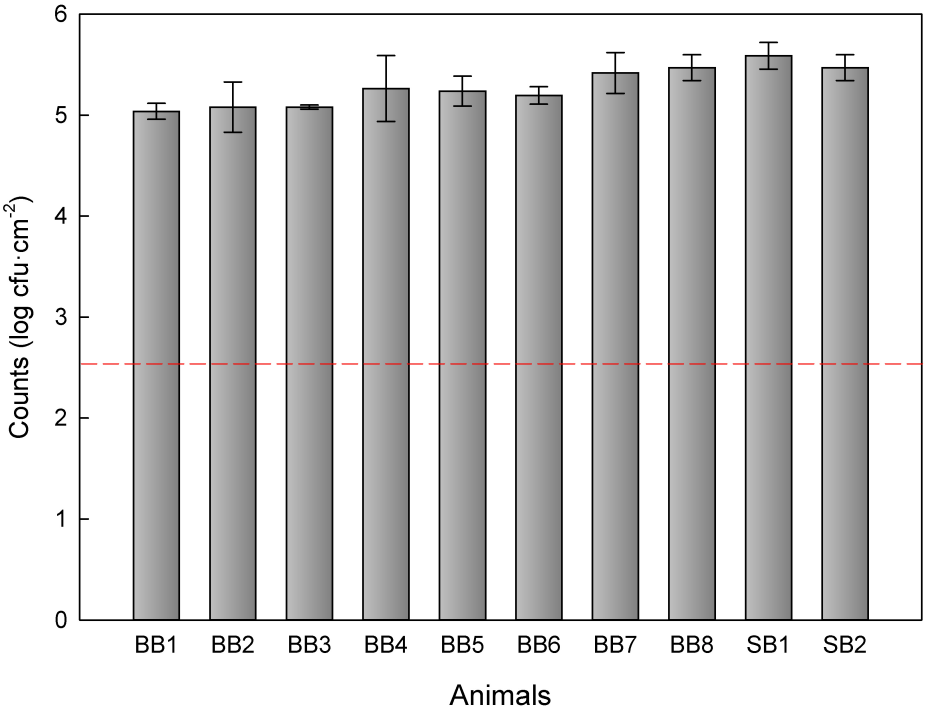

Figure 5.2 Prevalence of total coliforms (log cfu·cm^{-2}) on meat from Blesbok (BB) and Springbok (SB) harvested from Farm 2. Red line represents upper acceptable microbial limit according to DAFF regulations. Error bars were calculated based on a standard deviation at a 0.95 confidence interval.

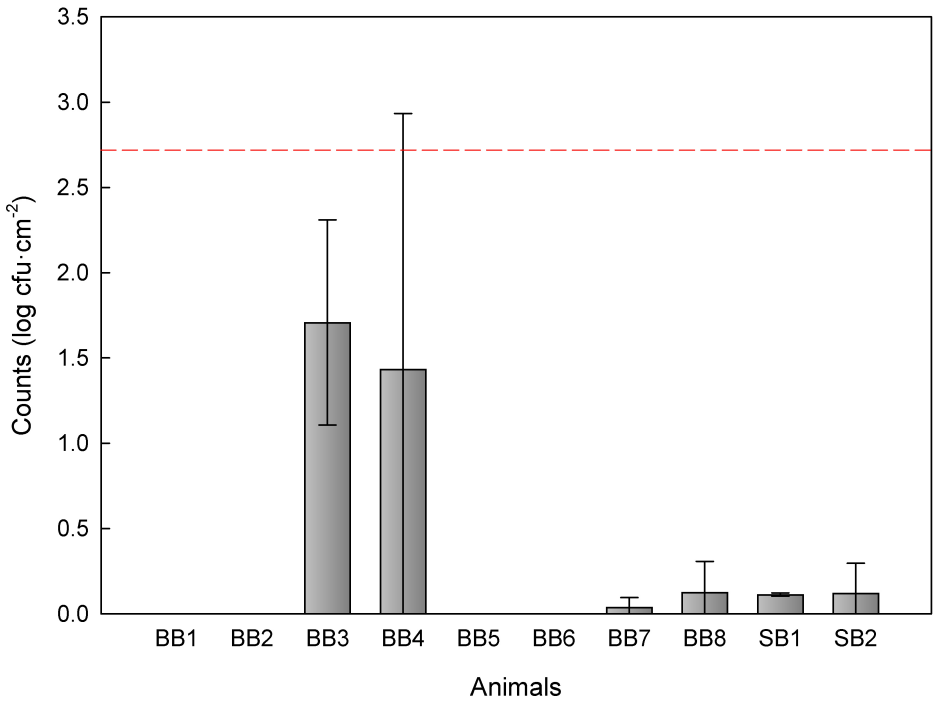

Figure 5.3 Prevalence of *Escherichia coli* (log cfu·cm^{-2}) on meat from Blesbok (BB) and Springbok (SB) harvested from Farm 2. Red line represents upper acceptable microbial limit according to DAFF regulations. Error bars were calculated based on a standard deviation at a 0.95 confidence interval.

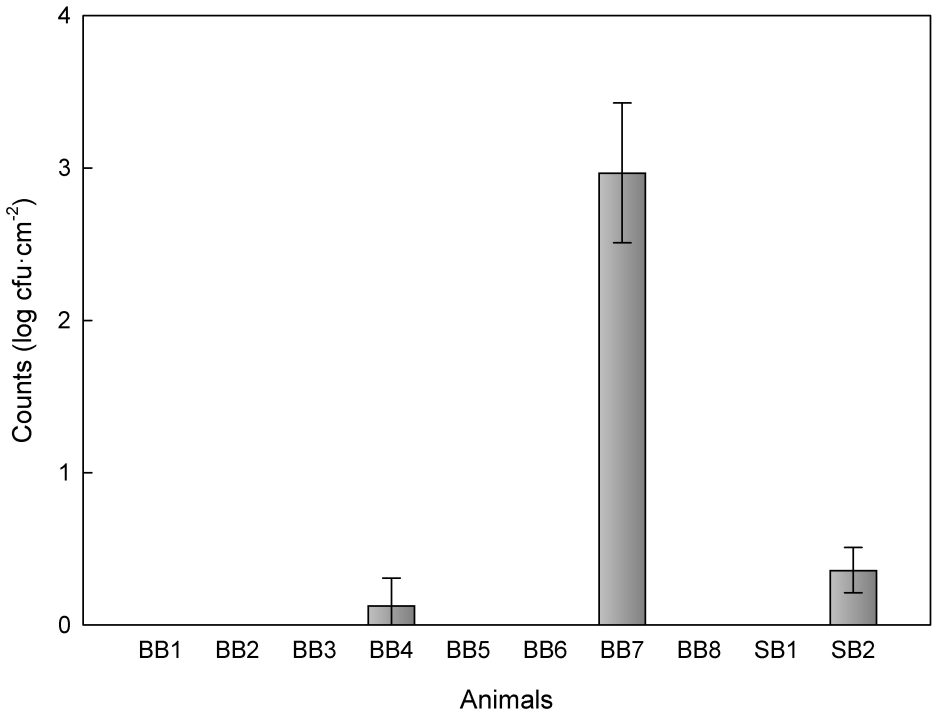

Figure 5.4 Prevalence of *Staphylococcus aureus* (log cfu·cm^{-2}) on meat from Blesbok (BB) and Springbok (SB) harvested from Farm 2. Error bars were calculated based on a standard deviation at a 0.95 confidence interval.

The prevalence of aerobic count (log cfu·g^{-1}) isolated from the faecal matter of animals harvested from Farm 2 is depicted in Figure 5.5. Aerobic count prevalence ranged from 5.78 log cfu·g^{-1} (BB6) to 6.44 log cfu·g^{-1} (SB1 and SB2). Total coliforms prevalence (log cfu·g^{-1}) isolated from the faecal matter of animals harvested from Farm 2 is depicted in Figure 5.6. The prevalence of total coliforms ranged from 6.53 log cfu·g^{-1} (BB2) to 7.04 log cfu·g^{-1} (BB8). *Escherichia coli* prevalence (log cfu·g^{-1}) isolated from the faecal matter of animals harvested from Farm 2 is depicted in Figure 5.7. The prevalence of *Escherichia coli* ranged from 3.00 log cfu·g^{-1} (BB1, BB6, BB7, BB8, SB1 and SB2) to 4.54 log cfu·g^{-1} (BB3). The prevalence of *Staphylococcus aureus* (log cfu·g^{-1}) isolated from the faecal matter of animals harvested from Farm 2 is depicted in Figure 5.8. *Staphylococcus aureus* prevalence ranged from 3.63 log cfu·g^{-1} (BB2, BB3, SB1 and SB2) to 4.40 log cfu·g^{-1} (BB4 and BB8).

The results obtained from the PCR amplification of the faecal samples showed that all samples tested negative for both *Salmonella* and *Listeria*. Diaz-Sanchez *et al.* (2013) found a *Salmonella* prevalence of 1.2% for wild boar and 0.3% for deer in their faecal matter. Avagnina *et al.* (2012) found a *Listeria* prevalence of 2.8% for a group of game tested (chamois, red deer, roe deer and wild boar) but no *Salmonella* was detected in any of the animals' faecal matter.

Considering the high log counts detected in the faecal matter for the above-mentioned bacteria, it is understandable that some of the carcasses had such high microbial contamination on them. The hides of wild animals naturally contains microbial contamination due to faecal matter present in the field as well as the environment they reside in (Bell, 1997). When skinning of the carcass is not performed properly, some of these microorganisms present on the hide can be transferred to the carcass, contaminating the meat (Blagojevic *et al.*, 2011; Nørrung & Buncic, 2008). It is postulated that this is what occurred in this trial, as the skinners were all inexperienced and cross contamination between faecal matter, the outer surface of the skin and the carcass could have occurred. Considering the high prevalence of both aerobic bacteria as well as coliforms in the faecal matter (Figures 5.5 and 5.6), the above stated speculation could thus explain the high prevalence of these bacteria on the meat after dressing.

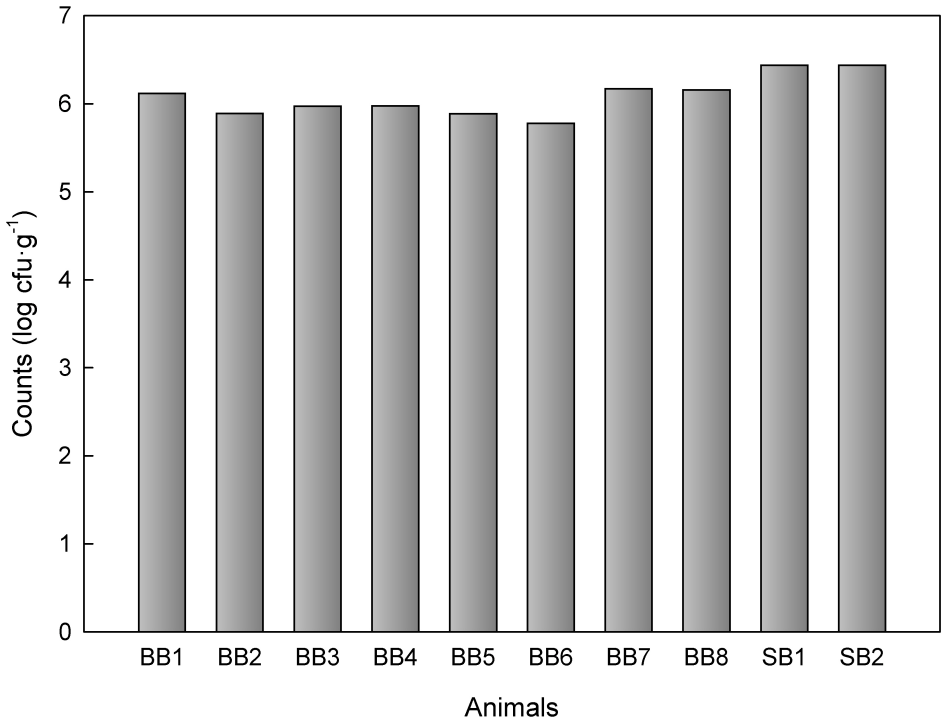

Figure 5.5 Prevalence of aerobic count (log cfu·g^{-1}) isolated from the faecal matter of from Blesbok (BB) and Springbok (SB) harvested from Farm 2.

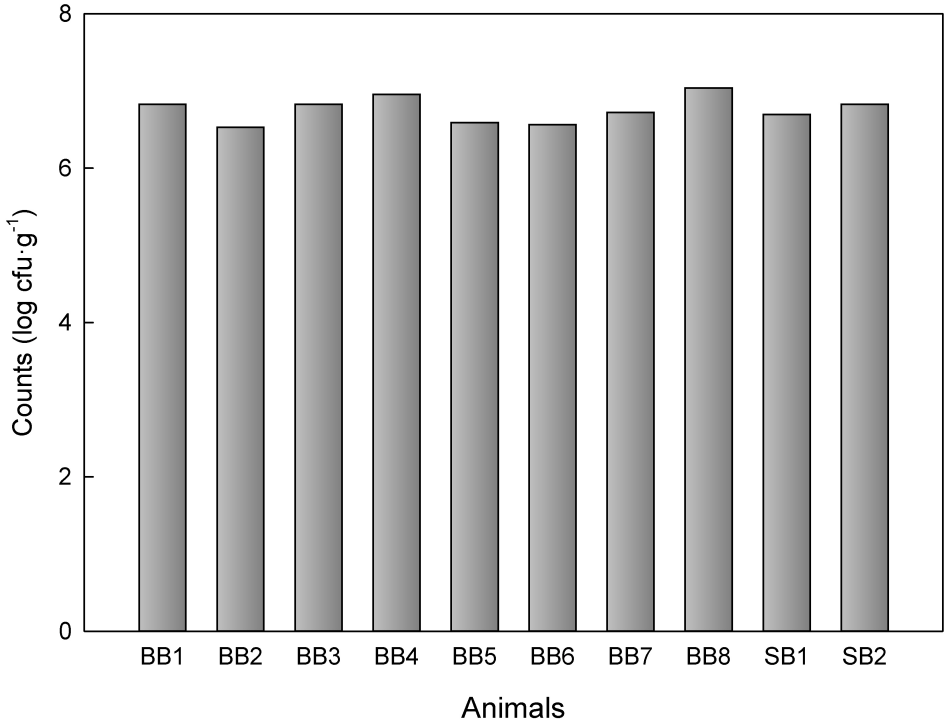

Figure 5.6 Prevalence of total coliforms (log cfu·g^{-1}) isolated from the faecal matter of from Blesbok (BB) and Springbok (SB) harvested from Farm 2.

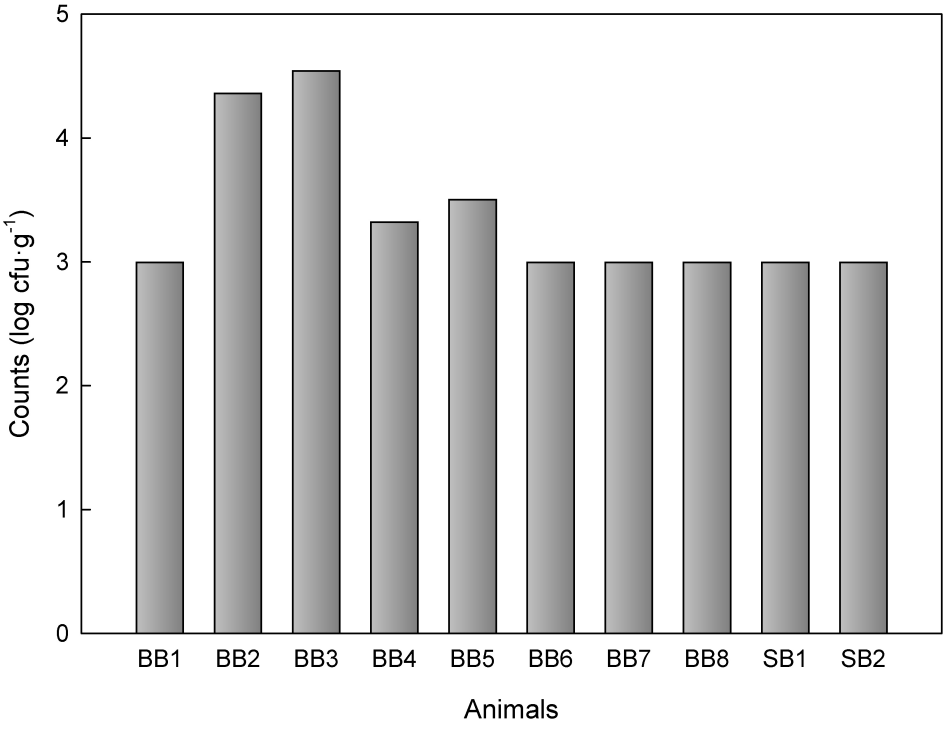

Figure 5.7 Prevalence of *Escherichia coli* (log cfu·g^{-1}) isolated from the faecal matter of from Blesbok (BB) and Springbok (SB) harvested from Farm 2.

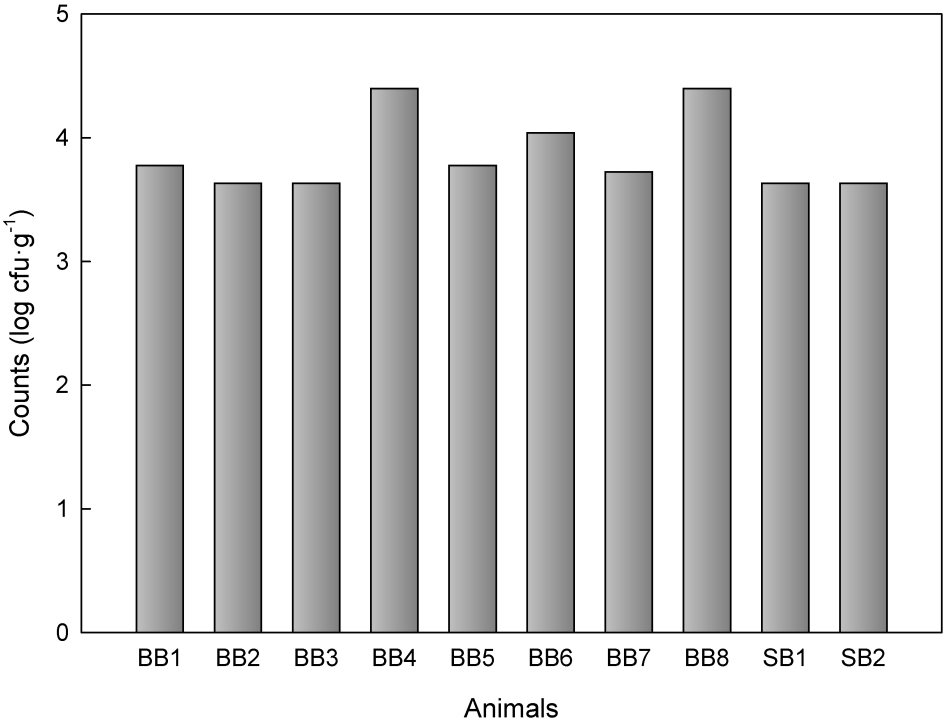

Figure 5.8 Prevalence of *Staphylococcus aureus* (log cfu·g^{-1}) isolated from the faecal matter of from Blesbok (BB) and Springbok (SB) harvested from Farm 2.

5.5 CONCLUSIONS

The aim of this study was to determine the microbial population present on South African game carcasses after dressing to determine whether the meat would meet the microbiological criteria in terms of food spoilage and food safety aspects.

Aerobic count prevalence on the meat ranged from 1.60 – 4.97 log cfu·cm^{-2} with BB1 and BB7 having the highest counts. The prevalence of total coliforms varied from 5.04 – 5.59 log cfu·cm^{-2}. *E. coli* prevalence ranged from 0.00 – 1.71 log cfu·cm^{-2} while *S. aureus* varied from 0.00 – 2.97 log cfu·cm^{-2}. The high *S. aureus* count seen for BB7 (2.97 log cfu·cm^{-2}) was due to contamination present on the carcass. Taking into consideration the results obtained for the meat as well as the upper limits for export regulations by DAFF, it can be noted that the meat is mostly uncompromised in terms of food spoilage. The aerobic counts were below the upper limits but the total coliforms count exceeded these limits. It was further also noted that all carcasses were within the upper limits in terms of food safety meaning all the meat was safe for consumption.

The aerobic count prevalence in the faecal matter of the animals ranged from 5.78 – 6.44 log cfu·g^{-1} and total coliforms ranged from 6.53 – 7.04 log cfu·g^{-1}. *E. coli* prevalence varied from 3.00 – 4.54 log cfu·g^{-1} while *S. aureus* ranged from 3.63 – 4.40 log cfu·g^{-1}. All the faecal samples tested negative for both *Salmonella* and *Listeria*. Considering these high counts detected in the faecal matter it is very possible that cross-contamination from the hide to carcass could have occurred during the dressing process.

Recommendations for future studies would include expanding the geographical range including more farms from around South Africa. As this study only included springbok and blesbok, it is recommended to include a wider variety of species as well as increase the sample size of each species. *Enterobacteriaceae* should also be included in future studies to be more comparable with legislation. A last aspect that could possibly be evaluated is if the level of contamination would change if the personnel underwent specific training on slaughtering hygienically.

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CHAPTER 6
DEVELOPMENT AND IMPLEMENTATION OF AN ORGANIC ACID SPRAY FOR THE REDUCTION OF MICROBIAL GROWTH ON GAME CARCASSES

6.1 ABSTRACT

As the demand for game meat grows globally, there is an increased pressure on the meat industry to ensure that the meat is microbiologically safe for human consumption. The aims of this study were to develop an organic acid spray that would be implemented in a field study to determine its efficacy in order to reduce microbial growth present on game carcasses. During optimisation trials, lactic acid, acetic acid, citric acid, octanoic acid, sodium benzoate as well as mixtures of these were tested to determine its efficacy to reduce bacteria suspended in Luria-Bertani broth. Lactic acid (2%) in combination with octanoic acid (0.5%) showed a 3.89 log cfu·mL^{-1} reduction for *Escherichia coli* (ATCC 25922) and a 7.60 log cfu·mL^{-1} reduction for *Salmonella* (ATCC 13076). This combination was used to determine its efficacy on artificially contaminated meat. The log reductions drastically decreased from those observed in the Luria-Bertani broth. The increased lactic acid concentration (5%) with the added sodium benzoate (0.1%) resulted in a 0.59 log cfu·g^{-1} reduction for *Escherichia coli* K-12, 1.33 log cfu·g^{-1} reduction for *Escherichia coli* (ATCC 25922) and a 0.33 log cfu·g^{-1} reduction for *Salmonella* (ATCC 13076), which were the highest achieved. This trend was not observed for *Staphylococcus aureus* (ATCC 25923) where a 0.16 log cfu·g^{-1} reduction was achieved. This organic acid mixture was implemented on meat samples (taken in the flank region) from eight Blesbok (*Damaliscus pygargus phillipsi*) and two Springbok (*Antidorcas marsupialis*) that were harvested from a farm in the Western Cape, South Africa. The desired log reductions were not achieved. *E. coli* reductions ranged from 0.088 – 1.831 log cfu·25 cm^{-2} whereas total coliforms achieved even lower log reductions ranging from 0.014 – 0.425 log cfu·25 cm^{-2}. Although reductions achieved for aerobic count was in line with that of *E. coli* and total coliforms, Blesbok 1 displayed a 3.369 log cfu·25 cm^{-2} reduction. *S. aureus* also followed the same trend as *E. coli* with reductions ranging from 0.161 – 1.567 log cfu·25 cm^{-2}. The overall log reductions achieved in the implementation study was not satisfactory. Further research should be conducted to improve on this mixture as well as explore different treatment application techniques.

6.2 INTRODUCTION

The consumption of game meat on a global scale is increasing with every year that passes. In this regard South Africa is no exception (Reilly *et al.*, 2003; Taylor *et al.*, 2015). These increases in

consumption give rise to a range of difficulties for the game meat industry in terms of the microbial safety of the meat. As game meat originates from animals that life in the wild, careful attention has to be given to comply with the strict health and safety regulations to ensure that the meat is safe for human consumption (Atanassova *et al.*, 2008).

The deep tissue of meat is generally accepted as being sterile, provided the animal is healthy (Mackey & Derrick, 1979). The harvesting process of animals can introduce microbial contaminations onto the once sterile meat. This is particularly relevant in cases where the animal is shot in the field and internal organs become damaged. This open wound provides the perfect environment for microbial contamination in the field setting (Mackey & Derrick, 1979; Gill, 2007). Further contamination of the meat can occur during dressing of the carcass (van Schalkwyk & Hoffman, 2016). The hides of wild animals naturally contain microorganisms. This is mostly due to their surroundings as well as contamination of the hide with faecal matter present in the field (Nørrung & Buncic, 2008; Blagojevic *et al.*, 2011).

Decreasing the microbial contamination on carcasses after dressing is an integral part in not only ensuring the meat is safe for consumption but also to decrease the possibility of spoilage. Three of the most widely used microbial interventions in the meat industry include hot water washes, steam pasteurisation of carcasses as well as organic acid carcass washes.

The use of hot water washes in meat processing plants are readily used to remove environmental contaminants such as soil and faecal matter from carcasses (Loretz *et al.*, 2011). Hot water washes are only effective if the water is heated to at least 75°C (Pipek *et al.*, 2005). Hot water treatments can be applied at two different processing steps. The first is application to the hide to remove as many contaminants as possible before skinning occurs. This will inherently decrease the amount of cross contamination from the hide to the carcass. The second is to the carcass after skinning to remove any contaminants that could have been carried over during the skinning process. The use of a combination of the two will lead to maximum reduction of contaminants (Small *et al.*, 2005; Sofos & Smith, 1998). Gill *et al.* (1995) showed a 2 log reduction of spoilage bacteria on pig carcasses that had been treated with 85°C water for 20 s. It is important to note that heat damage of the meat can occur if the water temperature is too high and the treatment time is too long (Pipek *et al.*, 2005).

Another effective method of reducing the microbial load on carcasses is the use of steam pasteurisation. This involves exposing the carcasses to steam at 82 – 97°C inside a chamber for approximately 6 – 12 s (Aymerich *et al.*, 2008). The advantage of steam pasteurisation is that there is a large amount of heat that is rapidly transferred to the carcass, increasing the surface temperature (James *et al.*, 2000). This is not the case with hot water washes. Aymerich *et al.* (2008) reported a 1 log cfu·cm^{-2} reduction for *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria innocua* on beef carcasses after steam at 93.3°C was applied for just over 6 s.

The use of organic acid washes in an abattoir setting has also proven to be a useful method to decontaminate carcasses. As organic acids are generally regarded as safe (GRAS) and are used in low concentrations (0.1 – 5%) it poses little risk to the consumer. The most widely used organic acids include lactic acid, acetic acid and citric acid (Barco *et al.*, 2015; Mani-López *et al.*, 2012; Brul & Coote, 1999; Hamby *et al.*, 1987).

Although the first two methods have been shown to work in an abattoir setting, the in-field use of these two methods is impractical. An intervention is required that is inexpensive to produce, requires little to no equipment, must be easy to use as well as be GRAS. The use of chemical agents such as organic acids or mixtures thereof possess the potential to reduce microbial growth on carcasses in the field.

The aim of this study was to develop an organic acid spray in laboratory trials to determine the decontamination efficacy of different organic acids and mixtures thereof. The second aim was to determine the efficacy of the developed organic acid spray in a field study on game carcasses.

6.3 MATERIALS AND METHODS

Optimisation of Organic Acid Spray

Trials 1 – 3

Two 25 mL Luria Bertani Broth's (LB-broth) (Biolab, Merck, South Africa) were prepared and inoculated with *Escherichia coli K-12* and *Salmonella enteritidi*s (ATCC 13076) respectively. The enrichments were incubated at 37°C for 24 h. After incubation, 5 mL aliquots of each enrichment were transferred to pre-sterilised McCartney bottles, one for the control and one for each treatment. The control sample received no treatment and the rest of the samples each received one of the treatments. The acid treatment concentration was based on the final concentration of the sample. After treatment, the control and treated samples were incubated at 37°C for 1 h. After incubation, serial dilutions (10^{-2} – 10^{-7}) were prepared for the control as well as the treatments. Plating was done in duplicate on Tryptic Soy Agar (TSA) (Biolab, Merck, South Africa) for all serial dilutions. All plates were incubated at 37°C for 24 h. The methodology for the first three trials were exactly the same, it was only the treatments that differed (Table 6.1).

Trials 4 – 5

Four 100 mL LB-broth's were prepared and inoculated with *Escherichia coli K-12, Escherichia coli* (ATCC 25922), *Salmonella enteritidi*s (ATCC 13076) and *Staphylococcus aureus* (ATCC 25923) respectively. The enrichments were incubated at 37°C for 24 h. Pieces of meat (± 10 g each) were prepared for the trials, one piece for the control and one piece for each treatment. These were prepared for each organism tested. After incubation the pieces of meat were submerged in the individual enrichments for 10 s. All meat pieces contaminated with the same organism were placed

into one bag and kept at 37°C for 30 min. After 30 min the pieces of meat received the treatments. During trial 4 every piece received 2 sprays (equal to 2 mL) of the acid treatment whereas in trial 5 they all received 5 sprays (equal to 5 mL) of acid treatment. The control sample received no treatment. After treatment, the control and treated samples were kept at 37°C for 1 h. After 1 h, serial dilutions (10^{-2} – 10^{-7}) were prepared for the control as well as the treatments. Plating was done in duplicate on TSA for all serial dilutions. All plates were incubated at 37°C for 24 h. The methodology for trials 4 – 5 were the same with only the treatments (Table 6.2) and treatment volumes that differed.

Table 6.1 Treatment composition for trials 1 – 3 of organic acid spray optimisation

Trial	**Treatment no.**	**Treatments**		
		Concentration (%)	Composition	Code
1	1	2	Acetic acid	AA
	2	2	Lactic acid	LA
	3	2	Citric acid	CA
2	1	2	Acetic acid	Mix 1
		2	Lactic acid	
		2	Citric acid	
	2	2	Acetic acid	Mix 2
		2	Lactic acid	
		2	Citric acid	
		0.5	Octanoic acid	
	3	0.5	Octanoic acid	OA
3	1	2	Lactic acid	Mix 3
		0.5	Octanoic acid	

Table 6.2 Treatment composition for trials 4 – 5 of organic acid spray optimisation

Trial	Treatment no.	Treatments		
		Concentration (%)	Composition	Code
4	1	2	Lactic acid	Mix 3
		0.5	Octanoic acid	
	2	2	Lactic acid	Mix 4
		0.1	Benzoic acid	
	3	2	Lactic acid	Mix 5
		0.5	Octanoic acid	
		0.1	Benzoic acid	
5	1	5	Lactic acid	Mix 6
		0.5	Octanoic acid	
		0.1	Sodium Benzoate	

Statistical Analysis

Statistical analyses of results were performed using Statistica 13.0 software (StatSoft, USA). Data was analysed by means of mixed model analysis of covariance (ANOVA). The Fisher least significant difference (LSD) test was also done to perform the different post hoc analyses. A 95% confidence interval was used to identify significant results i.e. a 5% significance level ($P \leq 0.05$) was used as the guideline.

Implementation of Organic Acid Spray

Harvesting

During this study, eight Blesbok (*Damaliscus pygargus phillipsi*) and two Springbok (*Antidorcas marsupialis*) were harvested from Farm 2 / Harvest 2 (refer to Chapter 4 for more details) in the Western Cape, South Africa. All animals were harvested according to the standard operating procedure (SU-ACUM14-001SOP) approved by the Stellenbosch University Animal Care and Use Committee.

Animals were shot and exsanguinated within a few minutes where after they were transported to the on-farm slaughtering facility. Carcasses were dressed (removal of head, legs and skin) and eviscerated according to the procedures described by van Schalkwyk & Hoffman (2010) and van Schalkwyk & Hoffman (2016). The slaughtering infrastructure on this farm was adequate to ensure that strict hygiene protocols could be followed. All skinned carcasses were transported back to the University of Stellenbosch in a cool truck (4°C). Upon arrival two pieces of meat (± 100 g each) were aseptically removed from the flank region of each carcass, vacuum packed and frozen (-18°C). These served as the control samples. The carcass then received the

Mix 6 (Table 6.2) treatment (carcasses were visibly wet), where after another two pieces of meat (± 100 g each) were aseptically removed from the flank region of each carcass, vacuum packed and frozen (-18°C). These served as the treated samples.

Sample preparation

Before analysis of the samples they were defrosted at 4°C overnight where after surface swabs (25 cm^2) of both the control and treated meat samples were taken and placed in 10 ml buffered peptone water (Biolab, Merck, South Africa). These samples were then used for analysis on the Tempo system (BioMérieux, South Africa) and Petrifilm (3M Company, St. Paul, MN, EUA).

Tempo system (BioMérieux)

Aliquots of the samples were transferred to the Tempo vials containing the reconstituted media. This was prepared by adding specific volumes of autoclaved distilled water to the media powder in the vials. *Escherichia coli* and *Staphylococcus aureus* tests were performed using one millilitre of the sample thus making a 1/40 Tempo dilution, whereas total coliforms test was performed using 0.1 ml of sample thus making a 1/400 Tempo dilution. The contents of the vials were transferred to the Tempo cards using the Tempo Filler. Each card consists of 48 wells, 16 of each of the three volumes (225, 22.5, 2.25 µL). *E. coli* and *S. aureus* cards were incubated at 37°C while totals coliform cards were incubated at 30°C (Owen *et al.*, 2010). All cards were incubated for 24 h where after the results were analysed on the Tempo Reader. This system makes use of software that detects which of the wells tested positive. The software uses the volumes of the positive wells as well as the dilution of the sample to mathematically calculate the $cfu \cdot cm^{-2}$ of the sample based on the Most Probable Number (MPN) tables.

Petrifilm

Aerobic count enumeration was done using the Petrifilm system. Using the samples used for the Tempo analyses a dilution series (10^{-2} – 10^{-7}) was prepared. One-millilitre aliquots of each dilution were inoculated onto AC (Aerobic count) petrifilms in duplicate. Using the spreader provided the inoculum was spread over the 20 cm^2 surface. The petrifilms were incubated at 30°C for 48 hours. All red colonies were counted as per the manufacturer's instructions.

6.4 RESULTS AND DISCUSSION

The aim was to develop an organic acid spray to be used on game carcasses to reduce microbial growth. When organic acids are introduced into the bacterial cells' environment, their main action is to disrupt the proton motive force (PMF) which plays an integral part in the survival of the cell (Willey *et al.*, 2011). In the electron transport chain, the PMF is generated at the final electron acceptor where the potential energy generated is used to synthesize ATP from ADP and P_i (Simon

et al., 2008). The PMF also functions as a transport mechanism transporting molecules directly into the cell without the use of ATP (Montville & Bruno, 1994).

The primary mechanisms by which organic acids influence the microbial activity of the bacterial cell includes the acidification of the cytoplasm leading to the interference of the energy production system as well as accumulation of dissociated acid anions till it reaches a toxic level (Mani-López *et al.*, 2012). A transmembrane gradient can be established when the pH of the environment is lower than that of the cellular cytoplasm. This occurs when non-dissociated acids diffuse through the microbial membrane creating an alkaline environment that in turn leads to the dissociation of the acid into an acid anion and a free proton (Ewadh *et al.*, 2013). The cell then tries to efflux these protons by exchanging them for other cations such as sodium or potassium (Mani-López *et al.*, 2012). It is believed that the cell makes use of active transport to efflux these protons, as the microbial membrane is impermeable to protons (Harold, 1972). This action is important to ensure the cellular interior maintains pH homeostasis (Brul & Coote, 1999).

The accumulation of the dissociated acid anions leads to the shifting of the internal pH to a range where it is no longer optimal for enzymatic activity, protein synthesis or DNA/RNA synthesis. Bacterial growth is greatly dependent on the pH and thus a reduction of the cytoplasmic pH in the bacterial cell can have a detrimental effect on the bacterium (Ewadh *et al.*, 2013). Further accumulation of the acid anion creates the driving force for cell inhibition as they ultimately hinder the PMF (Mani-López *et al.*, 2012; Willey *et al.*, 2011). The mode of action described above is for both Gram-negative and Gram-positive bacteria, although Gram-positive bacteria, such as *S. aureus*, have a defence mechanism to counteract the decrease in pH. This defence mechanism will be discussed at a later stage.

Optimisation

The log reductions obtained for *E. coli* K-12 for the first three trial's treatments are depicted in Figure 6.1. LA displayed the highest log reduction between the treatments of trial 1, although there was no difference ($p>0.05$) between AA, LA and CA. When observing the results of MIX 1, it can be noted that it had the same log reduction as the LA treatment although it was not significantly different ($p>0.05$) from AA, LA or CA. One might speculated that the AA and CA in MIX 1 might not have contributed as much as the LA. The addition of octanoic acid to MIX 1, creating MIX 2 showed higher log reductions with it being different ($p\leq0.05$) from the first four treatments. The OA treatment displayed the lowest log reduction of all treatments being different ($p\leq0.05$) from all other treatments. Based on the previous assumption that LA contributed the most to MIX 1 as well as the fact that the addition of octanoic acid to MIX 1 showed higher log reduction, MIX 3 was created being composed of only LA and OA. It can be noted (Figure 6.1) that MIX 3 displayed the highest log reduction differing ($p\leq0.05$) from all the other treatments.

The log reductions obtained for *Salmonella enteritidi*s (ATCC 13076) for the first three trial's treatments are depicted in Figure 6.2. During the first trial it can be noted that AA and LA displayed the highest log reductions but they were not different ($p>0.05$) from one another. CA displayed the lowest log reduction differing ($p\leq0.05$) from both AA and LA. During the second trial, all three treatments (MIX 1, OA and MIX 2) had the same log reduction being the highest of all the treatments. Although these three treatments did not differ ($p>0.05$) from one another, they differed ($p\leq0.05$) from all the other treatments. MIX 3 from trial 3 exhibited promising results in terms of log reductions for *Salmonella*. Although it was different ($p\leq0.05$) from those treatments in trial 2, the log reductions achieved were very satisfying. MIX 3 was chosen as the treatment to move onto the meat trials as it displayed the highest log reductions for *E. coli* K-12 as well it displaying promising results for *Salmonella.*

The fact that lactic acid (LA) produced such promising results was not unexpected as it is a small water-soluble molecule making it capable of moving through the water-filled porin proteins located on the outer membrane of bacteria (Alakomi *et al.*, 2000). It has also been suggested that LA has a potent outer-membrane disintegrating agent due to its ability to cause the release of lipopolysaccharides from the outer membrane. Lactic acid is also a natural compound produced by meat during post-mortem glycolysis. It has been suggested that the lactate anions are capable of slowing down the growth of microbes that may have survived, especially during the storage period (Pipek *et al.*, 2005).

The mechanism of medium chained fatty acids (MCFA) such as octanoic acid (OA) is not fully understood yet but it is believed they act as non-ionic surfactants, incorporating themselves into the cell membrane of the bacterium (Altieri *et al.*, 2009; Desbois & Smith, 2010). MCFA's can diffuse through the cell membrane of the bacterium creating pores, which then results in altered membrane permeability and ultimately cell death (Bergsson *et al.*, 2001).

Kim & Rhee (2013) performed a study where they combined a MCFA with an organic acid to determine the effect on the microbial cell membrane of *Escherichia coli* O157:H7. The use of octanoic acid alone resulted in a damaged cell membrane whereas the use of citric acid alone resulted in a cell membrane that was still fully intact. When these two were combined it resulted in a totally deformed cell membrane with its intracellular components being released. Following the results from this study, Kim & Rhee (2013) suggested that the use of a MCFA in combination with an organic acid might greatly increase the antimicrobial activity. As the MCFA damages the integrity of the cell membrane, the rate of organic acid diffusion into the cell rapidly increases. Damaging of the cell membrane also allows hydrogen ions to move freely into the cell, which would not have been possible if the cell membranes integrity was not compromised. The combined effect of organic acids being able to diffuse into the cell with greater ease and hydrogen ions being able to move into the cell freely aids in the accelerated acidification of the cells cytoplasm.

The log reductions obtained for *E. coli* K-12 for the fourth and fifth trial's treatments are depicted in Figure 6.3. All the treatments from trial 4 (MIX 3, MIX 4 and MIX 5) displayed negative log reductions meaning that the treated samples had higher microbial loads than the control samples. None of these three treatments differed ($p>0.05$) from one another. MIX 6 from trial 5 had the highest log reduction of all the treatments differing ($p\leq0.05$) from the other three treatments. Although MIX 6 displayed the highest log reduction, the 0.6 log cfu·g^{-1} reduction achieved was not sufficient.

The log reductions obtained for *Escherichia coli* (ATCC 25922) for the fourth and fifth trial's treatments are depicted in Figure 6.4. All the treatments from trial 4 (MIX 3, MIX 4 and MIX 5) displayed similar results with none of the three treatments differing ($p>0.05$) from one another. MIX 6 from trial 5 had the highest log reduction of all the treatments differing ($p\leq0.05$) from the other three treatments.

The log reductions obtained for *Salmonella enteritidi*s (ATCC 13076) for the fourth and fifth trial's treatments are depicted in Figure 6.5. From the graph it seems as if MIX 6 had the greatest log reduction whereas MIX 5 had a negative log reduction. This is once again due to the fact that the treated sample had higher microbial loads than the control sample. MIX 3 and 4 seems to be somewhere in the middle of these two previously mentioned treatments. Although these trends can be observed for the different treatments, it cannot be said with certainty that MIX 6 performed better than the rest. The reason for this is that there was no significant difference between any of the four treatments as the p-value of the F-test is greater than 0.05 ($p=0.13410$).

The log reductions obtained for *Staphylococcus aureus* (ATCC 25923) for the fourth and fifth trial's treatments are depicted in Figure 6.6. For *S. aureus,* MIX 3 displayed the highest log reduction differing ($p\leq0.05$) from all the other treatment. Although this treatment displayed the highest log reduction, the actual log reduction achieved was very low (0.434 log cfu·g^{-1}) and thus unsatisfying. MIX 4 showed the second highest log reduction but did not differ ($p>0.05$) from either MIX 5 or 6.

The reason for these insufficient results in terms of log reductions achieved may be due to *S. aureus'* ability to increase its internal pH combating the effects of the organic acid. Rode *et al.* (2010) found that when *S. aureus* cells were subjected to lactic acid treatment, they were able to increase the pH of the external environment and doing so increase their internal pH. It is believed they do this by metabolizing the acids and producing diacetyl (2,3-butanedione), ammonia and pyrazine. Headspace GC-MS analysis of lactic acid stressed *S. aureus* showed a 10-fold increase in the amount of pyrazines after a 20 h period.

In general, all the treatments for the fourth and fifth trials resulted in very low log reductions. It was speculated that this was due to the nature of the meat samples. As the meat pieces used did not have smooth surfaces, the enrichment containing the bacteria could have gone into the crevices on the surface of the meat where the organic acid treatment might not have reached

them. It was further speculated that the application of the spray onto the meat could have had an additional diluting effect making the organic acids used even weaker. The benzoic acid used for MIX 4 and MIX 5 in the fourth trial had difficulty dissolving which may have influenced the results. During the fourth trial, 2 mL of the organic acid mixture was applied to every treatment. It was speculated that this might not have been enough to effectively reduce the bacteria.

In the fifth trial (MIX 6) the benzoic acid was replaced with sodium benzoate, which fully dissolved. The volume of the organic acid mixture applied to each treatment was also increased to 5 mL. From figures 6.3 and 6.4 it can be noted that there were significant ($p \leq 0.05$) increases in the log reductions achieved for *Escherichia coli* K-12 and *Escherichia coli* (ATCC 25922) from MIX 5 to MIX 6. This same trend was also seen for *Salmonella enteritidis* (ATCC 13076) although these increases were not significant ($p > 0.05$). These trends were not the same for *Staphylococcus aureus* (ATCC 25923). MIX 6 was thus chosen as the treatment to be used during the implementation part of the organic acid spray study.

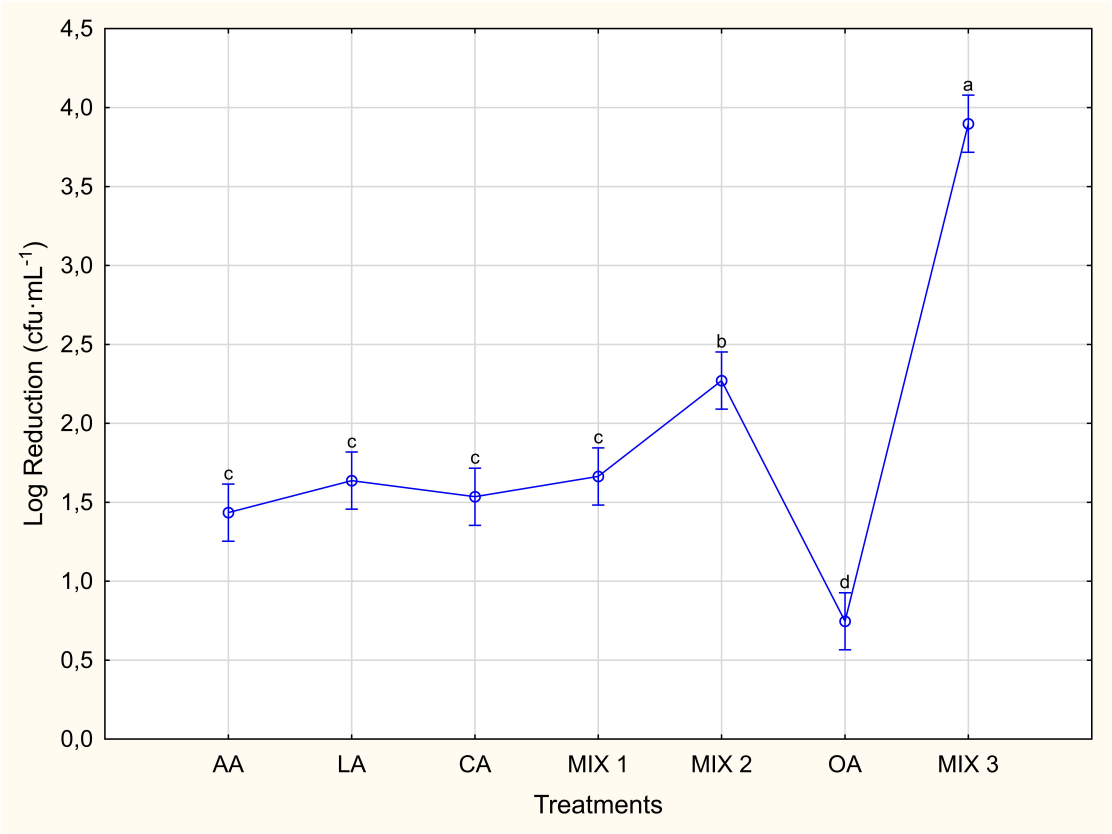

Figure 6.1 Log reductions obtained for *Escherichia coli* K-12 for treatments from Trials 1 – 3. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

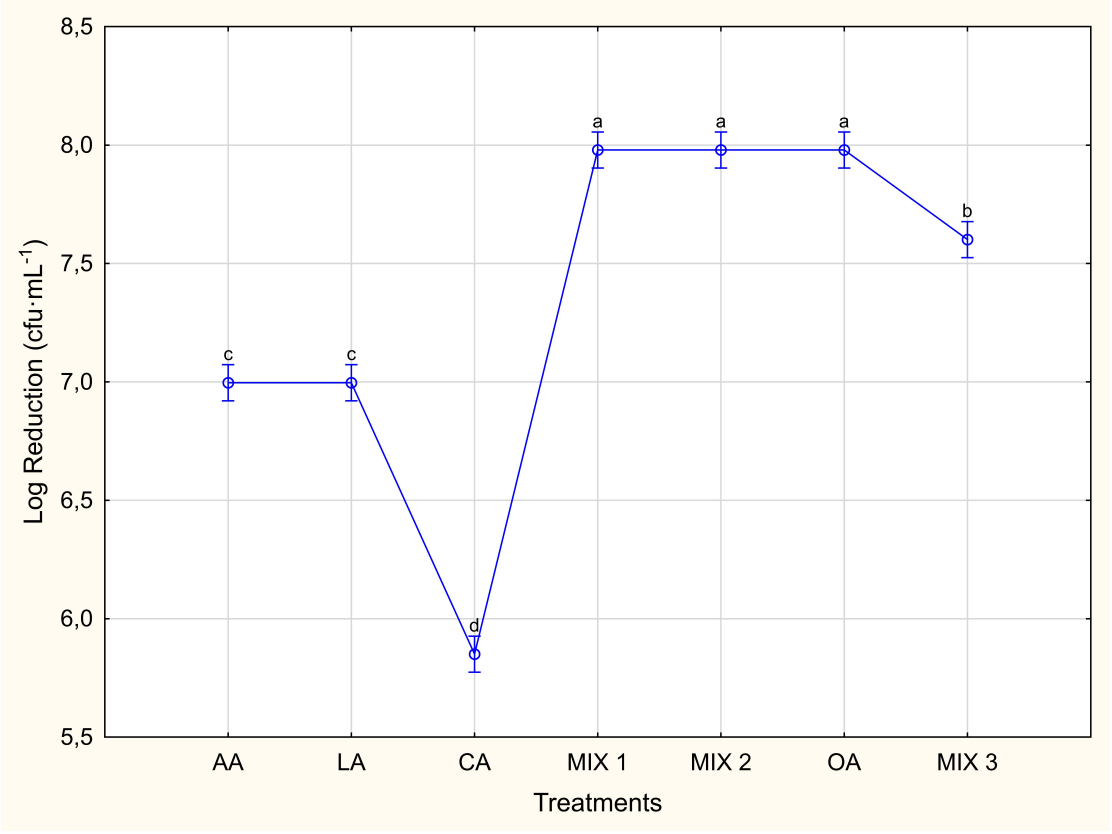

Figure 6.2 Log reductions obtained for *Salmonella enteritidis* (ATCC 13076) for treatments from Trials 1 – 3. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

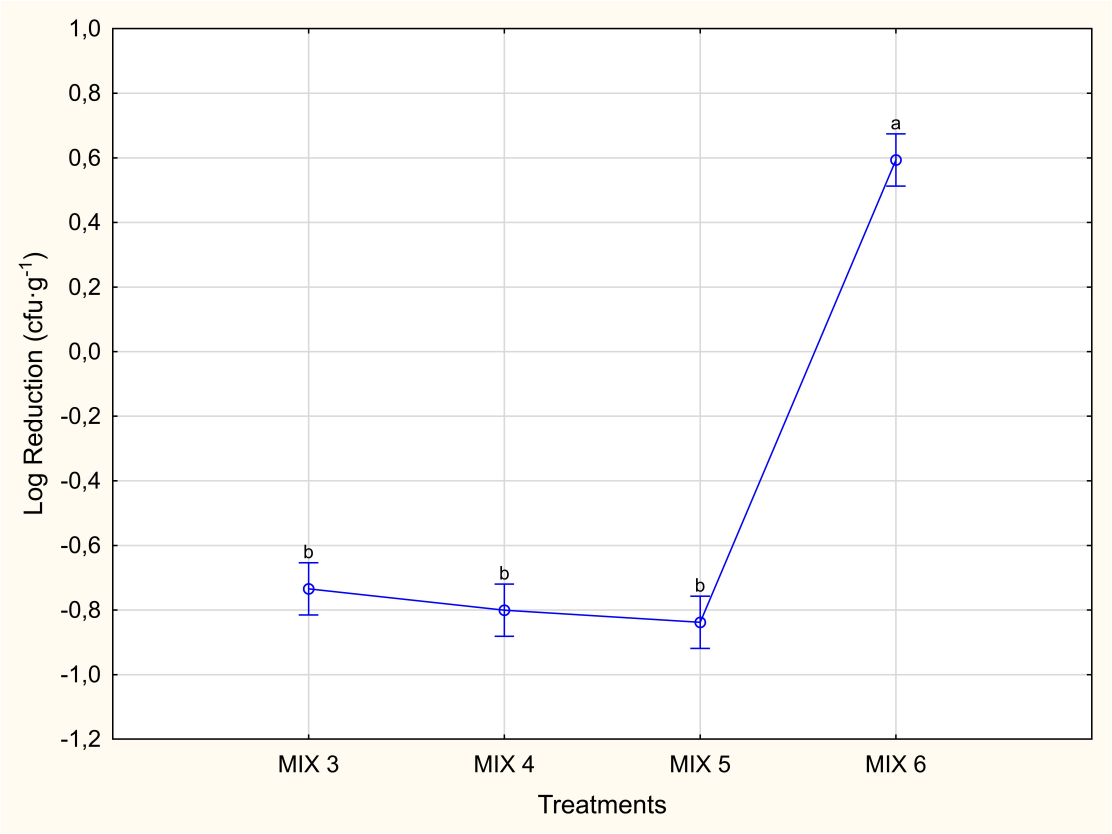

Figure 6.3 Log reductions obtained for *Escherichia coli* K-12 for treatments from Trials 4 – 5. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

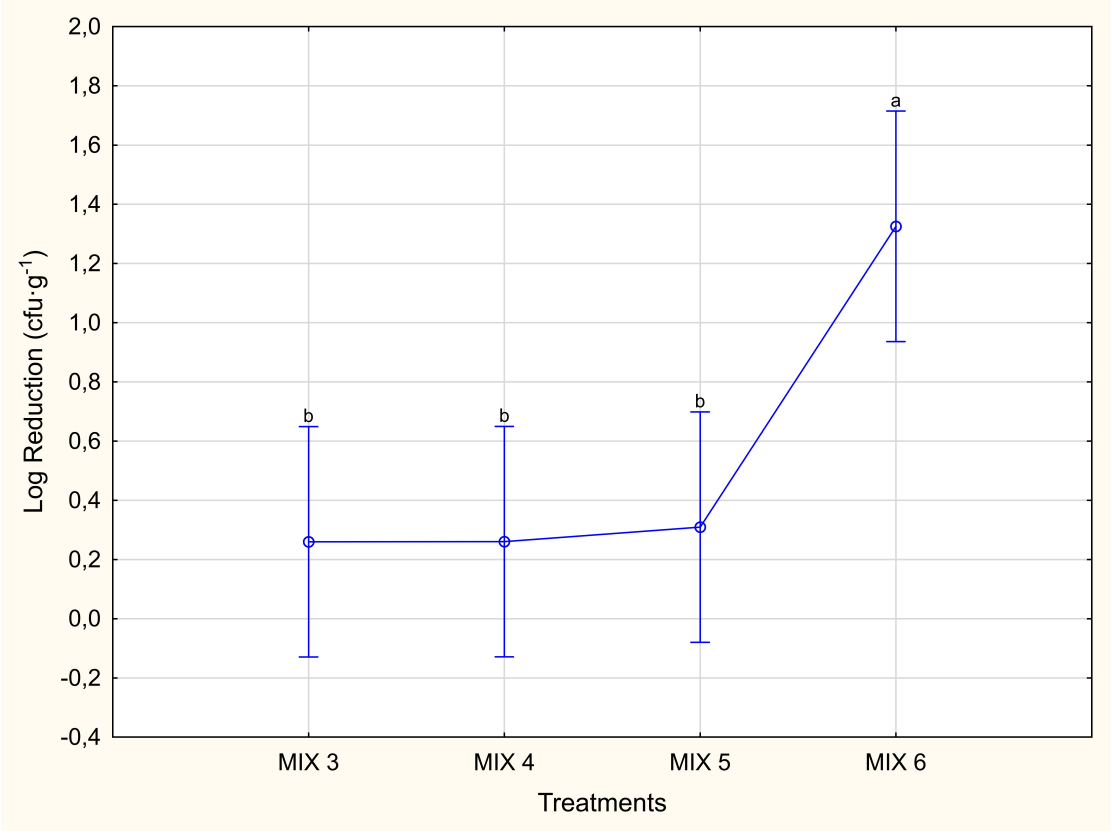

Figure 6.4 Log reductions obtained for *Escherichia coli* (ATCC 25922) for treatments from Trials 4 – 5. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

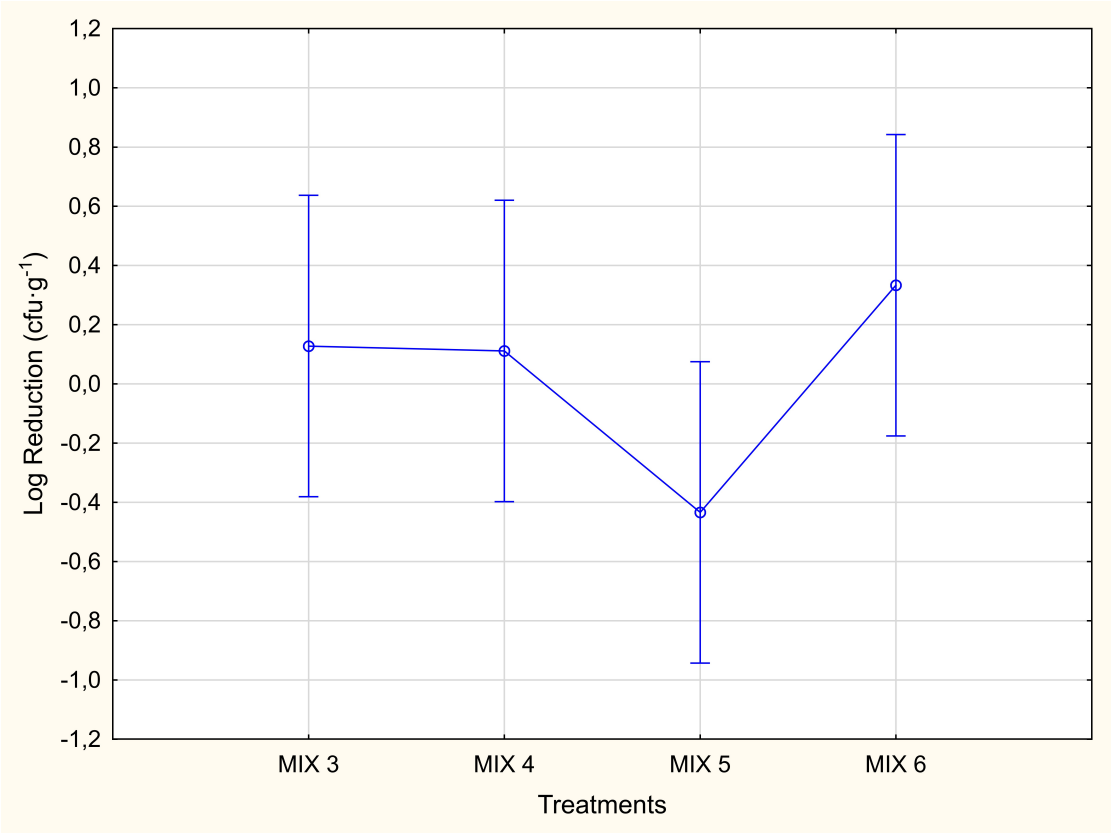

Figure 6.5 Log reductions obtained for *Salmonella enteritidi*s (ATCC 13076) for treatments from Trials 4 – 5. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

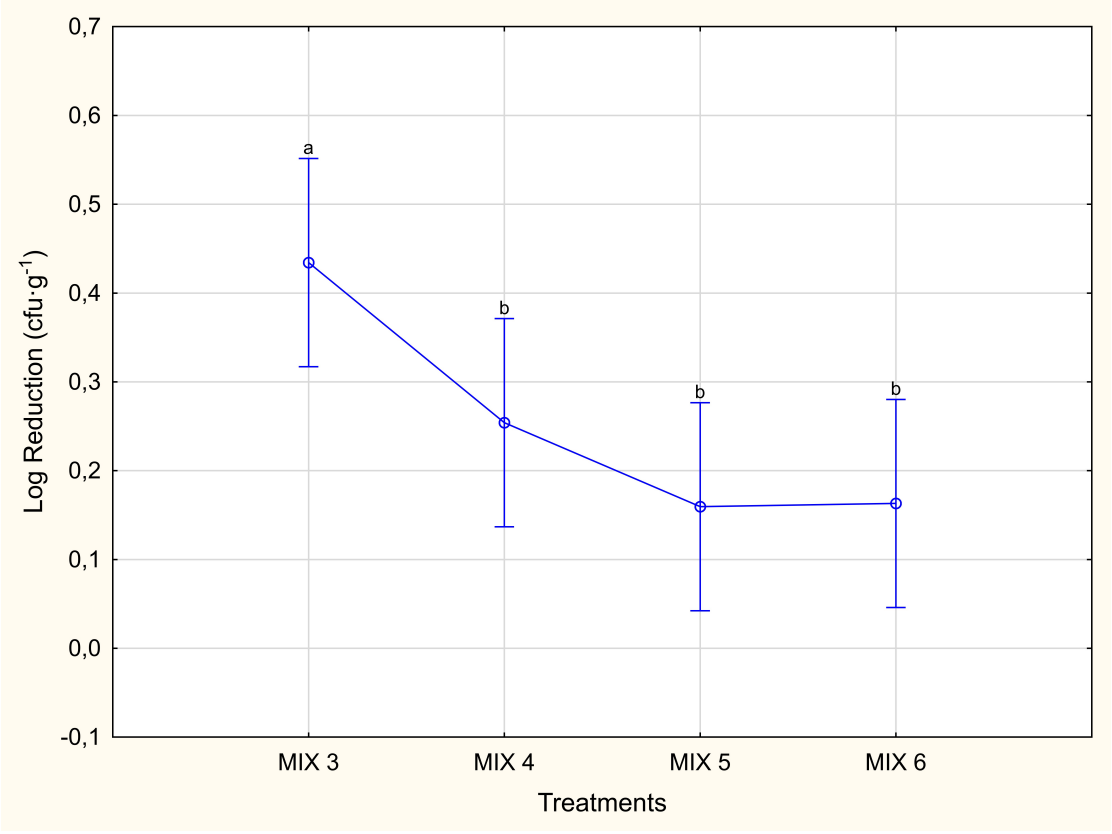

Figure 6.6 Log reductions obtained for *Staphylococcus aureus* (ATCC 25923) for treatments from Trials 4 – 5. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

Implementation

The log reductions (cfu·25 cm^{-2}) for *Escherichia coli* for the animals from Harvest 2 are depicted in Figure 6.7. The overall log reductions achieved were incredibly low ranging from 0.088 log (BB8) to 0.669 log (BB3) with BB4 being the outlier with a 1.831 log reduction. It was also noted that BB4 had a high standard deviation compared to that of the other animals. This variation can be explained in terms of the initial microbial load present on the two control samples. The one sample had a substantially higher initial microbial load than the other one. This variation can be expected as the microbial load distribution on a carcass can vary greatly from one site to another. It was further noted that BB2, BB5, BB6 and BB7 displayed no log reductions. This was due to the initial microbial load on the samples being less than 10 cfu·25 cm^{-2}. Log reductions cannot be obtained from such low initial counts.

Figure 6.8 depicts the log reductions (cfu·25 cm^{-2}) for total coliforms for the animals from Harvest 2. It can be noted that the overall log reductions were even lower than those reported for *Escherichia coli*. The log reductions ranged from 0.014 log (BB3) to 0.425 log (BB5). A large standard deviation can once again be noted for BB5, but as previously stated for *E. coli*, this deviation can be explained. Bosilevac *et al.* (2006) found a 1.0 log cfu·100 cm^{-2} reduction for *Enterobacteriaceae* (includes both *E. coli* and total coliforms) using 2% lactic acid applied at 42°C. This is comparable to results found for *E. coli* but was higher than that found for total coliforms. It was also found that the addition of a 74°C hot water wash for 5.5 s prior to the application of the lactic acid yielded a 2.5 log cfu·100 cm^{-2} reduction.

Aerobic count log reductions (cfu·25 cm^{-2}) for the animals from Harvest 2 are depicted in Figure 6.9. The overall log reductions achieved for aerobic count were in line with those for *E. coli* and total coliforms. The two exceptions where the log reductions were substantially higher than the rest were for BB6 that had the second highest log reduction (1.397 log) and BB1 that had the highest log reduction (3.369 log). It was further noted that BB2, BB3 and BB5 samples displayed no log reductions. The samples from these animals did not display the same trend as *Escherichia coli* where the initial load was too low. These samples all had sufficient initial microbial loads for log reductions to have taken place, but seeing as no reductions were observed it was speculated that the spray might not have worked on these samples. Bosilevac *et al.* (2006) found a 1.6 log cfu·100 cm^{-2} reduction for aerobic count using 2% lactic acid applied at 42°C. This reduction seems higher than that found in this study but taking into consideration that their sampling area was four times that used in this study, these results compare relatively well.

The log reductions (cfu·25 cm^{-2}) for *Staphylococcus aureus* for the animals from Harvest 2 are depicted in Figure 6.10. The overall log reductions achieved were, as all the other organisms, very low ranging from 0.161 log (BB3, BB5 and BB8) to 0.758 log (SB2) with BB7 having the highest log (1.567) reductions of all the animals. During analysis of the samples it was noted that one of the BB7 control samples was heavily contaminated with *Staphylococcus aureus* explaining

why the reduction achieved might have been so high. This can also explain the large standard deviation that can be observed for BB7. No log reductions were achieved for BB1, BB2, BB6 and SB1, as the initial microbial load on the samples were less than 10 cfu·25 cm^{-2}. Raftari *et al.* (2009) obtained a log reduction of 1.69 log $cfu \cdot g^{-1}$ for *S. aureus* using 2% lactic acid on artificially contaminated meat.

The overall reductions achieved for all samples were very low. One of the main reasons behind these low log reductions achieved could be that the surface of the meat is quite rough and has many pores. Bacteria get lodged into these pores and due to the surface tension on the meat the organic acid spray cannot enter these pores. The bacteria in these pores are thus unaffected and can proliferate as normal (Pipek *et al.*, 2005).

Another aspect that plays a crucial role in the efficacy of an organic acid treatment is the temperature at which the treatment is applied to the carcass. Cutter (1999) and Cutter & Rivera-Betancourt (2000) showed that 2% acetic acid applied at 35°C and 40°C to the surface of beef carcasses caused log reductions of 2.6 log $cfu \cdot cm^{-2}$ and 4.9 log $cfu \cdot cm^{-2}$ respectively for *Salmonella typhimurium.* During this study the organic acid mixture was applied at ambient temperature. Taking into account the results of the two studies stated above it can be speculated that if the application temperature was to be increased, instead of using ambient temperature, higher log reductions might be achieved. The application of the ambient temperature spray on the cold carcass could also have cooled down the spray reducing its effectiveness. Although the application of organic acid treatments at higher temperatures would produce higher log reductions, the in-field practicality of this is still a limiting factor.

It was further noted that the octanoic acid, even at a concentration as low as 0.5%, used in MIX 6 had a very unpleasant and pungent smell. In addition to the smell, it also caused discoloration of the subcutaneous fat on the carcass turning it light brown.

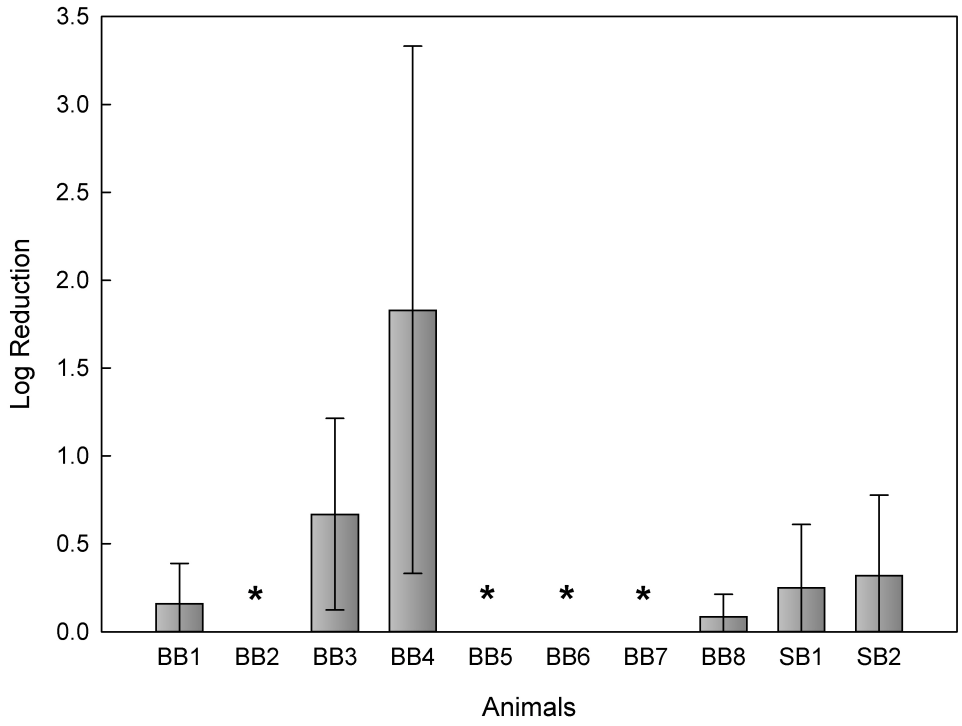

Figure 6.7 Log reductions (cfu·25 cm^{-2}) obtained for *Escherichia coli* for from Blesbok (BB) and Springbok (SB) from harvest two. Error bars were calculated based on standard deviation at a 0.95 confidence interval. Asterisk (*) indicates samples where the initial microbial load was too low for a reduction to have taken place.

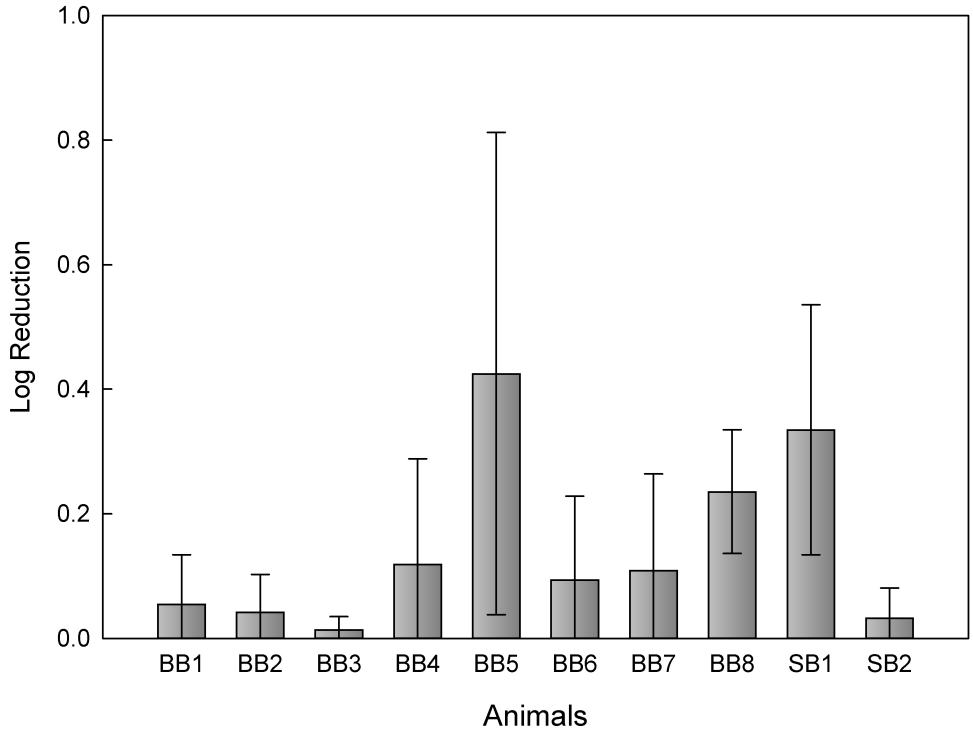

Figure 6.8 Log reductions (cfu·25 cm^{-2}) obtained for total coliforms for from Blesbok (BB) and Springbok (SB) from harvest two. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

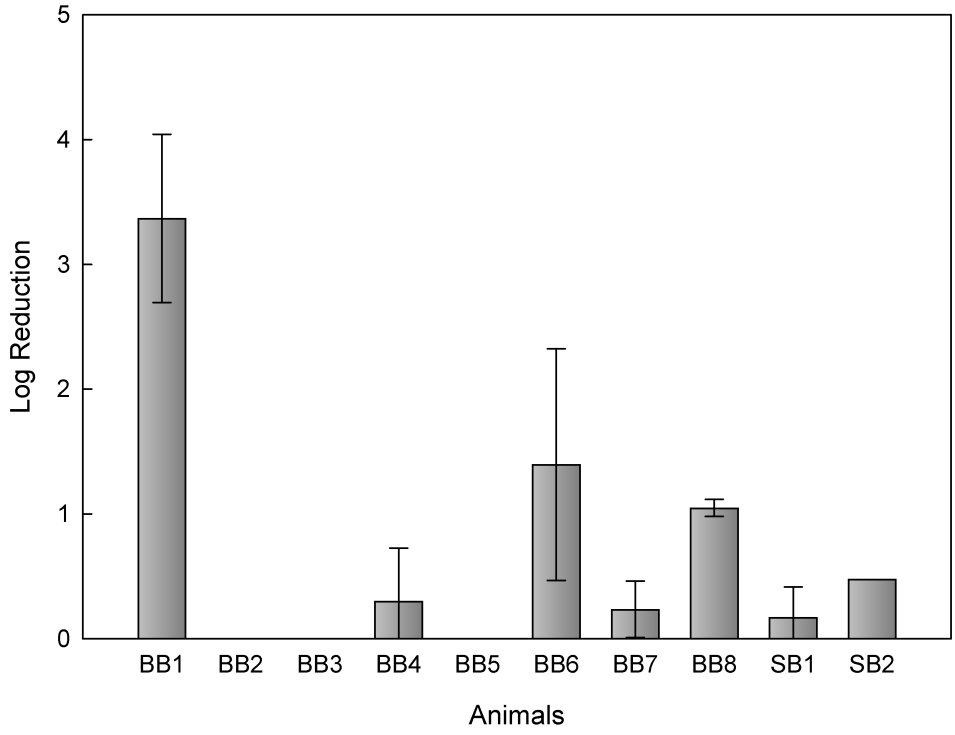

Figure 6.9 Log reductions (cfu·25 cm^{-2}) obtained for aerobic count for from Blesbok (BB) and Springbok (SB) from harvest two. Error bars were calculated based on standard deviation at a 0.95 confidence interval.

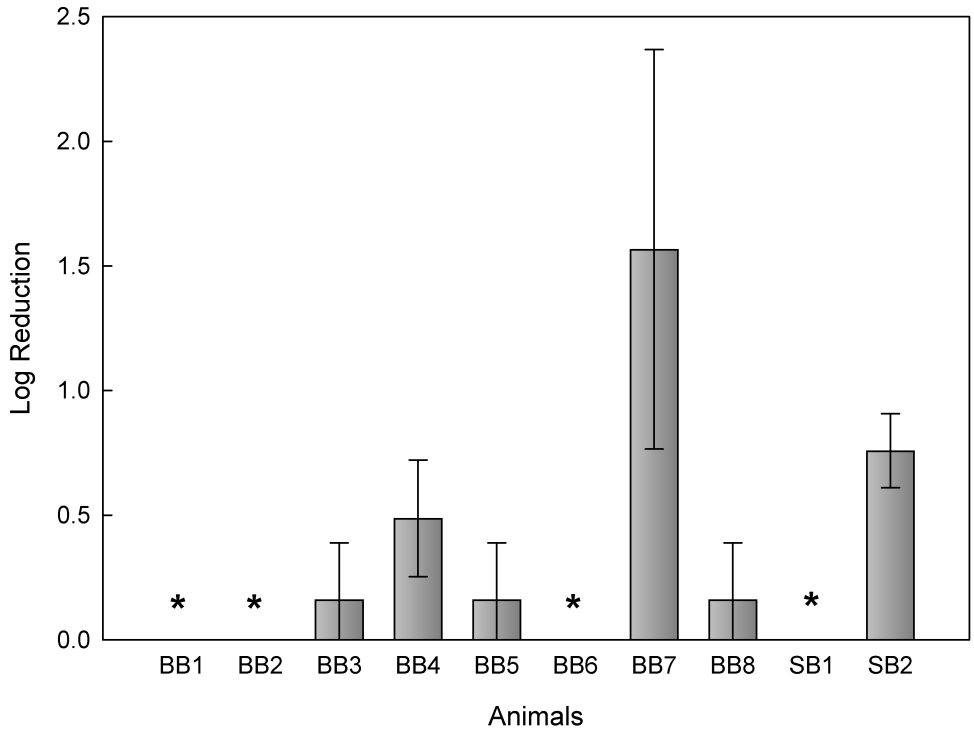

Figure 6.10 Log reductions (cfu·25 cm^{-2}) obtained for *Staphylococcus aureus* for from Blesbok (BB) and Springbok (SB) from harvest two. Error bars were calculated based on standard deviation at a 0.95 confidence interval. Asterisk (*) indicates samples where the initial microbial load was too low for a reduction to have taken place.

6.5 CONCLUSIONS

The aims of this study were to firstly develop an organic acid spray in laboratory trials to determine the decontamination efficacy of different organic acids and mixtures thereof. The second aim was to determine the efficacy of the developed organic acid spray in a field study on game carcasses.

The development study consisted of five separate trials. Trials 1 – 3 were conducted in enrichment mediums. Results for *E. coli* K-12 showed that no differences were seen for the LA, AA, CA and MIX 1 treatments. The addition of octanoic acid in MIX 2 significantly improved the efficacy of the MIX 1 treatment. In the third trial AA and CA was removed from MIX 2 creating MIX 3, which had the highest log reduction of all the treatment. These same trends were not observed for *Salmonella*, as the AA and LA treatments performed much better than the CA treatment. No differences were observed for the MIX 1, OA and MIX 2 treatments. The log reductions obtained for MIX 3 was slightly lower than that of the previously mentioned treatments. Despite this, MIX 3 was chosen as the treatment to move onto the meat trials (trials 4 – 5). During the meat trials there was a clear trend that could be observed. MIX 3, 4 and 5 all displayed similar log reductions but MIX 6 surpassed them all and displayed the highest log reductions for *E. coli* K-12, *E. coli* and *Salmonella*. *S. aureus* was the only bacterium that did not follow this trend but this could be expected due to its different cell wall structure. As MIX 6 displayed the most promising results, it was chosen to move onto the next part of the study.

During the second part of the study MIX 6 was implemented on 10 game animals harvested in the Western Cape. The results obtained were insufficient in terms of log reductions achieved. *E. coli* reductions ranged from 0.088 – 1.831 log cfu·25 cm^{-2} whereas total coliforms achieved even lower log reductions ranging from 0.014 – 0.425 log cfu·25 cm^{-2}. Although reductions achieved for aerobic count was in line with that of *E. coli* and total coliforms, BB1 displayed a 3.369 log cfu·25 cm^{-2} reduction. *S. aureus* also followed the same trend as *E. coli* with reductions ranging from 0.161 – 1.567 log cfu·25 cm^{-2}. As no results were obtained from samples with low initial microbial contamination, it does not necessarily mean that the spray was ineffective.

The unsatisfactory results from the implementation study gave rise to some recommendations that could possibly improve future studies. As fatty acids have shown promising results, octanoic acid could be replaced with another fatty acid such as capric acid or lauric acid. An increase in the organic acid treatment temperature is also suggested although the in-field practicality might be difficult. The use of equipment that can deliver the treatment at higher pressures seems more realistic for in-field use and higher pressures might be able to penetrate the pores in the meat where bacteria reside. A final recommendation would be to apply a hot water wash, or even cold water if hot water is not available, before the organic acid treatment as a combination treatment has been shown to work more effectively in reducing the microbial population present on the meat.

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CHAPTER 7
GENERAL DISCUSSION AND CONCLUSIONS

The world global population was estimated to be around 7.4 billion people during 2016 with 54 million residing in South Africa (Anonymous, 2016). The United Nations Department of Economic and Social Affairs (UN DESA) estimated that this number could increase to 9.7 billion people by 2050, indicating a drastic increase in population size (UN DESA, 2015). The rate at which the global population is increasing each year poses great challenges to the food and meat industry in terms of food security. It becomes somewhat of a juggling act trying the feed an increasing population while still conserving the world's natural resources (Cooper, 1995). Game meat as an alternative protein source to that of livestock may lighten the load on the meat industry (Hoffman & Cawthorn, 2013).

Game meat consumption globally, as well as in South Africa, is increasing every year (Taylor *et al.*, 2015). Game meat is known for its health benefits, such as being low in cholesterol, and with consumers becoming more health conscious about the products they consume it is clear why global consumption is increasing (Hoffman & Wiklund, 2006).

As game meat originates from animals that live in the wild, strict health and safety regulations have to be in place to ensure the meat is of good quality and safe for human consumption. This places further pressure on the game meat industry (Atanassova *et al.*, 2008). The harvesting process can introduce microbial contamination onto the once sterile meat. Contamination can occur at several stages, from the shot wound all the way through to the dressing of the carcass (van Schalkwyk & Hoffman, 2016; Gill, 2007). Microbial contamination of meat can introduce an array of different bacteria onto the meat ranging from spoilage bacteria to pathogens such as Shiga-toxin producing *Escherichia coli* (STEC). Microbial interventions are thus an integral part in ensuring meat that is safe for consumption (Loretz *et al.*, 2011).

The first aim of this study was to optimise a DNA extraction protocol for the isolation of the STEC positive control as well as optimizing the PCR conditions and reaction composition for the amplification of the *stx*$_1$, *stx*$_2$ and *eaeA* virulence genes. The DNA extraction protocol optimisation started with a basic crude extraction having no additional DNA washing steps. This protocol delivered unsatisfying results, as too many PCR inhibitors were still present. This led to the inclusion of a 99.9% ethanol wash to purify the DNA of any PCR inhibitors that may be present after the extraction process. The ethanol wash in addition to the re-suspension of the DNA in sterile distilled water produced the most PCR inhibitor free DNA (DNA-T3). The optimisation of the PCR reaction compositions depended greatly on the primer concentration. The initial primer concentration of 1 µM resulted in non-specific binding, causing the formation of primer-dimers that led to a decrease in PCR amplification product that formed. When the primer concentration was

decreased to 0.2 µM, satisfactory PCR amplification results were obtained with little primer-dimer formation.

The optimised DNA extraction protocol, using the lower primer concentration, were subjected to comparison testing to determine which of the PCR amplification conditions produced the optimum amplification results. It was noted that the first set of conditions had a too high annealing temperature, which lowered the efficacy of the PCR amplification process. The second set of conditions had a too low annealing temperature that inhibited the primers from effectively binding to the single stranded DNA. When the annealing temperature was chosen in between the two previous temperatures, satisfying amplification results were achieved (PCR-T3). Although these optimised processes worked well for the STEC positive control, it was noted that extracting inhibitor free DNA from a faecal sample would not be possible due to the nature of the sample. Two commercially available DNA extraction kits were tested and it was found that the ZR Fecal DNA MiniPrep kit produced inhibitor free DNA that resulted in PCR products with high intensity bands when visualised on an agarose gel. The above-mentioned optimised protocols were thus used during the subsequent study.

The second aim of the study was to determine the prevalence of STEC contamination in game species from two different farms in the Western Cape, South Africa. Faecal and meat samples were gathered from six game species located on the two different farms. All analyses were conducted using the optimised protocols from the before mentioned study. The faecal samples gathered from Farm 1 showed that two Zebras and two Black Wildebeest harboured STEC. The meat samples on the other hand showed that all four species (Zebra, Impala, Black Wildebeest and Eland) contained STEC. It was speculated that cross contamination could have occurred during the slaughter and dressing process whereby the STEC was transferred from the hide to the meat. It was further speculated that cross contamination could also have occurred during the transport of the carcasses in the cool truck due to the close proximity of the carcasses.

The faecal samples of the game species harvested from Farm 2 (Blesbok and Springbok) all tested negative for STEC. Cattle are known to be one of the main reservoirs of STEC and it has also been established that STEC can be transferred between animals on a farm. An additional study was conducted on faecal matter collected from cattle on this farm to determine if the cattle possess the organism. The cattle results displayed the same trend as that observed for the game species, as none of the cattle possessed the STEC organism. This could possibly explain why the game animals from the second farm tested negative. As the cattle did not harbour the organism, they could not have transferred it to the game animals. Overall, a high prevalence of STEC positive animals (26.9%) was observed. This could have dire financial implications on the game meat export market, especially meat exported to the European Union, in the long run.

The third aim of this study was to determine the microbial population present on South African game carcasses after dressing to determine whether the meat would meet the microbiological criteria in terms of food spoilage and food safety aspects. It was further aimed to determine the microbiological population found in the faecal matter of these animals so as to determine whether cross contamination could have occurred. Only species from Farm 2 (Blesbok and Springbok) were used in this part of the study.

Aerobic bacteria prevalence on the meat ranged from 1.60 – 4.97 log cfu·cm^{-2} whereas total coliforms varied from 5.04 – 5.59 log cfu·cm^{-2}. *E. coli* prevalence ranged from 0.00 – 1.71 log cfu·cm^{-2} while *S. aureus* varied from 0.00 – 2.97 log cfu·cm^{-2}. When these results were compared to the upper limits for export regulations by DAFF it was noted that the meat was mostly uncompromised in terms of food spoilage. The aerobic bacteria counts were below the upper limits but the total coliforms count exceeded these limits. It was also noted that all carcasses were within the upper limits in terms of food safety (*E. coli* and *S. aureus*) meaning all the meat was safe for consumption.

Aerobic bacteria prevalence in the faecal matter of the animals ranged from 5.78 – 6.44 log cfu·g^{-1} and total coliforms ranged from 6.53 – 7.04 log cfu·g^{-1}. *E. coli* prevalence varied from 3.00 – 4.54 log cfu·g^{-1} while *S. aureus* ranged from 3.63 – 4.40 log cfu·g^{-1}. None of the faecal samples tested positive for *Salmonella* and *Listeria*. Considering these high microbial counts detected in the faecal matter it is very possible that cross contamination from the hide to the carcass could have occurred during the dressing process. The high prevalence of both aerobic bacteria as well as coliforms in the faecal matter could explain the high prevalence of these bacteria on the meat after dressing.

The fourth aim of this study was to develop an organic acid spray in laboratory trials to determine the decontamination efficacy of different organic acids and mixtures thereof. It was further aimed to determine the efficacy of the developed organic acid spray in a field study on Blesbok and Springbok from Farm 2. The study consisted of five separate trials. Trials 1 – 3 were conducted in enrichment mediums whereas Trials 4 – 5 were on meat.

During the first three trials the results for *E. coli* K-12 showed no log reduction differences for the LA, AA, CA and MIX 1 treatments. The addition of octanoic acid in MIX 2 greatly improved upon the efficacy of the MIX 1 treatment. The elimination of AA and CA from MIX 2 creating MIX 3, displayed the highest log reduction of all the treatment. The trends seen for *E. coli* K-12 were not observed for *Salmonella*, as the AA and LA treatments performed much better than the CA treatment. No differences were observed for the MIX 1, OA and MIX 2 treatments. The log reductions obtained for MIX 3 was slightly lower than that of the previously mentioned treatments. Despite this, MIX 3 was chosen as the treatment to move onto the meat trials (trials 4 – 5).

Throughout the meat trials a clear trend could be observed. MIX 3, 4 and 5 all displayed similar log reductions however MIX 6 surpassed them all, displaying the highest log reductions for

E. coli K-12, *E. coli* and *Salmonella*. *S. aureus* was the only bacterium that did not follow this trend. This could be expected as it is a Gram-positive bacterium and its cell wall structure differs from that of Gram-negative bacteria. MIX 6 was selected for use in the field trial section of the study due to its promising results.

During the second part of the study MIX 6 was implemented on 10 game animals harvested from Farm 2 in the Western Cape. The results obtained were insufficient in terms of log reductions achieved. *E. coli* reductions ranged from 0.088 – 1.831 log cfu·25 cm^{-2} whereas total coliforms achieved even lower log reductions ranging from 0.014 – 0.425 log cfu·25 cm^{-2}. The reductions achieved for aerobic count was in line with that of *E. coli* and total coliforms whereas *S. aureus* followed the same trend as *E. coli.* Some samples displayed no log reductions as the initial microbial contamination on the sample was too low, but this does not necessarily mean that the spray was ineffective. It was also noted that the octanoic acid had a very unpleasant and pungent smell in addition to the fact that it caused discoloration of the subcutaneous fat on the carcass turning it light brown

Following the results obtained from this study some recommendations have been made for future studies. General recommendations pertaining to the study, as a whole, would be to expand the geographic area tested to include a wider variety of farms in South Africa as well as to increase the sample sizes used in this study and also include a larger variety of species. Concerning the microbial population section of the study it was recommended that future studies include the testing of *Enterobacteriaceae* as well as evaluate whether the level of contamination would decrease if the personnel underwent specific training on slaughtering hygienically. Recommendations pertaining to the organic acid spray study would be to replace the octanoic acid with another fatty acid such as capric acid or lauric acid; to use equipment that can deliver the treatment at higher pressures to penetrate the pores in the meat where bacteria reside and lastly to apply a hot/cold water wash before the organic acid treatment, as a combination treatment has been shown to work more effectively in reducing the microbial population present on the meat.

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