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THE MICROBIAL ECOLOGY OF BILTONG IN SOUTH AFRICA DURING PRODUCTION AND AT POINT-OF-SALE

Keshia Naidoo

THE MICROBIAL ECOLOGY OF BILTONG *IN SOUTH AFRICA DURING PRODUCTION AND AT POINT-OF-SALE*

Keshia Naidoo

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Masters of Science.

Johannesburg 2010.

DECLARATION

I hereby declare, that this is my own, unaided work. It is being submitted for the degree of Masters of Science in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____
KESHIA NAIDOO
0402012F

_____ *Day of* _____ 2010.

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PREFACE

Some aspects of the work conducted for this dissertation have or will be presented as publications elsewhere:

CHAPTER 2:

Naidoo, K. and Lindsay, D. (2010). Potential cross-contamination of the Ready-to-eat dried meat product, *biltong*. *British Food Journal*, **112 (4)**.

CHAPTER 3 and 4.1:

Naidoo, K. and Lindsay, D. (Accepted). Pathogens associated with *biltong* product, and their *in vitro* survival of hurdles used during production. *Journal of Food Protection Trends*.

CHAPTER 4.2:

Naidoo, K., and Lindsay, D. (2010). Survival of *Listeria monocytogenes,* and enterotoxin-producing *Staphylococcus aureus* and *Staphylococcus pasteuri*, during two types of *biltong* manufacturing processes. *Journal of Food Control*, **21 (7)**: 1042-1050.

ABSTRACT

The aim of this work was to determine the microbial ecology of *Biltong*, a South African national snack commodity at point-of-sale and during production. It was established that *biltong* at point-of-sale carried bacterial counts ranging from *ca.* 6- 7 Log CFU/ g of aerobic mesophilic, *ca.* 2.5- 4 Log CFU/ g of Enterobacteriaceae, *ca.* 1.5- 3 Log CFU/ g of coliforms, *ca.* 1- 3 Log CFU/ g of presumptive *Staphylococcus* and *ca.* 1 Log CFU/ g of *Escherichia coli* populations in descending order. Furthermore, foodborne pathogens such as *Listeria monocytogenes*, *Shigella dysenteriae* and enterotoxin-producing *Staphylococcus aureus* were prevalent in low incidences (0.5- 2 %) in *biltong* product at point-of-sale and highlighted the potential of *biltong* as a reservoir for potential foodborne pathogens. It was shown that the type of *biltong* preparation method utilised in the production of *biltong* significantly influenced the survival of potential foodborne pathogens on the final product. In particular, enterotoxin-producing *Staphylococcus* strains were shown to survive throughout. Although, processing had a significant effect on the survival of bacterial pathogens on *biltong* product, the environment and conditions employed at point-of-sale further contributed to cross-contamination of *biltong* product prior to consumption.

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A verse from the Tamil Scripture: The Thirukural that should not be forgotten:

“

391. கற்க கசடறக் கற்பவை கற்றபின்
நிற்க அதற்குத் தக.

பிழை இல்லாதவற்றைத் தனது குறைகள் நீங்குமளவுக்குக் கற்றுக்கொள்ள வேண்டும். க அதன்படி நடக்கவேண்டும்.

Lore worth learning, learn flawlessly
Live by that learning thoroughly.

Translation of verse 391: Learn perfectly all that you learn, and there after keep your conduct worthy of that learning.”

DEDICATED

TO MY BELOVED FAMILY

IN LOVING MEMORY OF

MY GRANDFATHERS

(MR V. NAIDOO &

MR G. R. NAIDOO)

CHAPTER 1:

INTRODUCTION

1.1 INTRODUCTION TO FOODBORNE DISEASE

With the worldwide increase in demand for food and food products, there is an increase in diseases that are associated with these commodities (Kaferstein *et al*., 1997). Foodborne diseases have ultimately been associated with either infections or intoxications (Jay *et al*., 2005). On a global scale, foodborne diseases are the major cause of millions of illnesses and thousand of deaths annually (Tauxe, 1997; Powell *et al*., 2001; Jay *et al*., 2005; de Souza, 2008; CBC News, 2009). In the USA alone, it has been estimated that food causes 76 million illnesses, 300 000 hospitalisations and 5000 deaths annually (De Waal, 2003; Jay *et al*., 2005; Widdowson *et al*., 2005). In 2007, a total of 6647 outbreaks associated with food (Table 1.1) were reported in the USA and ultimately resulted in 128 370 illnesses (Gerner-Smidt and Whichard, 2007). Furthermore, the World Health Organisation (WHO) have recently estimated that in Africa and East Asia, foodborne diseases attribute to 1.2 million fatalities per annum in people over the age of 5 (MacInnis, 2009).

To date it has been shown that bacterial pathogens may contaminate and proliferate within food commodities at any point in the production, processing, packaging, transportation, storage and consumption (Sofos, 2008; Sauders *et al*., 2009). Several reports have shown that inadequate processing involving sub-lethal hurdle steps and improper storage conditions favoured the proliferation of pathogenic bacterial populations (Keene *et al*., 1997; Sofos, 2008). Ingesting foods that have not only been contaminated with pathogenic bacterial cells, but have also been contaminated with bacterial toxins are the main causes of microbial foodborne disease (Mead *et al*.,

1999; Jay *et al*., 2005; Gerner-Smidt and Whichard, 2007; CDC, 2009a). In order for foodborne illness to occur, an adequate amount of pathogenic bacterial cells or pre-formed toxins have to be ingested. Once ingested, bacterial cells in the gastrointestinal tract can invade the host or cause the proliferation of various toxins depending on the bacterial pathogen (Jay *et al*., 2005). Consumption of bacterial cells and toxins are responsible for causing hundreds of different diseases (MacInnis, 2009) which have been associated with several common symptoms including; nausea, vomiting, diarrhoea, abdominal cramps and fever, while less commonly observed symptoms include meningitis, septicaemia, endocarditis, kidney failure and even death (Tauxe, 1997; Ray, 2001; Tauxe, 2002; CDC, 2005; Gerner-Smidt and Whichard, 2007; Gerner-Smidt and Whichard, 2008). There are several bacterial species that are commonly responsible for causing foodborne infections and the above-mentioned symptoms and are summarised in Table 1.2.

Whenever foodborne illness occurs, it does not only affect society in terms of health, but also affects the associated economy as a result of decreased productivity and high costs associated with 1) medical treatments for illness, 2) litigation, 3) product recalls from suppliers and 4) the destruction of contaminated food products (Altekruse *et al*., 1997; Medeiros *et al*., 2001; Powell *et al*., 2001; Ray, 2001; Salin and Hooker, 2001; Whyte *et al*., 2004; Jay *et al*., 2005; Normanno *et al*., 2005; Zhu *et al*., 2005; Singh *et al*., 2006; CBC News, 2009; Maki, 2009). For example in Toronto alone, it has currently been reported that foodborne illness in 437 000 people is equivalent to losses equating to $500 million as a result of the loss of productivity and medical costs (CBC News, 2009).

1.2 READY-TO-EAT (RTE) FOODS

In our current society, it has become a culture to spend less time in the kitchen preparing meals, which has lead to an increased demand for processed foods, such as ready-to-eat (RTE) food products (Collins, 1997). RTE meats and meat products are commodities that are produced using one or more pathogen reduction processes and are considered safe for human consumption without any further culinary practices or processing after purchase (Angulo *et al*., 1998; Kaneko *et al*., 1999; Lake *et al*., 2002; Jay *et al*., 2005). These products are often produced from meats such as beef, lamb, pork and poultry or mixtures of such meats (Lake *et al*., 2002; Jay *et al*., 2005). Processing of these meats generally involves either heating (pasteurisation, cooking, baking, boiling or steaming), curing, smoking, fermentation, drying, vacuum or modified atmosphere packaging and refrigerated or frozen storage, either alone or in combinations (Borch and Arinder, 2002; Lake *et al*., 2002; Jay *et al*., 2005; Toldra, 2006; Arnau *et al*., 2007). Recent studies have shown that RTE foods, in particular meat products, are becoming increasingly popular reservoirs of potential foodborne pathogens (Lake *et al*., 2002; Jay *et al*., 2005; Bohaychuk *et al*., 2006) (Table1.2).

1. Bacterial foodborne pathogens associated with RTE meats and meat products

Although RTE meats are produced under microbial limiting steps or processes (hurdles), several bacterial pathogens (Table 1.2) have commonly been associated with these products, and the subsequent incidences of foodborne illness. The characteristics of these pathogens will be further discussed below.

a. Listeria* (*L.*) *monocytogenes

Listeria monocytogenes is a Gram-positive, motile, rod-shaped bacterium, ubiquitously found in food processing, distribution and retail environments (Lake *et al*., 2002; Nelson *et al*., 2004; Sauders *et al*., 2009). Since this bacterium is a psychrotroph, it is capable of growth between 1 - 45 ºC and is known to survive and proliferate at refrigeration temperatures (Doyle *et al*., 2001; Ray, 2001; Nelson *et al*., 2004; Jay *et al*., 2005; Jemmi and Stephan, 2006). However, while growth is observed at 25 - 37 ºC, maximum replication is observed between 7 - 10 ºC (Lou and Youseff, 1999).

In certain foods, *L. monocytogenes* cells are capable of withstanding freezing, drying, high salt concentrations, water activity (a_w) of 0.90 and pH values of 5 and above (Sergelidis *et al*., 1997; Lou and Youseff, 1999; Norrung, 2000; Glaser *et al*., 2001; Peiris *et al*., 2008; Skandamis *et al*., 2008). This ubiquitous organism readily attaches to the surfaces of raw or unprocessed meats and is difficult to eradicate once attachment has taken place (Pal *et al*., 2008; Sauders *et al*., 2009). In 1929, *L. monocytogenes* was identified as a human pathogen however, it was only in the 1980's that this pathogen was identified specifically as a foodborne pathogen (Cox *et al*., 1989; Meng and Doyle, 1998; Slutsker and Schuchat, 1999; Buncic and Avery, 2004). As such, this organism is a potential threat to the safety of food commodities (Pal *et al*., 2008).

L. monocytogenes has frequently been isolated from the surfaces and interior muscle of several meats highlighting its presence prior to processing (Buncic and Avery,

2004; Sauders *et al*., 2009). Cooking, smoking, fermenting and drying are methods often employed for the production of RTE meat products and have been shown to successfully eradicate *L. monocytogenes* (Uyttendaele *et al*., 1999; Ingham *et al*., 2004; Ingham *et al*., 2006a). However, the prevalence of *L. monocytogenes* has been observed in several RTE meats and meat products at point-of-sale, including meat pates, vacuum-packaged beef, jerky, salami, fermented sausage, cooked sausage, uncooked hot dogs, turkey franks, sliced ham, and cold cut meats (Uyttendaele *et al*., 1999; Doyle *et al*., 2001; Ray, 2001; Lake *et al*., 2002; Seman *et al*., 2002; Buncic and Avery, 2004; Vitas and Garcia-Jalon, 2004; CDC, 2005; Jay *et al*., 2005; Zhu *et al*., 2005; Gibbons *et al*., 2006; Grinstead and Cutter, 2007; Sauders *et al*., 2009) (Table 1.2). The prevalence of this organism in food commodities prior to consumption is a cause for concern, especially since it is the etiological agent of the foodborne disease listeriosis (Glaser *et al*., 2001; Nelson *et al*., 2004; Yildirium *et al*., 2004; Zhu *et al*., 2005; Sauders *et al*., 2009). Although, 13 serotypes of *L. monocytogenes* have been identified, serotypes 1/2a, 1/2b and 4b have commonly been associated with outbreaks and have accounted for 95 % of infections (Manzano *et al*., 1998; Slutsker and Schuchat, 1999; Buncic and Avery, 2004; Nelson *et al*., 2004; Yildirim *et al*., 2004; Jemmi and Stephan, 2006).

Over the last 3 decades the incidences of listeriosis have drastically increased and were associated with high mortality rates (Czajka and Batt, 1994; Sergelidis *et al*., 1997; Buncic and Avery, 2004; Yildirim *et al*., 2004; Grinstead and Cutter, 2007; Sheen and Hwang, 2008). This foodborne disease accounts for about 2500 cases of illness, of which 91 % of illnesses resulted in hospitalisation and 20 % in fatalities in the USA, while in Europe 0.5 to 7.5 cases per million of the population and 20 - 30 %

fatalities are reported per year (Buncic and Avery, 2004; Nelson *et al*., 2004; Grinstead and Cutter, 2007; Peiris, *et al*., 2008). Several immuno-compromised populations, including toddlers and the elderly, pregnant women and their foetuses, cancer patients, diabetics, hepatic patients and those with HIV and AIDS infections have a greater risk of succumbing to fatal listeriosis (Slutsker and Schuchat, 1999; Buncic and Avery, 2004; Jacquet *et al*., 2004; Yildirim *et al*., 2004; Esteban *et al*., 2009).

Listeriosis often results after the ingestion of contaminated foods particularly RTE food commodities (Buncic and Avery, 2004; Gibbons *et al*., 2006). The exact amount of bacterial cells that need to be consumed for listeriosis to occur has not been stipulated and is highly variable (Lake *et al*., 2002). Upon ingestion of contaminated foods, *L. monocytogenes* propagates from the intestinal lumen to the central venous and nervous systems and in the case of pregnant women, to the fetoplacental unit (Henderson *et al*., 2000; Glaser *et al*., 2001; Buncic and Avery, 2004; Jacquet *et al*., 2004). The incubation period for disease and onset of symptoms may range from a day to several months (Lake *et al*., 2002; Buncic and Avery, 2004; Jay *et al*., 2005). Symptoms often associated with listeriosis include headache, fever, vomiting, diarrhoea, meningitis/ meningoencephalitis, endocarditis, septicaemia and abortion or stillbirths (in pregnant women) (Slutsker and Schuchat, 1999; Lake *et al*., 2002; Seman *et al*., 2002; Buncic and Avery, 2004; Jacquet *et al*., 2004; Nelson *et al*., 2004; Jay *et al*., 2005; Esteban *et al*., 2009; Sauders *et al*., 2009). If infection is detected and diagnosed, several antibacterial treatments can be administered including trimethophrim-sulfamethoxazole, penicillin and ampicillin (Slutsker and Schuchat, 1999; Lake *et al*., 2002; Buncic and Avery, 2004).

***b. Salmonella* spp.**

Salmonella spp. are Gram-negative, facultatively anaerobic, non spore-forming, rod shaped bacteria and are often motile by peritrichous flagella (Jay *et al*., 2005; Prescott *et al*., 2005). These organisms have readily shown growth in pH ranges between 6.6 - 8.2 and temperature ranges of 5 - 45 ºC however, these mesophilic bacteria grow optimally at temperatures of between 35 - 37 °C (Jay *et al*., 2005). *Salmonella* cells are capable of surviving long durations of freezing as well as drying, however, are unable to survive at low pH (less than 4.5) and replicate at a_w 0.94 (Ray *et al*., 2001; Jay *et al*., 2005). Members of this genus are normally associated with the intestinal tracts of birds, animals and humans (Jay *et al*., 2005; Prescott *et al*., 2005).

Salmonella spp. have been shown to survive the processing steps involved in the production of RTE products (Portocarrero *et al*., 2002a; Gormley *et al*., 2009; Hwang *et al*., 2009; Lucke, 2009). Recently the prevalence of *Salmonella* spp. in several RTE meat products, such as corned beef, sliced ham, jerky, *biltong*, meat salads, meat spreads, pates, sliced salami, salami sticks, chicken franks, roast beef, dried ham, chorizo, pork sausages and dry/semi-dried fermented sausages have been observed and have led to foodborne illness in some instances, in addition to the development of heat, acid and salt tolerant microorganisms (Neser *et al*., 1957; Harrison and Harrison, 1996; Meng and Doyle, 1998; Levine *et al*., 2001; Moore, 2004; Nummer *et al*., 2004; Prescott *et al*., 2005; Buege *et al*., 2006; Gibbons *et al*., 2006; Singh *et al*., 2006; Siriken *et al*., 2006; Gormley *et al*., 2009).

Salmonella spp. are the casual agents of salmonellosis, a disease commonly reported and accounts for 69 % of foodborne illnesses each year (CDC, 1995; Alterkruse *et al*., 1997; Collins, 1997; Murphy *et al*., 2004; CDC, 2006). Globally salmonellosis has been responsible for 1.3 billion cases of acute gastroenteritis and results in 3 million deaths per annum (Zhao *et al*., 2006). Although all of the species in this genus have been considered human pathogens, serotypes of *Salmonella* Enterica, *Salmonella* Typhimurium and *Salmonella* Heidelburg have most often been associated with human foodborne disease (Altekruse *et al*., 1997; Jay *et al*., 2005; Prescott *et al*., 2005; CDC, 2006; CDC, 2007). In order for salmonellosis to occur, an individual has to consume approximately 10^5 to 10^{10} bacterial cells. However, the ingestion of as little as 10 - 100 cells of some highly infectious strains have also been reported (Jay *et al*., 2005; Prescott *et al*., 2005). Upon ingestion, the bacterium invades the mucosa of the small intestine and produces enterotoxins and cytotoxins which causes inflammation and the subsequent destruction of intestinal epithelia (Altekruse *et al*., 1997; Jay *et al*., 2005; Prescott *et al*., 2005). Symptoms of salmonellosis are often exhibited within 8 - 48 hours after ingestion and include nausea, vomiting, abdominal pain, headache, chills and diarrhoea, prostration, muscular weakness, faintness, moderate fevers, restlessness and drowsiness (Meng and Doyle, 1998; Jay *et al*., 2005; Prescott *et al*., 2005; Gerner-Smidt and Whichard, 2008). In addition, reactive arthritis has also been observed in several incidences (Meng and Doyle, 1998; Gerner-Smidt and Whichard, 2008). Symptoms usually persist for 2 - 8 days and fatality rates associated with disease are relatively low except in immuno-compromised populations (Meng and Doyle, 1998; Prescott *et al*., 2005; Norrung and Buncic, 2008; CDC, 2009b).

As salmonellosis has progressed over the decades, resistance to treatments of antimicrobial agents such as ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/ sulfisoxazole and tetracycline have developed within *Salmonella* spp. and has led to the prevalence of multi-drug resistant strains (Alterkruse *et al*., 1997; Borch and Arinder, 2002; Gerner-Smidt and Whichard, 2008; Sofos, 2008).

c. Escherichia (E.) coli 0157:H7

Escherichia coli 0157:H7 is a Gram-negative, non spore-forming, motile rod shaped bacterium that grows rapidly at temperatures of 30 to 42 °C, while growth appears to be staggered at temperatures of 44 to 46 °C (Dykes, 2004). Although this organism has been shown to be heat and salt sensitive, cells are capable of surviving in food commodities at a temperature of –20 °C (Ray, 2001; Dykes, 2004). Furthermore, this organism is capable of surviving at low pH (4.5 and below) and varying a_w and is known to have an unusual tolerance to acid and drying (Park *et al*., 1999; Doyle *et al*., 2001; Ray, 2001; Campbell *et al*., 2004; Dykes, 2004; Jay *et al*., 2005; Singh *et al*., 2006; Montet *et al*., 2009).

In 1982, *E. coli* 0157:H7 had first been recognised as a foodborne pathogen, and has become increasingly predominant, causing approximately 20 000 illnesses and 250 deaths annually in the USA alone (Armstrong *et al*., 1996; Montet *et al*., 2009). Low doses, for example, only 10 - 50 cells of this bacterium, are capable of causing disease (Armstrong *et al*., 1996; Doyle *et al*., 2001; Dykes, 2004; Strachan *et al*., 2005; Silagyi *et al*., 2009). Associated with the onset of disease, is the production of the enterotoxin verotoxin (VTI) or shiga-toxin (ST) (Armstrong *et al*., 1996; Park *et al*., 1999; Ray, 2001; Dontorou *et al*., 2003; Jay *et al*., 2005; Strachan *et al*., 2005; Singh

et al., 2006). There can be a single or several toxins that are generally associated with disease and symptom development (Park *et al*., 1999). Symptoms of infection include hemorrhagic colitus, haemolytic uraemic syndrome (HUS) and thrombic thrombocytopenic purpura (TTP) (Park *et al*., 1999; Doyle *et al*., 2001; Ray, 2001; Dontorou *et al*., 2003; Alam and Zurek, 2004; Dykes, 2004; Jay *et al*., 2005; Singh *et al*., 2006; Montet *et al*., 2009). Symptoms associated with the haemolytic colitis include abdominal cramps, watery diarrhoea which becomes bloody as a result of destruction of the large intestine lining, and vomiting (Ryu *et al*., 1999; Dykes, 2004; Strachan *et al*., 2005). The toxins that are produced cause the lysis of red blood cells, which ultimately cause blood clotting within the blood vessels of the kidney. The kidneys as a result are damaged and in certain cases kidney failure has been observed and have resulted in several fatalities (Hilborn *et al*., 1999; Park *et al*., 1999; Dykes, 2004). Symptoms associated with TTP include brain clots, seizers, and coma and often result in death (Park *et al*., 1999; Doyle *et al*., 2001; Ray, 2001; Dykes, 2004; Jay *et al*., 2005). For the treatment of illness, antibiotics are often avoided in order to prevent the formation of additional toxins from the dying bacterial cells (Dyke, 2004; Sofos, 2008). Populations mostly affected include the young (< 5 years old), the elderly and immuno-compromised (Park *et al*., 1999).

RTE commodities where *E. coli* 0157:H7 were found as a contaminants and have been implicated in foodborne illness implications include semi-dried and dried fermented meats, fermented sausages, venison jerky, dry-cured salami and pre-sliced salami (Armstrong *et al*., 1996; Faith *et al*., 1998; Hilborn *et al*., 1999; Park *et al*., 1999; Ryu *et al*., 1999; Albright *et al*., 2003; Strachan *et al*., 2005; Ferreira *et al*., 2007; Montet *et al*., 2009).

d. Staphylococcus (S.) aureus

Staphylococcus aureus is a small, non-motile Gram-positive, facultative anaerobic coccoid shaped bacterium ubiquitously found in the mucous membranes of the nasal cavity as well as the skin of most mammals (Doyle *et al*., 2001; Jay *et al*., 2005; Ingham *et al*., 2006a; Ingham *et al*., 2006b). This mesophile is capable of surviving at temperatures between 6 - 47 °C while the optimum temperature for bacterial growth is 37 °C (Pearson and Dutson, 1986; Hudson, 2004; Jay *et al*., 2005; Prescott *et al*., 2005). Furthermore, *S. aureus* proliferates freely in pH ranges of 4.2 - 9.3 and low a_w (0.85) (Hudson, 2004; Ingham *et al*., 2006b). Several strains of *S. aureus* have shown to be tolerant to heat, drying and high salt concentrations (7 - 25 %) (Portocarrero *et al*., 2002b; Hudson, 2004; Valero *et al*., 2009) and have subsequently been found as contaminants of country hams, jerky, *charqui*, *alheiras*, semi-dried and dried fermented sausages (CDC, 1995; Levine *et al*., 2001; Portocarrero *et al*., 2002b; Lara *et al*., 2003; Moore, 2004; Nummer *et al*., 2004; Normanno *et al*., 2005; Sumner *et al*., 2005; Yoon *et al*., 2005; Valero *et al*., 2009).

In 1894, the first account of *S. aureus* as a foodborne pathogen was reported (Portocarrero *et al*., 2002a; Hudson, 2004; Jay *et al*., 2005). Manufacturing processes often readily kill members of this species, however it is the toxins they produce that are resilient to processing (Jay *et al*., 2005). Foodborne disease arises from the consumption of 1 or more of 13 enterotoxins. These toxins may be grouped into subclasses A, B, C, D, E, G, H, I and J (Balaban and Rasooly, 2000; Dinges *et al*., 2000; Hudson, 2004; Jay *et al*., 2005). Although several classes of enterotoxins are produced, enterotoxins A and D have most commonly been associated with foodborne

illness (Bergdoll, 1990; Balaban and Rasooly, 2000; Portocarrero *et al*., 2002b; Hudson, 2004; Jay *et al*., 2005). Toxins are produced under aerobic conditions between 10 - 45 ºC and pH ranges of 5.3 - 7. These low molecular weight compounds are heat stable and resistant to proteolytic enzymes, gamma irradiation and pH extremes (Bergdoll, 1990; Marrack and Kappler, 1990; Balaban and Rasooly, 2000; Hudson, 2004; Valero *et al*., 2009).

To date, staphylococcal intoxication contributes to a high incidence of global foodborne disease and is considered to be the second most commonly recorded illness related to food (Lara *et al*., 2003; Normanno *et al*., 2005; de Souza, 2008; Valero *et al*., 2009). It has recently been reported that 35 outbreaks of staphylococcal intoxication had resulted in 777 cases of illness, 14 hospitalisations and 1 fatality in Spain, while in the USA 6 to 80 million illnesses, 9000 fatalities and $5 million dollar losses are reportedly observed per annum (Balaban and Rasooly, 2000; Valero *et al*., 2009). Salted meat products, such as hams, have previously contributed to 24 % of staphylococcal intoxication (Portocarrero *et al*., 2002b). Ingestion of $\leq$ 1.0 µg of enterotoxin has been shown to sufficiently initiate staphylococcal intoxication (Balaban and Rasooly, 2000; Hudson, 2004; Jay *et al*., 2005; Valero *et al*., 2009). Upon ingestion, toxins stimulate the transmission of signals from the receptors of the intestinal tract to the medullary emetic centre of the brain (Hudson, 2004; Jay *et al*., 2005). Symptoms associated with staphylococcal intoxication are often apparent 0.5 - 7 hours after the ingestion of toxins and include nausea, frequent vomiting and abdominal pains, diarrhoea has less frequently been observed along with headaches, fever and faintness (Dinges *et al*., 2000; Hudson, 2004; Normanno *et al*., 2005;

Huong *et al*., 2010). Staphylococcal intoxication is self-limiting in most cases and therefore the administration of antimicrobials is often limited (Jay *et al*., 2005).

2. Sources of contamination of RTE meat and meat products

As previously mentioned several RTE meats and meat products have been implicated in several incidences of foodborne illness, it is suggested that several sources of contamination could have been the contributing agents aiding in these illness implications (Aycicek *et al*., 2006; Hutchison *et al*., 2006; Tebutt *et al*., 2007). Contamination of RTE products is considered to originate from several sources including the ingredients used to prepare commodities, raw materials, poorly cleaned (sanitised) surfaces, utensils used to prepare and dispense RTE foods, the hands and apparel of food handlers and poor hygiene practices of associated personnel who are in direct contact with food commodities (Borche and Arinder, 2002; Reij *et al*., 2004; Jay *et al*., 2005; Aarnisalo *et al*., 2006; Aycicek *et al*., 2006; Lues and Van Tonder, 2007; Tebutt, 2007; Todd *et al*., 2007; Christison *et al*., 2008; Sauders *et al*., 2009; Todd *et al*., 2009).

a. Ingredients used to prepare RTE meats and meat products

The prevalence of bacterial pathogens in meats and meat products may be attributed to 2 major sources, i.e. the animal harbouring the pathogen itself and the cross-contamination from the processing environment and humans (Borche and Arinder, 2002). Contaminated raw materials or the ingredients, which are added, throughout the production of RTE meat products have previously been implicated as sources of contamination (Reij *et al*., 2004). Contaminants have often been found to include

Salmonella spp., *Yersinia enterocolytica* and *Clostridium botulinum* (Borch and Arinder, 2002; Reij *et al*., 2004). Furthermore cross-contamination from raw meats to processed meats has also been recognised as a vector aiding in the dissemination of bacterial pathogens (Dempster *et al*., 1973; Reij *et al*., 2004; Todd *et al*., 2009).

b. **Utensils used to prepare and dispense RTE foods**

Processing equipment in food preparation and point-of-sale environments have previously been highlighted as sources of contamination (Mosupye and von Holy, 2000; Borch and Arinder, 2002; Reij *et al*., 2004; Aarnisalo *et al*., 2006; Christison *et al*., 2007). Such contamination arises when bacteria attach to the surfaces of processing equipment, such as packaging machines, cutting (slicing and dicing) machines, spoons, knives and cutting boards as a result of poor cleaning regimes (Medeiros *et al*., 2001; Lunden *et al*., 2002; Reij *et al*., 2004; Sneed *et al*., 2004; Haysom and Sharp, 2005; Jay *et al*., 2005; Barros *et al*., 2007; Christison *et al*., 2008; Sauders *et al*., 2009). Furthermore, such attachment leads to the formation of biofilms on the surfaces of equipment and is known to facilitate the dissemination of microorganisms including *S. aureus*, *Salmonella* spp., *E. coli* 0157:H7 and *L. monocytogenes*, onto food products that may subsequently come into contact with these surfaces (Lunden *et al*., 2002; Reij *et al*., 2004; Sauders *et al*., 2009; Silagyi *et al*., 2009). *L. monocytogenes*, due to its ubiquitous nature, is known to readily attach to and form biofilms on processing equipment, such as the blades of mechanical slicers, and have subsequently been disseminated into RTE meat products (Senczek *et al*., 2000; Lunden *et al*., 2002; Reij *et al*., 2004; Sheen and Hwang, 2008; Gormley *et al*., 2009; Sauders *et al*., 2009), which have resulted in severe incidences of foodborne illness (Lunden *et al*., 2002; Riej *et al*., 2004; Sauders *et al*., 2009). For example, in

2008, an outbreak of listeriosis associated with the consumption of cold cut meats in Toronto, resulted in 20 fatalities and several cases of illness. In this outbreak, it was shown that the cutting devices used to slice meat were the primary reservoir for foodborne *L. monocytogenes* (Byrne, 2008).

c. The hands of food handlers

It has been recognised that food handlers have played a significant role in the incidences of foodborne illness (Reij *et al*., 2004; Jay *et al*., 2005; Lues and Van Tonder, 2007; Todd *et al*., 2007; Todd *et al*., 2008; Todd *et al*., 2009) and have contributed to 81 foodborne outbreaks to date (Reij *et al*., 2004; Lues and Van Tonder, 2007). Bare hand contact by the handler and inadequate hand washing practices have ultimately been the main factors associated with foodborne illness (Reij *et al*., 2004; Lues and Van Tonder, 2007). It is suspected that pathogens such as *Salmonella* spp., *Shigella* and *S. aureus* have been readily transmitted from food handlers, while pathogens such as *Campylobacter*, *E. coli* 0157:H7 and *Vibrio cholerae* are not as easily transmitted (Todd *et al*., 2008). Types of cross-contamination aiding in the dissemination of these pathogens include hand to surface, surface to hand, food to hand, hand to food and combinations of these contact points (Todd *et al*., 2009). Furthermore, it has recently been shown that long and artificial nails are associated with higher microbial loads and are potential reservoirs of food and faecal matter, especially when proper hand washing techniques are not implemented (Todd *et al*., 2009). In addition, rings and other jewellery have also been shown to increase the microbial loads associated with the hands of food handlers (Todd *et al*., 2009).

1.3 RTE DRIED MEAT PRODUCT *BILTONG*

1. What is *Biltong*?

The word *biltong* is derived from the Dutch words "*bil*" which refers to buttock, rump or meat and "*tong*" which refers to tongue or strip (Lewis *et al*., 1957; Van den Heever, 1970). *Biltong* is the name given to strips of meat that have been cured or spiced and air-dried at ambient temperatures (Van den Heever, 1970; Van de Riet, 1982; Prior, 1984). This RTE meat product is somewhat similar to *carne seca* from Mexico, *charqui* from South America, jerky in USA, *kilshi* in Sahel and *rou gan* from China (Van den Heever, 1970; Leistner, 1987; Collignan *et al*., 2001; Smelser *et al*., 2004; Sweeney, 2005) (Fig 1.1).

2. History of *biltong*

Biltong is a commodity that has been around for centuries. It has been reported that the Voortrekkers who migrated around South Africa, would mass kill Springbok (Game). The hides of these animals were cured while the flesh was either spiced and placed on the saddles of horses and allowed dry or were sun-dried and thereafter cured for future consumption (Halliburton, 1902; Lewis *et al*., 1957; Van de Reit, 1982). In addition, during the migration of African herders it was also common practice to cure and sun-dry meats for preservation purposes (Van de Reit, 1982; Jones *et al*., 2001; Sweeney, 2005).

3. Culture and tradition of *biltong*

Biltong is considered to be the national snack food commodity of South Africa (Van den Heever, 1970; Nortje *et al*., 2006). There is no flea market, supermarket, tuck shop, convenience store, petrol station, party, bar or event where this commodity is absent. *Biltong* has become a staple snack in every household and is enjoyed by all ages, sexes and races. It is believed that South Africans acquire the taste for this delicacy as toddlers during the teething stage of growth (Sweeney, 2005; Farrer and van Schoor, 2008) (Fig 1.2).

With the ever-increasing population, as well as sporting events, there is an elevated demand for *biltong* both nationally and internationally (Attwell, 2003; Konieczny *et al*., 2007; Royer, 2009). *Biltong* sales have not only increased but have led to international markets investing in this commodity since it is "Proudly South African" (Sweeney, 2005). Additionally, the emigration of South African citizens worldwide has led to the introduction and production of "traditional" South African *biltong* in several countries such as New Zealand, Australia, United States of America and United Kingdom (Read, 2004; Sweeney, 2005; Berry, 2007; del Grosso, 2009). For these reasons as well as the relative ease in producing *biltong*, many South Africans produce this commodity in the confines of their homes (Attwell, 2003; Exzanian, 2009), where microbial safety is a concern (Albright *et al*., 2003; Konieczny *et al*., 2007).

4. Process of producing *biltong*

The methods and recipes for producing traditional *biltong* are often passed down from generation to generation (Dzimba *et al*., 2007) and therefore, several methods are currently available and utilised. Although several methods exist, there are several basic steps associated with producing *biltong*, including the selection of meats that will be used, vinegar marinating, spicing and drying (Fig 1.3) (Van Tonder and Van Heerden, 1992; Burnham *et al*., 2008; Castle Lager East Cape *Biltong* Festival, 2008; Tymon, 2008).

a. Meat selection

Biltong can be created from various meats such beef, venison, ostrich, springbok, chicken and certain types of fish (Neser *et al*., 1957; Van den Heever, 1970; Auerswald *et al*., 2006; Van Wyk, 2007; Tymon, 2008). Although several meats can be used, beef is often most preferred. In order to produce *biltong*, silverside, topside, flank, fillet and the eye muscles that run down both sides of the backbone of beef are preferred (Lewis *et al*., 1957; Van Tonder and Van Heerden, 1992; Sweeney, 2005; Dzimba *et al*., 2007; Anonymous, 2009a) (Fig 1.4a). Once the meats have been selected (Fig 1.3) it is then cut into strips (Van Tonder and Van Heerden, 1992) (Fig 1.4a).

It should be remembered that several bacterial species are capable of causing foodborne diseases and are associated with meats, particularly that of bovine meats. These include *S. aureus*, *Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolytica*, *L. monocytogenes* and *E. coli* 0157:H7 (Pearson and Dutson, 1986; Jensen *et al*., 1998; Harrison *et al*., 2001; Mayrhofer *et al*., 2004; Nummer *et al*., 2004; Sumner *et al*., 2005; Nortje *et al*., 2006; Norrung and Buncic, 2008).

b. Vinegar soak

Once the beef portions have been sliced they are placed into one of several diluted or undiluted vinegars (acetic acid) depending on personal preference. Apple cider vinegar is most often used (Hopkins *et al*., 2004). Other vinegars that are commonly used include normal spirit vinegars (brown and white), balsamic vinegars and red and white wine vinegars (Tymon, 2008; Anonymous 2009b). The vinegar soak step is responsible for the partial eradication of sinew and binding tissue in meat portions, it serves as a tenderiser, prevents extensive weight loss of the final product and contributes to the aroma, flavouring, texture and colour of meat during the production of *biltong* (Marshall, 2003; Olivier and Proust, 2008; Anonymous, 2009c).

Not only does the vinegar soak improve the taste and appearance of *biltong* that will be produced, it also contributes to the destruction of potential foodborne bacterial pathogens (Stivarius *et al*., 2002; Marshall, 2003), products containing acetic acids such as vinegars and wines display bacteriostatic and bacteriocidal properties (Entani *et al*., 1998; Steinkraus, 2002; Stivarius *et al*., 2002; Marshall, 2003; Johnston and Gaas, 2006). The antibacterial properties of these organic acids cause the inhibition of bacterial enzymes, membrane functioning, transportation of nutrients and overall metabolic processes (Chang and Fang, 2007). Therefore, higher concentrations of vinegar and increased durations of soaking should ideally reduce 99 % of bacterial pathogens (Marshall, 2003). The problem however, is that certain bacterial strains including *E. coli* 0157:H7, *Salmonella* spp. and *L. monocytogenes* have reportedly shown survival in the presence of low concentrations of organic acids and the development of acid tolerance in some instances (Berry and Cutter, 2000; Merrell and

Camilli, 2002; Calicioglu *et al*., 2002; Calicioglu *et al*., 2003; Marshall, 2003; Chang and Fang, 2007; Stopforth *et al*., 2007; Skandamis *et al*., 2008).

c. Spice marinating

For several centuries, a group of "esoteric food adjuncts" (Srinivasan, 2005) known as spices have been used to augment the taste of, and preserve food commodities (Sherman and Billing, 1999; Srinivasan, 2005; Shan *et al*., 2007). There are several of these basic adjuncts that form the constituents of the traditional *biltong* spice mixture, which is used in the marination of meat slices (Fig 1.4b). The fundamental spice constituents of this mixture includes, salt (NaCl), brown sugar, black pepper and coriander. Additional spices, such as chilli, onion powder, ground ginger, mustard powder or garlic powder, are often added depending on the flavour of *biltong* that is desired (Lewis *et al*., 1957; Leistner, 1987; Sweeney, 2005; Dzimba *et al*., 2007). In addition, nitrites and saltpetre may also be included in spice mixture for the purpose of enhancing the colour of the final *biltong* product (Van den Heever, 1970; Dzimba *et al*., 2007).

Although spices are used to enhance the flavour of food commodities, they also aid in the tenderisation of meats, and often possess antimicrobial properties (Briozzio *et al*., 1989; Billing and Sherman, 1998; De *et al*., 1999; Sherman and Billing, 1999; Bjorkroth, 2005; Srinivasan, 2005; Papachan *et al*., 2007). Each of the spices used in the basic *biltong* spice mixture performs a particular function in the process of producing *biltong*. For example, salt is one of the oldest adjuncts used to preserve meats due to its ability to inhibit the growth of bacteria (Van den Heever, 1970) by altering the osmotic potential, as well as reducing the moisture content of the meats.

However, at certain concentrations, bacterial species such as *S. aureus* and *Salmonella* spp. are able to develop a tolerance to this adjunct (Van den Heever, 1970; ICMSF, 1980; Chesneau *et al*., 1993; Ryu *et al*., 1999; Sherman and Billing, 1999; Jay *et al*., 2005).

The vinegar soak step ultimately gives meat an acidic flavour; brown sugar is therefore added in order to produce *biltong* that is more acceptable to the palate (Bjorkroth, 2005). In addition, although black pepper and coriander are also used to enhance the flavour of *biltong*, these condiments have shown antimicrobial activity against several bacterial species including *S. aureus*, *Salmonella* spp., *E. coli* and *Bacillus cereus* (Pruthi, 1980; Zaika, 1988; Perez and Aneseni, 1994; Billing and Sherman, 1998; De *et al*., 1999; Delaquis *et al*., 2002; Chaudhry and Tariq, 2006; Burdock and Carabin, 2009). Although spices are associated with antimicrobial properties, it has recently been recognised that bacterial pathogens are not as greatly affected or inhibited by these antimicrobials as they were before (Elgayyar *et al*., 2001; Calicioglu *et al*., 2003). For example, it has recently been shown that black pepper and coriander were not inhibitory to strains of *E. coli* in particular *E. coli* 0157:H7 (De *et al*., 1999; Takikawa *et al*., 2002; Shan *et al*., 2007). Furthermore, it has been shown that spices themselves are reservoirs of bacterial pathogens and contaminants (das Chagas Oliveira Freire and Offord, 2002; Jay *et al*., 2005; Vij *et al*., 2006; Sagoo *et al*., 2009).

d. Refrigeration

This step is essential for allowing the saturation of spices into the meat slices. Furthermore, the storage of meat slices at 4 °C for more than 20 hours aids in

reducing the presence of bacterial cells on the meat, as it is aimed at cold shocking bacterial cells (ICMSF, 1980). The foodborne pathogen *L. monocytogenes* is known to withstand and proliferate at low temperatures (Mellefont *et al*., 2008; Cunningham *et al*., 2009; Zhang *et al*., 2009), thus this stage of processing holds a particular cause for concern with regard to this pathogen.

e. Drying

For several centuries, drying had been the only method employed for the traditional preservation of meat products against the growth of microbial populations and subsequent spoilage prior to the advent of refrigeration (Mothershaw *et al*., 2003; Hierro *et al*., 2004; Trystram, 2004; Jay *et al*., 2005; Toldra, 2006). Preservation is attained by the extraction or binding of moisture which results in the reduction of water activity (a_w) within the meat commodity (Jay *et al*., 2005; Rahman *et al*., 2005; Dzimba *et al*., 2007). This extraction occurs as water molecules move from the internal cells of meat, through the cell membranes and disperse into the environment (Panyawong and Devahastin, 2007). The reduction of a_w (< 0.90) consequently results in the inhibition of the microbial enzymes that are responsible for growth, degradation of food and general metabolism (Mothershaw *et al*., 2003; Jay *et al*., 2005; Rahman *et al*., 2005).

The drying step of the *biltong* manufacturing process may be considered as the most important step, especially since it is the last processing step prior to the consumption of *biltong*. In addition, drying should ideally eradicate any pathogenic and spoilage populations that may have survived the hurdles imposed by the vinegar and spice marination steps.

Throughout the centuries, the drying of meats for the production of *biltong* has changed and evolved significantly. Initially, meats were dried out in the open environment whereby the heat from the sun, and wind were responsible for removing moisture (Lewis *et al*., 1957). Today however, spiced meats are hung in environmentally controlled rooms or units, which often contain electrical devices that supply heat and air circulation. Although, drying meats in a controlled room (Fig 1.5) are common practices used by large-scale commercial *biltong* producers, there are several devices and methods that are commonly used by small-scale or home *biltong* producers for drying, including; home-made enclosed cabinets, home-made *biltong* boxes and commercially produced *biltong* dehydrator units (Fig 1.6).

❖ *Home-made enclosed cabinets*

Home-made *biltong* boxes are generally wooden framed cabinets containing metal rods that run across the width and have mesh screens on the doors or sides of the cabinet (Fig 1.6) (Anonymous, 2002). The use of meshing as screens on doors or side walls allows air to migrate through the cabinet and serves as a protective barrier against rodents, insects and external contaminants (Anonymous, 2002). Home producers often use these units to produce *biltong* on a small-scale.

❖ *Home-made* biltong *boxes*

These devices are made from sealed wooden or cardboard boxes containing steel rods that have been modified in several ways (Anonymous, 2002; Anonymous, 2009d). These modifications include the 1) addition of a light bulb at the base of the box, 2) placement of a sheet of wood containing perforations above the light bulb and 3)

production of holes in the sides and lid of the boxes to allow the movement of air through the box. This system uses the light bulb to produce heat, which is introduced into the box and thereby reduces the time taken for meat to dry (Stathakis, 1993; Anonymous, 2002; Klein-SA, 2008; Anonymous, 2009d). As it is easy to manufacture these systems, this has become the more favoured device in the drying of meat in the home and small-scale production of *biltong*. It has recently been suggested that steel boxes should replace wooden boxes, as wood is known to support the growth of spoilage organisms (Hirsh, 2006) and as such may potentially support the growth of bacterial pathogens.

❖ *Commercially produced* biltong *dehydrator units*

These devices operate on the same principles as the home-made *biltong* boxes, however these units are made of perspex, plastic or steel and often only have perforations on the roof and base of the dryer (Fig 1.6). Several of these systems still use a light bulb for a source of heat, while more modern units have heating elements or coils in the base (Anonymous, 2009a; Anonymous, 2009e). Home, small-scale and large-scale *biltong* producers often use these units.

❖ *Environmentally controlled rooms*

For large-scale *biltong* production, *biltong* producers often allocate a specific room for drying. These rooms often contain thermal sources in the form of heaters and mechanically oscillating devices, which create and allow the circulation of air within the room (Fig 1.5).

5. Composition, nutrition and physiochemical properties of *biltong*

Biltong is composed of 65 % protein, 1.9 % fat, 7.6 % glucose, 6.8 % glycogen, 5 - 10 % salt (NaCl), 10 - 860 ppm nitrate (Lewis *et al*., 1957; Van den Heever, 1970; Taylor, 1976; Osterhoff and Leistner, 1984; Prior, 1984) and has a_w of 0.77 and pH of ≤ 5.5 (Van den Heever, 1970; Osterhoff and Leistner, 1984; Dzimba *et al*., 2007). These properties make this commodity microbiologically stable. It has recently been reported that *biltong* with increased moisture (a_w ranging from 0.85 - 0.93) is more popular with consumer markets (Nortje *et al*., 2006; Dzimba *et al*., 2007).

6. Microbiology associated with *biltong*

Previous studies have indicated that commercially available *biltong* carried microbial loads of 5 - 7.5 Log CFU/ g (Van den Heever, 1970; Taylor, 1976; Prior, 1984; Leistner, 1987) and included populations of salt tolerant micrococci such as *Micrococcus luteus* and *Micrococcus varians*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Lactobacillus*, *Bacillus* and several yeasts (Neser *et al*., 1957; Bokkenheuser, 1963; Taylor, 1976; Leistner, 1987). In addition, *Salmonella* spp. and strains of *E. coli* in particular *E. coli* 0157:H7 have also previously been isolated from *biltong* (Neser *et al*., 1957; Bokkenheuser, 1963; Van den Heever, 1965; Van den Heever, 1970; Leistner, 1987; Wolter *et al*., 2000; Abong'o and Momba, 2008; Abong'o and Momba, 2009). As the home production of *biltong* is currently increasing, a cause for concern also arises, as there is a higher risk of cross-contamination with coliform and pathogenic bacteria from these environments during the production of this commodity (Konieczny *et al*., 2007). Furthermore, in addition to bacterial and yeasts populations, moulds and fungi have also been prevalent in *biltong* (Van den Heever, 1970; Osterhoff and Leistner, 1984; Leistner, 1987). To

date there appears to be limited research within the literature regarding the presence and prevalence of bacterial pathogens in *biltong*. The most recent microbial evaluations of *biltong* have been conducted several decades ago. In addition, there appears to be no legislation in South Africa governing the acceptable levels of microbial populations associated with *biltong* (Bokkenheuser, 1963; Van den Heever, 1970).

7. Foodborne outbreaks associated with *biltong*

Five reported cases of foodborne illness associated with consumption of contaminated *biltong* appear in the literature (Neser *et al*., 1957; Durbach, 1957; Bennett and Hook, 1959; Bokkenheuser, 1963; Botes, 1966; Van den Heever, 1970; Tshekiso, 2002). From 4 of the outbreaks, *Salmonella newport*, *Salmonella lomita*, *Salmonella anatum* and *Salmonella* Typhimurium were the suspected causal agents of salmonellosis which resulted in 1 fatality and several cases of illnesses (Neser *et al*., 1957; Bokkenheuser, 1963; Van den Heever, 1965; Botes, 1966; Van den Heever, 1970). Symptoms associated with these outbreaks often included fever, loss of appetite, vomiting, nausea, headaches, diarrhea, acute gastro-enteritis and 2 cases of acute appendicitis (Neser *et al*., 1957; Botes, 1966; Van den Heever, 1970). It is reported that the latter outbreaks occurred as a result of using meats of diseased animals to produce *biltong* (Lewis *et al*., 1957; Neser *et al*., 1957; Van den Heever, 1965). The last outbreak occurred in Good Hope, an area located in Botswana and 17 people exhibited illness. Symptoms associated with illness, included fever, loss of appetite, abdominal pain, diarrhoea and vomiting. No fatalities were reported and the causal agent of the foodborne illness was not published (Tshekiso, 2002).

For comparative purposes, several outbreaks have been associated with jerky. Jerky is an American RTE product with some similarities to *biltong*. Both products are RTE, dried and spiced meat commodities. However, jerky is usually heated or cooked prior to being dried, and no vinegar is used in its production (Hanes, 2006; Abong' o and Momba, 2009; Romeis, 2009; Anonymous, 2009a; Anonymous, 2009f).

Since 1966 there have been 9 outbreaks of foodborne disease associated with the consumption of contaminated jerky, 6 of which involved *Salmonella* spp. as casual agents (CDC, 1995, Keene *et al*., 1997; Eidson *et al*., 2000; CDC, 2002; Nummer *et al*., 2004). Symptoms associated with these outbreaks had included diarrhoea, bloody diarrhoea, fever, abdominal pain and vomiting (Smelser *et al*., 2004). In 1995, the biggest outbreak occurred and a total of 111 people succumbed to salmonellosis (CDC, 1995). The strains responsible for this outbreak were identified as *Salmonella* Typhimurium, *Salmonella* Montevideo and *Salmonella* Kentucky (Eidson *et al*., 2000; Nummer *et al*., 2004; Jay *et al*., 2005). Contamination was thought to have occurred as a result of incorrect drying temperatures. However, the latter was never confirmed as the plant that manufactured the jerky shut down on a voluntary basis (CDC, 1995). In 2003, beef jerky produced in Mexico was again the vehicle of salmonellosis, in this incidence 22 people were infected. This outbreak however showed that even under good manufacturing practices and correct drying procedures bacterial pathogens are able to persist in this RTE commodity (Eidson *et al*., 2000; Nummer *et al*., 2004).

The prevalence of *S. aureus* has been observed in beef jerky and has resulted in 2 outbreaks of staphylococcal food poisoning in 1982 and 1995. It was suspected that

the outbreaks had arisen due to improper drying practices associated with the production of this commodity (Eidson *et al*., 2000; Nummer *et al*., 2004). In 1996, there were 6 confirmed cases of *E. coli* 0157:H7 illness associated with venison jerky. Foodborne illness had been due to the ingestion of venison jerky that had been contaminated with this bacterial pathogen (Keene *et al*., 1997: Nummer *et al*., 2004). The cause of the latter contamination was attributed to the survival of *E. coli* 0157:H7 as a result of sub-lethal drying conditions (Keene *et al*., 1997; Nummer *et al*., 2004). Although no incidences of listeriosis have been associated with jerky, a 52 % prevalence of *L. monocytogenes* has been recently observed (Levine *et al*., 2001; Nummer *et al*., 2004).

1.4 MOTIVATION

Biltong, the national RTE snack is associated with sporting events, parties and social gatherings and is most importantly a staple snack in most South African households. The safety of *biltong* poses a particular cause for concern as not only has this commodity been implicated in outbreaks of foodborne illness, but is also consumed by South African populations who are immuno-compromised in terms of age, pregnancy, chronic illness and HIV/AIDS infection. The latter is particularly important, as members of these populations are more susceptible to severe infections by bacterial pathogens such as *L. monocytogenes*, *S. aureus*, *Salmonella* spp. and *E. coli* 0157:H7 (Sneed *et al*., 2004).

As the demand for *biltong* is on the increase both nationally and internationally, a dramatic increase in the production of this commodity at a commercial, small-scale

and household level has been observed. To date, there appears to be limited knowledge associated with the production of this commodity in terms of hygiene, good manufacturing practices and ensuring microbial safety by the individuals producing *biltong* on the home front and within small establishments. Several studies have shown that the production, processing and point-of-sale environments and practices associated with food commodities are 1) sources of cross-contamination and 2) vectors aiding in the dissemination of bacterial pathogens, which have in certain instances resulted in foodborne illness implications. To date, there are no such studies associated with the production, processing and point-of-sale environments associated with *biltong* with regard to reducing and preventing the survival of pathogenic bacteria.

Studies evaluating the microbiology and safety of *biltong* at point-in-sale in South Africa are limited and were only conducted during 1955 - 1977. In addition, there have been no recently published studies within the literature regarding the changes in microbial populations during the process of producing *biltong*. As the progression of time has lead to the emergence of new foodborne pathogens (Tauxe, 2002), it is essential to evaluate the current prevalence of these organisms in *biltong* at point-of-sale. Literature on other products with similarities to *biltong*, for example jerky, has shown the survival of various foodborne pathogens in dried and spiced RTE meats. The recent detection of *E. coli* 0157:H7 in South African *biltong* (Abong'o and Momba, 2008; Abong'o and Momba, 2009) has further questioned the prevalence of other emerging pathogens such as *L. monocytogenes*. Such studies are currently lacking within the literature.

Table 1.1: Summary of the microorganisms that are often associated with foodborne diseases.

Foodborne Microorganism	Specific Examples	References
Bacteria	*Escherichia coli* 0157:H7, Verotoxigenic *E. coli* (VTEC), *Salmonella* spp., *Yersinia* spp., *Staphylococcus aureus, Listeria monocytogenes, Campylobacter, Bacillus cereus, Shigella, Vibrio cholerae, V. parahaemolyticus, Clostridium botulinum, C. perfringens, Aeromonas*.	Todd, 1996; Kaferstein *et al.*, 1997; Levine *et al*., 2001; Adak *et al*., 2002; Tauxe, 2002; Hughes *et al*., 2007.
Viruses	Norwalk-like viruses, Adenoviruses, Astrovirus, NLV, Rotovirus, SLV, Norovirus, Hepatitis A.	Todd, 1996; Ray, 2001; Adak *et al*., 2002; Widdowson *et al*., 2005; Hughes *et al*., 2007.
Pathogenic parasites	*Cryptosporidium parvum, Cyclospora cayatenensis Giardia duodenalis*.	Adak *et al*., 2002; Hughes *et al*., 2007.

Table 1.2: Summary of the bacterial pathogens, symptoms and RTE meat products associated with foodborne illness.

Bacterial Pathogen	Gram	Cell Shape	RTE meat products that have been contaminated	Symptoms associated with illness	Toxin produced	References
Listeria* (*L.*) *monocytogenes	+	Rod	Pates, vacuum packaged beef, jerky, salami, fermented sausage, cooked sausage, uncooked hotdogs, turkey franks, sliced ham and cold cut meat.	Flu-like, mild fever, abdominal cramps, nausea, vomiting, diarrhoea, headaches, miscarriage and still births.	Haemolysin listeriolysin O.	Levine *et al.*, 2001; Moore, 2004; Nummer *et al.*, 2004; Prescott *et al.*, 2005; Jay *et al.*, 2005; Buege *et al.*, 2006; Gibbons *et al.*, 2006; Singh *et al.*, 2006; Siriken *et al.*, 2006; Gormley *et al.*, 2009
***Salmonella* spp.**	-	Rod	Corned beef, sliced ham, jerky, meat salads, meat spreads and semi and dried fermented sausages.	Abdominal cramps, diarrhoea, headaches, nausea, vomiting, chills, fever and prostration.	Enterotoxins and cytotoxins	
***Escherichia (E.) coli* 0157:H7**	-	Rod	Semi and dried sausages, meats, venison jerky, pre-cured and pre-sliced salami.	Hemorrhagic colitus, haemolytic uremic syndrome (HUS), Thrombocytopenic pupura (TTP), abdominal cramps, diarrhoea, vomiting, seizures, kidney failure and brain clots.	Verotoxin(VTI), Shiga (ST).	
Staphylococcus (S) aureus	+	Cocci	Jerky and semi and dried fermented sausages.	Nausea, diarrhoea, abdominal pain and prostration.	13 Types of Enterotoxins.	

+ represents Gram-positive bacterial pathogens; - represents Gram-negative bacterial pathogens

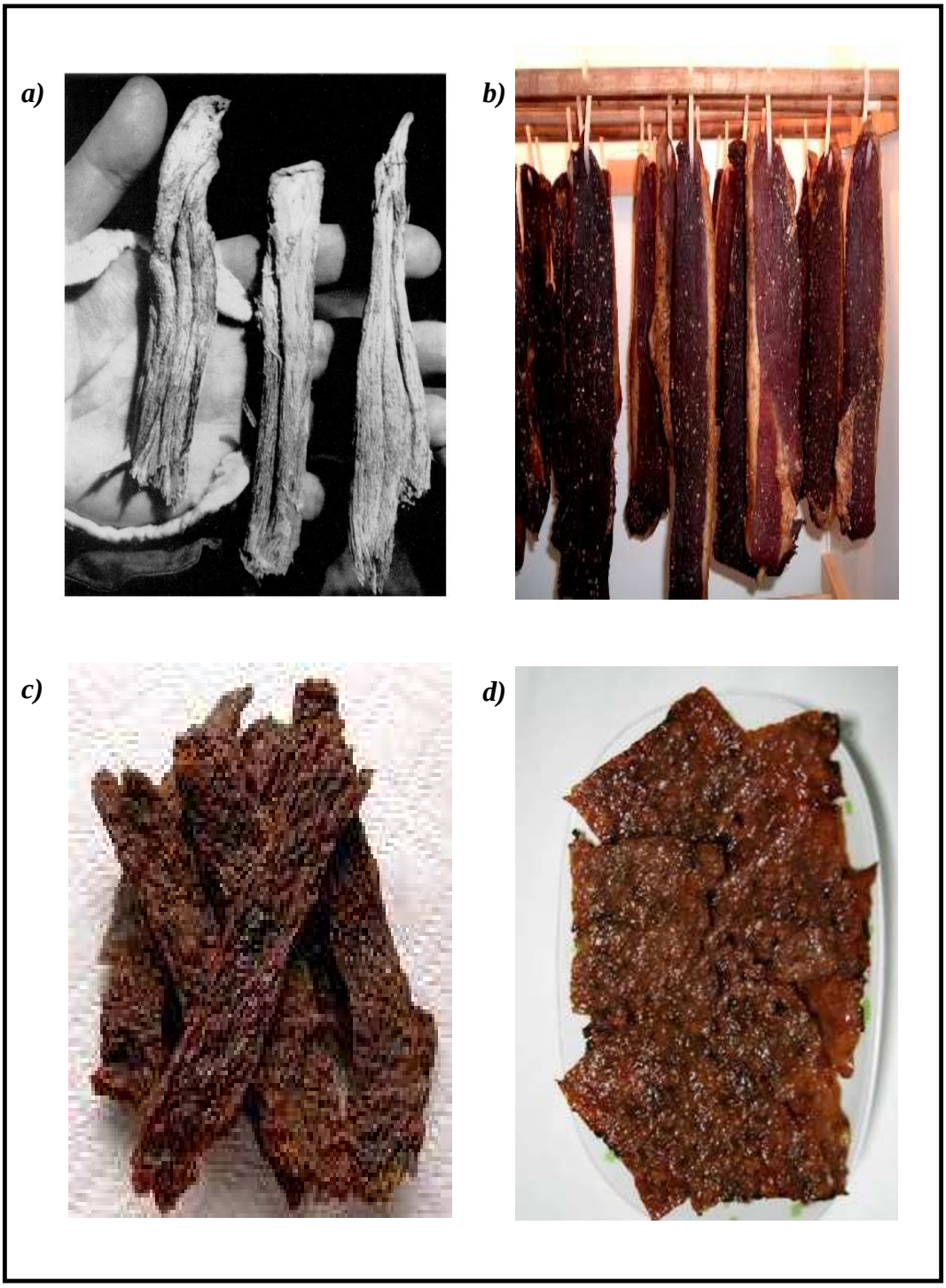


Figure 1.1: Examples of similar RTE dried meat commodities. *a*) Traditional *biltong* in 1957 (Lewis *et al*., 1957), *b*) modern *biltong* (Exzanian, 2009), *c*) jerky (Liechty Buffalo Ranch, 2009) and *d*) *rou gan* (Waren, 2009).

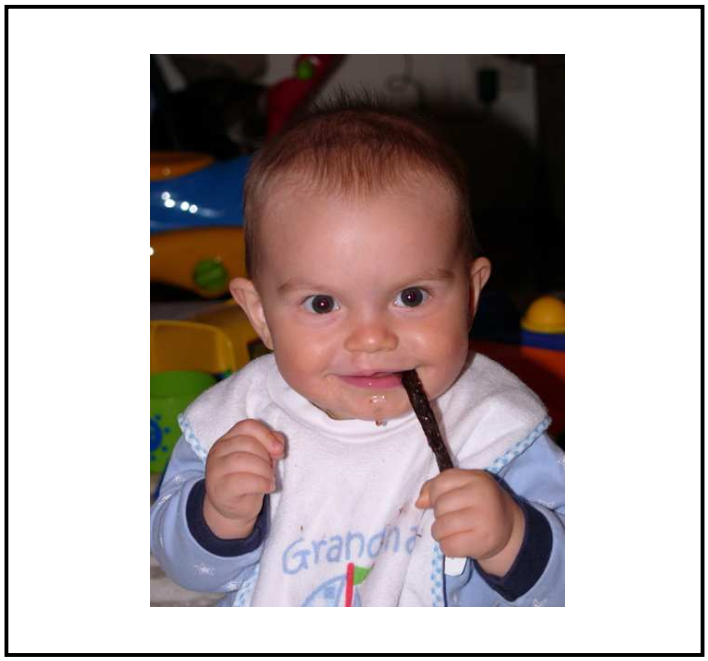


Figure 1.2: *Biltong* sticks as traditionally used by South Africans to replace teething rings (Anonymous, 2006).

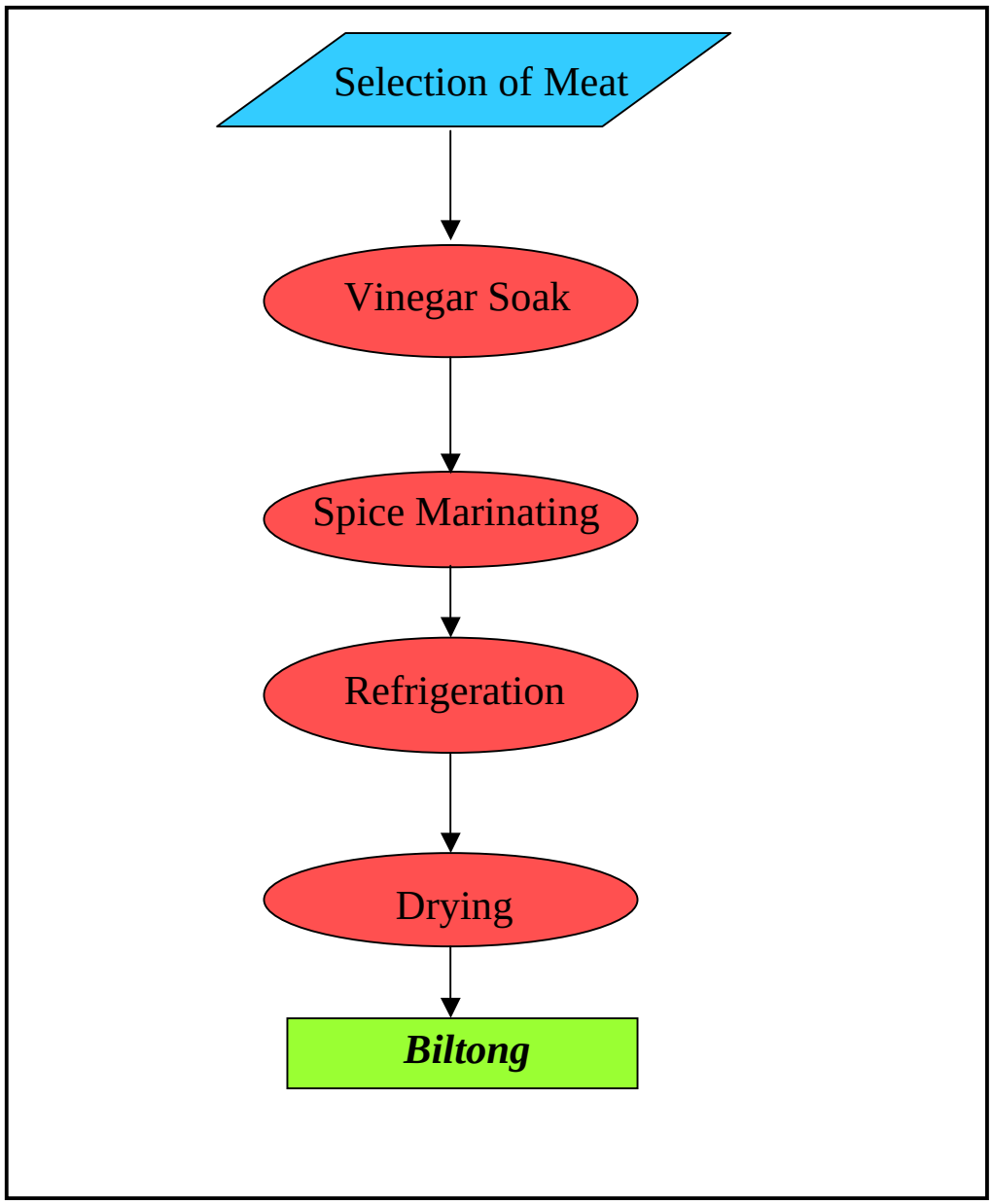


Figure 1.3: Process flow representation of the *biltong* manufacturing process. Represents steps in the process that are expected to be hurdles for bacterial pathogens.

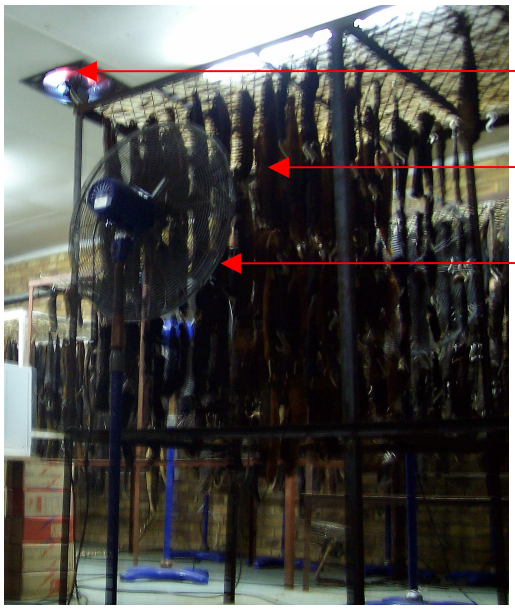
a)



b)



Figure 1.4: Beef portions commonly used for the commercial production of *biltong*. *a)* Rinsed portions ready-for-use, *b)* marination of beef portions with traditional *biltong* spice mixtures.





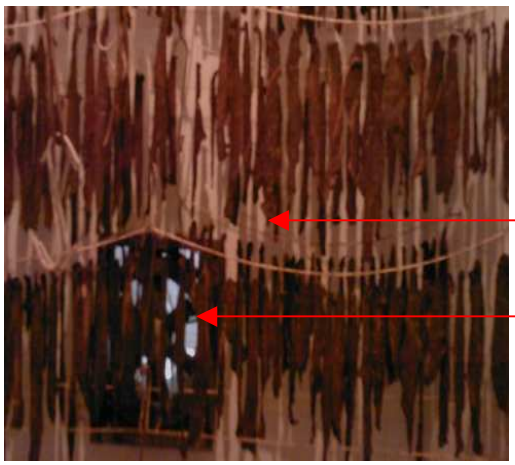


Figure 1.5: Drying of meat portions in controlled rooms for large-scale commercial *biltong* production.

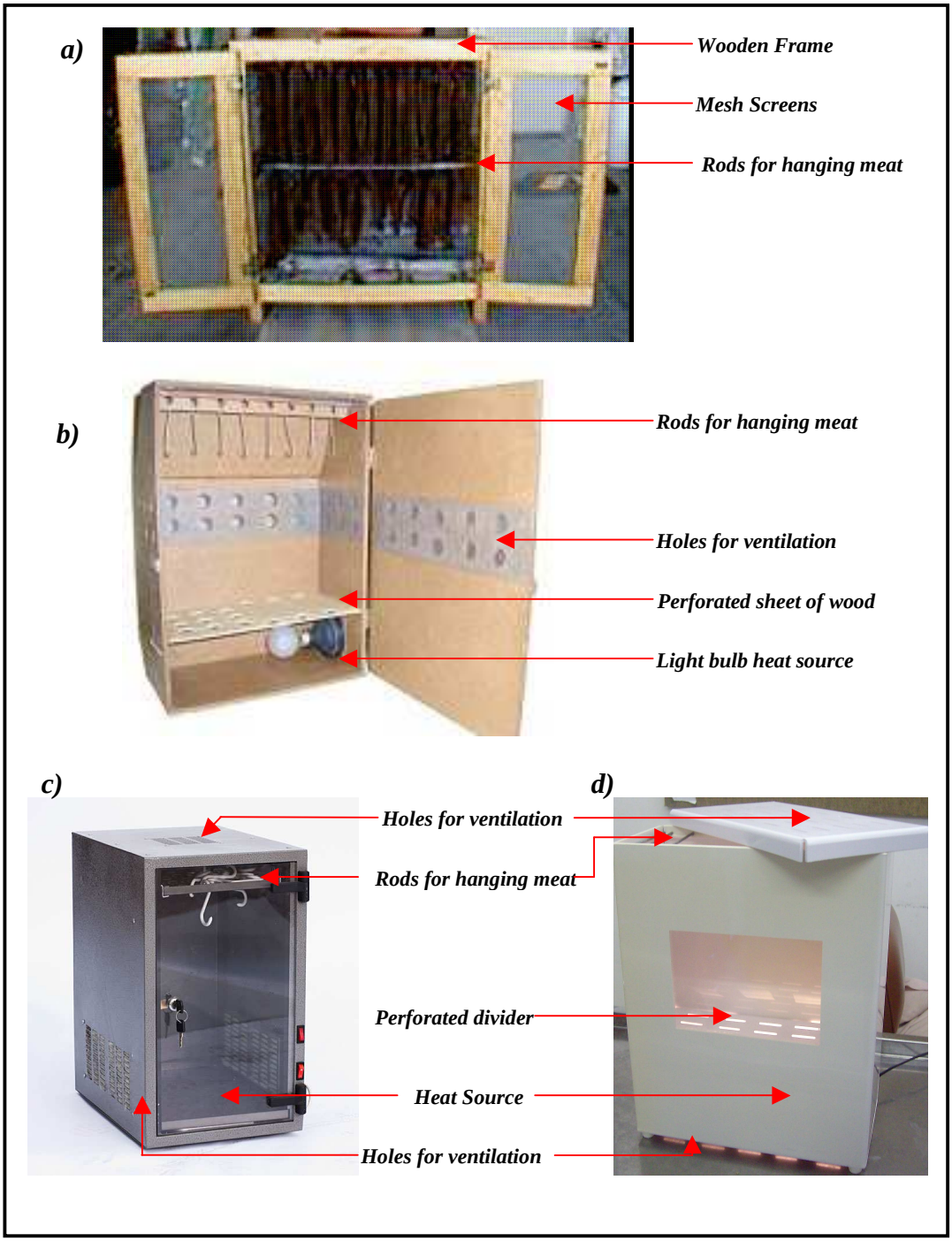


Figure 1.6: *Biltong* boxes commonly used for the drying of meats. *a)* Home-made enclosed cabinet (Anonymous, 2002), *b)* wooden home-made *biltong* box (Anonymous, 2002), *c)* commercially produced steel *biltong* dryer with a filament heat source (Anonymous, 2009e) and *d)* commercially produced perspex *biltong* dryer with a light bulb heat source.

CHAPTER 2:

POTENTIAL CROSS-CONTAMINATION OF THE READY-TO-EAT, DRIED MEAT PRODUCT, **BILTONG,** *AT POINT-OF-SALE IN JOHANNESBURG, SOUTH AFRICA.*

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ABSTRACT

The aim of this study was to evaluate the hygiene of surfaces that come into direct contact with *biltong* product at point-of-sale in three different *biltong* retailers in Johannesburg, South Africa, by investigating the presence of indicator organisms. Samples were collected and plated in duplicate for aerobic plate, Enterobacteriaceae, coliform and *Escherichia coli* counts using standard methods. Typical *E. coli* colonies on Rapid *E. coli* 2 Agar ™ were selected and further identified using 16S rDNA molecular sequencing methods. Bacterial counts associated with *biltong* product ranged between 6 - 7 Log CFU/ g, while counts from swab samples of cutting utensils ranged between 5 - 6 Log CFU/ cm^2. Overall, the lowest counts were associated with swab samples of the display cabinets (2 - 6 Log CFU/ cm^2). Predominant populations were often similar between *biltong* product and the swab samples of various surfaces, indicating potential cross-contamination. Results from 16S rDNA sequence analysis showed that *E. coli* strains isolated from *biltong* product and correspondingly from the swab samples of cutting utensils, were 100 % genetically similar. Strains of potential pathogens belonging to the *Shigella dysenteriae* group (99 %) were also identified. This study highlighted that surfaces in direct contact with *biltong*, an increasingly popular dried meat commodity worldwide, may act as potential sources of 1) cross-contamination of product and 2) potential foodborne pathogens and may hold foodborne illness implications.

1. INTRODUCTION

Biltong is a traditional South African ready-to-eat (RTE), dried and spiced meat product, and is also regarded as a national snack commodity (Nortje *et al*., 2006; Dzimba *et al*., 2007). This RTE meat product is somewhat similar to *carne seca* from Mexico, *charqui* from South America, jerky from USA, *kilshi* from Sahel and *rou gan* from China (Jones *et al*., 2001; Dzimba *et al*., 2007). In the UK in particular, *biltong* has accompanied the migration of South African populations to the British Isles and a variety of retailers specialising in this commodity are becoming commonplace (Henley Standard, 2008).

On a global scale RTE meat products are important vehicles of potential foodborne pathogens. In the case of *biltong*, five documented outbreaks have been published (Neser *et al*., 1957; Bokkenheuser, 1963; Botes, 1966; Van den Heever, 1970; Tshekiso, 2002). The first outbreak killed 1 and sickened 20 individuals who had consumed *biltong*, which was contaminated with *Salmonella newport* (Neser *et al*., 1957). Furthermore the second, third and fourth outbreaks were also attributed to the consumption of *biltong* contaminated with *Salmonella* spp. and had caused severe gastro-enteritis (Bokkenheuser, 1963; Botes, 1966; Van den Heever, 1970). The latest outbreak occurred in Botswana whereby 17 individuals succumbed to illness after the consumption of *biltong*. In this case, however, no causal agent was disclosed (Tshekiso, 2002). The most recent study into the food safety aspect of traditional South African *biltong* was conducted in 1963 (Bokkenheuser, 1963). In addition, no

modern studies on *biltong* contact surfaces appear in the literature to date. By contrast, there are several reported outbreaks associated with jerky, the American RTE dried meat product similar to *biltong*. Since 1966 to date there have been a total of 9 recorded outbreaks associated with the consumption of contaminated jerky whereby *Salmonella* spp. were the casual agents in 6 of these, while *Staphylococcus aureus* caused 2 outbreaks and *Escherichia coli* 0157:H7 was the causal agent of 1 outbreak (CDC, 1995; Keene *et al*., 1997; Eidson *et al*., 2000; Nummer *et al*., 2004).

Previous studies conducted on other RTE products have shown that the hands of food handlers, aprons, utensils, work surfaces, gloves, mops and cloths are all potential reservoirs for bacterial contamination of RTE food products. This was determined by evaluating, among others, the presence of indicator organisms (Christison *et al*., 2007; Lues and Van Tonder, 2007; Tebbutt *et al*., 2007; Christison *et al*., 2008). Indicator organisms included aerobic populations, coliforms, *Escherichia* (*E.*) *coli* and members of the Enterobacteriaceae family, which all provided insight into hygiene and sanitary practices (Aycicek *et al*., 2006; Lues and Van Tonder, 2007).

Therefore the aim of this study was to evaluate the hygiene of the surfaces that come into direct contact with *biltong* product at point-of-sale in three different *biltong* retailers by investigating the presence of indicator organisms.

2. MATERIALS AND METHODS

2.1 Description of retailers and samples collected.

Three *biltong* retailers (A, B, C) in Johannesburg, South Africa were selected for this survey and visited on four separate occasions, during the months of April to June 2008. Retailer A was a medium-scale butchery, in which *biltong* was produced, cut, stored and sold on-site. Retailer B was a boutique distributor where *biltong* was produced off-site, and was often a home-made product, cut, stored (Fig 2.1) and sold on these premises. Retailer C was a medium-scale industrial *biltong* manufacturer where *biltong* was produced, cut, stored sold on-site and distributed to various retail outlets.

From the three retailers the following samples were obtained upon each visit at point-of-sale: (1) three different *biltong* variants including varieties: of beef, game or chicken; spiced with chilli, salt and vinegar or traditional spices such as coriander, black pepper and brown sugar; dry product *ca.* a_w 0.77 (Nortje *et al*., 2006; Dzimba *et al*., 2007); medium product *ca.* a_w 0.85 (Nortje *et al*., 2006; Dzimba *et al*., 2007) and wet product *ca.* a_w 0.93 (Nortje *et al*., 2006; Dzimba *et al.*, 2007), (2) air samples within display cabinets and (3) swab samples of various surfaces including the cutting utensils (cutting apparatus (Fig 2.2) and associated preparation surface), 3 inner walls of the *biltong* display cabinets in contact with *biltong* product (Fig 2.3), and both hands of *biltong* handlers (Fig 2.4). All samples were transported at 4 °C to the laboratory and processed on the same day of collection. A total of 36 *biltong* samples, 36 environmental air samples and 84 surface swab samples were collected in this study.

2.2 Sample collection, processing and analysis

For each *biltong* sample, 20 g of the *biltong* product was transferred into a Whirl-Pak bag (Nasco, USA), combined with 180 ml diluent [0.1 % Bacteriological Peptone (BioLab, Midrand, South Africa) + 0.85 % sodium chloride] and homogenised for 2 min using a Colworth 400 Stomacher (Geornaras and von Holy, 2000; Mosupye and von Holy, 2000; Christison *et al*., 2008). The homogenised *biltong* samples were serially diluted in diluent and plated in duplicate using standard plating methods onto several types of media (Table 2.1), in order to enumerate aerobic bacteria (APC), Gram-Negative bacteria (GNC), Enterobacteriaceae bacteria (EBC), coliform bacteria (CC) and *E. coli* (EC).

Environmental air samples were obtained by means of triplicate settle plates (Pasquarella *et al*., 2000) of Tryptone Soy agar (TSA) (BioLab), which had been placed within the *biltong* display cabinets of each retailer for a duration of 10 min (Geornaras *et al*., 1996) and transported to the laboratory and incubated for 48 h at 37 °C.

For *biltong* contact surfaces, sterile polyester tipped Copan swab rinse kits (Copan, Brescia-Italy), were used to swab an area of 25 cm^2 for each surface in each *biltong* retailer (3 inner walls of display cabinet, cutting device and work surface) (Geornaras and von Holy, 2000). Hand samples of *biltong* handlers (Fig 2.4) were obtained by rolling the swab over the palm as well as in between the fingers and thumb for *ca.* 15 sec (Christison *et al*., 2008). All swabs were transported to the laboratory at 4 °C, where the polyester tip was aseptically cut from the applicator stick and inserted along with the contents of the rinse kit into a sterile test tube and vortexed for 1 min (Eisel

et al., 1997; Christison *et al*., 2008). Following 20 min recovery, samples were serially diluted in diluent and plated in duplicate onto various growth media (Table 2.1) (Mosupye and von Holy, 2000) in order to enumerate APC, GNC, EBC, CC and EC counts.

2.3 Enumeration

Duplicate plates containing between 30 to 300 colonies, or the highest number if below 30 were enumerated (Kunene *et al*., 1999; Christison *et al*., 2007; Christison *et al*., 2008) and expressed as Log colony forming units (Log CFU) per gram for *biltong* samples, per cm^2 for surface samples or per hand for *biltong* handlers.

2.4 Characterisation of predominant populations

Four colonies were selected from duplicate APC plates of each sample showing bacterial growth on the highest dilution. A total of 129 isolates were obtained from the *biltong* product (43 per retailer) and overall 230 isolates were obtained from surface swabs (76 from retailer A, 76 from retailer B and 78 from retailer C). Overall a total of 359 strains had been isolated. Colonies were isolated and purified using standard purification methods previously described (von Holy and Holzapfel, 1988; Kunene *et al*., 1999; Kubheka *et al*., 2001; Christison *et al*., 2007; Christison *et al*., 2008) and thereafter characterised using dichotomous keys (Faller and Schleifer, 1981; Fischer *et al*., 1986).

2.5 Statistical analysis

Counts obtained for the various *biltong* samples were compared in order to determine statistical significance using the analysis of variance (ANOVA) at 95 % confidence interval (STATGRAPHICS, STSC Inc. and Statistical Graphics Corporation).

2.6 Identification of presumptive *E. coli* using 16S rDNA sequencing

Typical *E. coli* colonies (8 isolates), which grew as purple colonies on Rapid *E. coli* 2 agar™ from *biltong* product and swab samples from contact surfaces were selected for further molecular identification using 16S rDNA sequencing. Polymerase chain reaction (PCR) amplification was carried out as previously described using the primer sets U1392R (5'- ACG GGCGGT GTG TRC-3') and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3') in combination with Fermentas 2x PCR Master Mix (Fermentas Life Science, www.fermentas.com). The purified PCR product was sequenced and analysed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against the 16S rDNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). A homology tree highlighting the clustering of the presumptive *E. coli* isolates was then constructed using DNAMAN version 4, Lynnon Biosoft (Christison *et al*., 2007).

2.7 Scanning electron microscopy of *biltong* product

In order to evaluate the bacterial strains attached to the *biltong* product surface, Scanning Electron Microscopy had been utilised. *Biltong* product from each retailer was selected and prepared as previously described (Lindsay *et al*., 1996; Lindsay *et al*., 2002). Samples were fixed for 24 h in 3 % glutaraldehyde, followed by a series of ethanol dehydrations, critical point dried, mounted and coated with carbon and gold-palladium. Prepared *biltong* samples were visualised under the JSM-840 Scanning Electron Microscope (Lindsay *et al*., 1996; Lindsay *et al*., 2002).

3. RESULTS AND DISCUSSION

3.1 Overall hygienic evaluation of retailers

Overall, results from this study indicated that retailer B had consistently showed the highest bacterial counts associated with all *biltong* product (Fig 2.5), swabs from contact surfaces (Fig 2.6) and air samples, while retailer A had showed the lowest significant ($p < 0.05$) bacterial counts associated with all samples (Fig 2.5, 2.6).

3.2 Bacterial counts associated with *biltong* samples

Biltong, an RTE dried meat product, is a staple snack commodity in most South African households (Lewis *et al*., 1957; Dzimba *et al*., 2007). Previous studies have shown that *biltong* carries bacterial APC loads of between 5 - 7.5 Log CFU/ g (Prior, 1984). When comparing the bacterial counts observed in this study, it was evident that the *biltong* product obtained from retailers A and C corresponded to that of literature reports as APCs ranged from *ca.* 6 - 7.5 Log CFU/ g (Fig 2.5a, c). By contrast, APCs obtained from *biltong* product sampled from retailer B (Fig 2.5b) were significantly

higher ($p < 0.05$) than that of retailers A (Fig 2.5a) and C (Fig 2.5c) and had also generally exceeded the previously published ranges by *ca.* 0.50 - 1 Log CFU/ g (Fig 2.5b).

Modern consumer markets favour moister *biltong*, which is considered to be more appealing to the pallet (Nortje *et al*., 2006; Dzimba *et al*., 2007). Traditionally, dried *biltong* has a water activity (a_w) of 0.77, however, the favoured *biltong* has 40 % more moisture than traditional *biltong* and has an a_w of between 0.85 and 0.93 (Nortje *et al*., 2006; Dzimba *et al*., 2007). An increased a_w may result in the increased survival of bacterial pathogens, such *Staphylococcus aureus*, which are capable of growth and enterotoxin production at a_ws of ≥ 0.85 (Nortje *et al*., 2006). Indeed, APCs from this study (Fig 2.5c) indicated that wet *biltong* product (*ca.* a_w 0.93) obtained from Retailer C carried a higher bacterial load when compared to the drier product (*ca.* a_w 0.77) (Fig 2.5c). The latter highlights the possibility that higher a_w values are associated with higher loads of microbial populations (Jay *et al*., 2005).

EBC, CC and EC were used in this study to assess product hygiene as high EBC and ECs are indicative of enteric pathogens (Barros *et al*., 2007). Retailer A had the highest EBC (*ca.* 3 - 4 Log CFU/g) (Fig 2.5a) while *biltong* product obtained from retailer C had the lowest associated EBC (*ca.* 3 Log CFU/ g) (Fig 2.5c). The highest CC (*ca.* 2.5 to 4 Log CFU/ g) and ECs (*ca.*1 - 1.5 Log CFU/ g) were recorded from *biltong* samples obtained from retailer B. Although ECs obtained from retailer A and C were below the lower detection limit (1 Log CFU/ g) (Fig 2.5a, c), the presence of 1 or 2 purple colonies on Rapid *E. coli* 2 ™ agar per 20 grams had been apparent. The

presence of *E. coli* is an index organism for the potential presence of other bacterial pathogens such as *Salmonella*, *Shigella* and *E. coli* 0157:H7 which are all capable of contaminating and surviving in the same environmental niches (Moore and Griffith, 2002; Li *et al*., 2005; Lues and Van Tonder, 2007).

3.3 Bacterial populations associated with surface samples

According to Lues and Van Tonder (2007), the South African National Health Regulation stipulates that a working surface or any surface that comes into direct contact with a food commodity should not contain more than 100 viable microorganisms per cm^2 (2 Log CFU/ cm^2) to be considered clean and safe. When comparing the results obtained in this study, it was evident that swab samples from all the surfaces obtained from retailers A, B and C (Fig 2.6) had exceeded this stipulated acceptance level by a range of *ca.* 0.25 to 1.5 Log CFU/ cm^2.

Overall, the highest APCs were evident from swab samples of the cutting utensils while the lowest observed APCs were associated with swabs samples from *biltong* display cabinets (Fig 2.6). A similar trend had been observed for EBCs and CCs from retailers A and C (Fig 2.6a, c). The highest EBCs and CCs observed had been associated with the swab samples obtained from the hands of the *biltong* handler in retailer A (Fig 2.6a), the display cabinet from retailer B (Fig 2.6b) and the cutting utensils from retailer C (Fig 2.6c). The generally accepted levels for CCs on food contact surfaces are reportedly < 2.5 CFU/ cm^2 (0.40 Log CFU/ cm^2) (Lues and Van Tonder, 2007). In this study it was evident that none of the sampled surfaces had

conformed to this standard, indicating that all surfaces were deficient in hygiene practices.

ECs between all 3 retailers conformed to the accepted level of 1 CFU/ cm^2 (0.40 Log CFU/ cm^2) (Lues and Van Tonder, 2007), with the exception of the swab sample from the cutting utensils in retailer A (Fig 2.6a), were all below the lower detection limit (Fig 2.6). However, the growth of 1 or 2 purple colonies on Rapid *E. coli* 2 ™ agar per 25 cm^2 was apparent from swab samples taken from cutting utensils in retailers A and C and were selected for further identification.

3.4 Bacterial populations associated with environmental air samples

When evaluating the counts obtained from the TSA settle plates, retailer B was the only retailer with counts (34 colonies/ 10min) within the countable range as stipulated by Mossel *et al*. (1995) (20 –200 colonies), while retailer A (6 colonies/ 10 min) and C (4 colonies/ 10min) were below the countable range. In retailer B, constant movement of people within this boutique was noted and as such this air movement may have been a contributing factor in the contamination of product within this retailer.

3.5 Predominant populations associated with each retailer.

Of the 359 predominant isolates that were collectively associated with *biltong* product and swab samples of contact surfaces, Gram-positive isolates especially members of the *Bacillus, Staphylococcus* and Micrococcaceae genera proved to be dominant (about 82.8 %) (Fig 2.7). Generally, Gram-negative populations constituted 8.5 % of

the predominant populations, while yeasts comprised of 9.2 % of predominant populations (Fig 2.7).

Predominant populations obtained from *biltong* product and swab samples from the various contact surfaces highlighted the importance of the environment in potential cross-contamination of *biltong* product. For example, similar trends in predominant populations were observed between the product and swab samples from all surfaces in retailer A (Fig 2.7). Predominant populations specifically those associated with *biltong* product and the swab samples of the cutting utensils, were the same with the exception of Gram-negative rod populations and occurred in very similar frequencies as seen in the incidences of yeast (8 % associated with *biltong* and 10 % associated with cutting utensils), *Bacillus* (31 % associated with *biltong* and 37 % associated cutting utensils) and *Staphylococcus* (27 % associated with *biltong* and 33 % associated cutting utensils) populations (Fig 2.7). Similarly, in retailers B and C *biltong* product and swab samples from the hands of the *biltong* handler or the cutting utensils were shown to share similar predominant populations types (Fig 2.7). Again, the *Staphylococcus* genera were the dominant populations associated with *biltong* product (67 and 63 %) and either the hands of the *biltong* handler or cutting utensils (48 and 45 %) (Fig 2.7). Qualitatively, observations using Scanning Electron Microscopy supported these findings as both rod and coccoid-shaped cells were observed on *biltong* product surfaces (Fig 2.8).

The hands of RTE product handlers as well as cutting utensils have previously been identified as vectors aiding in the contamination of RTE food products (Tebbutt,

1986; Little and de Louvois, 1998; Lunden *et al*., 2002; Tebbutt *et al*., 2007; Todd *et al*., 2007; Todd *et al*., 2008; Sauders *et al*., 2009; Todd *et al*., 2009). Cutting devices are of a particular importance as the blades are capable of disseminating high numbers of microbial populations with every slice, which may also include bacterial pathogens with every slice (Tebbutt, 1986; Tebbutt *et al*., 2007; Sauders *et al*., 2009). Since the trend observed in predominant populations associated with the *biltong* product and swab samples from the cutting utensils in this study were similar (Fig 2.7), it may be suggested that the cutting utensils potentially contributed to the cross-contamination of the *biltong* product.

To date, several studies have implicated cutting utensils as the sources of cross-contamination of RTE products (Tebbutt, 1986; Little and de Louvois, 1998; Lunden *et al*., 2002; Tebutt *et al*., 2007; Christison *et al*., 2007; Christison *et al*., 2008; Pal *et al*., 2008; Sauders *et al*., 2009). In some instances these devices have aided in the dissemination of bacterial pathogens (Lunden *et al*., 2002; Pal *et al*., 2008), which have recently lead to severe foodborne illness implications as a result (Bouzane and Wylie, 2008).

3.6 Identification of presumptive *E. coli* isolates

In order to confirm the potential cross-contamination between *biltong* product and associated surfaces, typical *E. coli* (purple colony from Rapid *E. coli* 2 agar ™) colonies were selected from *biltong* product and swab samples of surfaces including isolates KesE99 (accession number EU919568) and KesE3 (accession number EU884312) (retailer A), KesE4 (accession number EU884313), KesE6 (accession

number EU884314), KesE7 (accession number EU888568) (retailer B) and KesE8 (accession number EU888639), KesE9 (accession number EU888640), KesE10 (accession number EU888641) (retailer C) for 16S rDNA sequencing.

Results from molecular analysis indicated that of the 8 presumptive *E. coli* strains, only 5 strains were confirmed as positive *Escherichia coli* (KesE99, KesE6, KesE8, KesE9, KesE10), while 2 strains were identified as *Shigella dysenteriae* (KesE4, KesE7) and 1 was identified as *Buttiauxella agrestis* (KesE3) (Fig 2.9). Identification of these isolates by 16S rDNA sequencing further confirmed the suggestion of potential cross-contamination between *biltong* product and the environment. For example isolate KesE8, which was isolated from wet *biltong* product (a_w 0.93 - Dzimba *et al*., 2007), and strain KesE10, isolated from the swab sample of the cutting utensils (retailer C) were 100 % genetically similar to each other and were identified as *E. coli* (99 %) strains (Fig 2.9). In addition, isolate KesE9, from medium *biltong* product (a_w 0.85 – Dzimba *et al*., 2007) (retailer C) was 99 % genetically similar to the latter. This had further confirmed the role that the cutting utensil might have played in the cross-contamination of the wet and medium *biltong* products, or *vice versa*. Previous studies have indeed shown that cutting utensils act as sources of RTE product cross-contamination with other bacterial pathogens such as *Listeria monocytogenes* (Aarnisalo *et al*., 2006; Gibbons *et al*., 2006; Pal *et al*., 2008; Sauders *et al*., 2009).

In addition, strains KesE4, KesE6 and KesE7, obtained from different *biltong* product types in the same retailer (retailer B) were 100 % genetically similar to each other and

99 % similar to the *Shigella dysenteriae*/ *boydii* cluster (Fig 2.9), which strongly suggested potential product to product cross-contamination. Of an additional concern was the presence of possible *Shigella* strains, which are enteric pathogens capable of causing classic bacillary dysentery at cell numbers of as little as 10 CFU (Johnson *et al*., 2005; Li *et al*., 2005). In addition several strains of these species have previously been associated with foodborne illness (Li *et al*., 2005) and have a relative ease in transmission via the faecal oral route indicating deficient hygiene practices (Li *et al*., 2005). Furthermore, the presence of *Shigella* indicated that bacterial pathogens such as *Salmonella* and *E. coli* 0157:H7 may have also potentially been present in these *biltong* products as these species are all capable of occupying the same environmental niches (Li *et al*., 2005).

The findings of this study raised concern with regard to cleaning regimes within all three retailers. It also validated the aim of this study and showed that surfaces that come into contact with *biltong* at point-of-sale may be vectors aiding in the dissemination of pathogens especially if they aid in the dissemination of hygiene indicators. It is therefore essential that improved cleaning regimes and enclosed *biltong* cabinets should be introduced and implemented within all retailers surveyed in order to reduce the risk of product contamination and spread of potential pathogens.

4. CONCLUSION

It is apparent that surfaces that are in direct contact with *biltong* product at point-of-sale are potential reservoirs in contributing to the cross-contamination of the

final product. The presence of *Shigella dysenteriae* on *biltong* product has questioned the presence of bacterial pathogens such as *Listeria monocytogenes*, *E. coli* 0157:H7 and *Salmonella* as they may share the same environmental niches. This has initiated further research into evaluating the presence of these pathogens in *biltong* product at point-of-sale (Chapter 3).

Table 2.1: Culture methods, media and the aerobic incubation time and temperatures utilised in the analysis of all *biltong* and swab samples of selected surfaces obtained from the three retailers in this study.

Population Tested for	Time (h)	Temperature (°C)	Plating Method	Bacterial Growth Media
Aerobic Plate Counts (APC)	48	37	Pour (10^1), Spread	Tryptone Soy agar (TSA) (BioLab,Midrand South Africa).
Gram Negative Counts (GNC)	48	37	Spread	Violet Red Bile Glucose agar (VRBG) (Oxoid,London).
Coliform Count (CC) (blue, green colonies)	48	37	Spread	Rapid *E. coli* 2 agar™ (Bio-Rad, France).
***E. coli* Counts (EC) (purple/ indigo colonies)**	48	37	Spread	Rapid *E. coli* 2 agar™ (Bio-Rad, France).
Enterobacteriaceae Counts (EBC) (purple, green and white colonies)	48	37	Spread	Rapid *E. coli* 2 agar™ (Bio-Rad, France).



Figure 2.1: The refrigerated storage of different variants of *biltong*, prior to and after trading hours in retailer B.



Figure 2.2: Cutting apparatus (1) as well as the blades of apparatus (2) used to slice the *biltong* product that was surveyed in this study from retailers A (*a*), B (*b*) and C (*c*).

a)



b)



c)



Figure 2.3: The *biltong* display cabinets, which were surface swabbed during this survey from retailers A (*a*), B (*b*) and C (*c*).



Figure 2.4: *Biltong* product being dispensed after slicing by *biltong* handler without using gloves in retailer A.

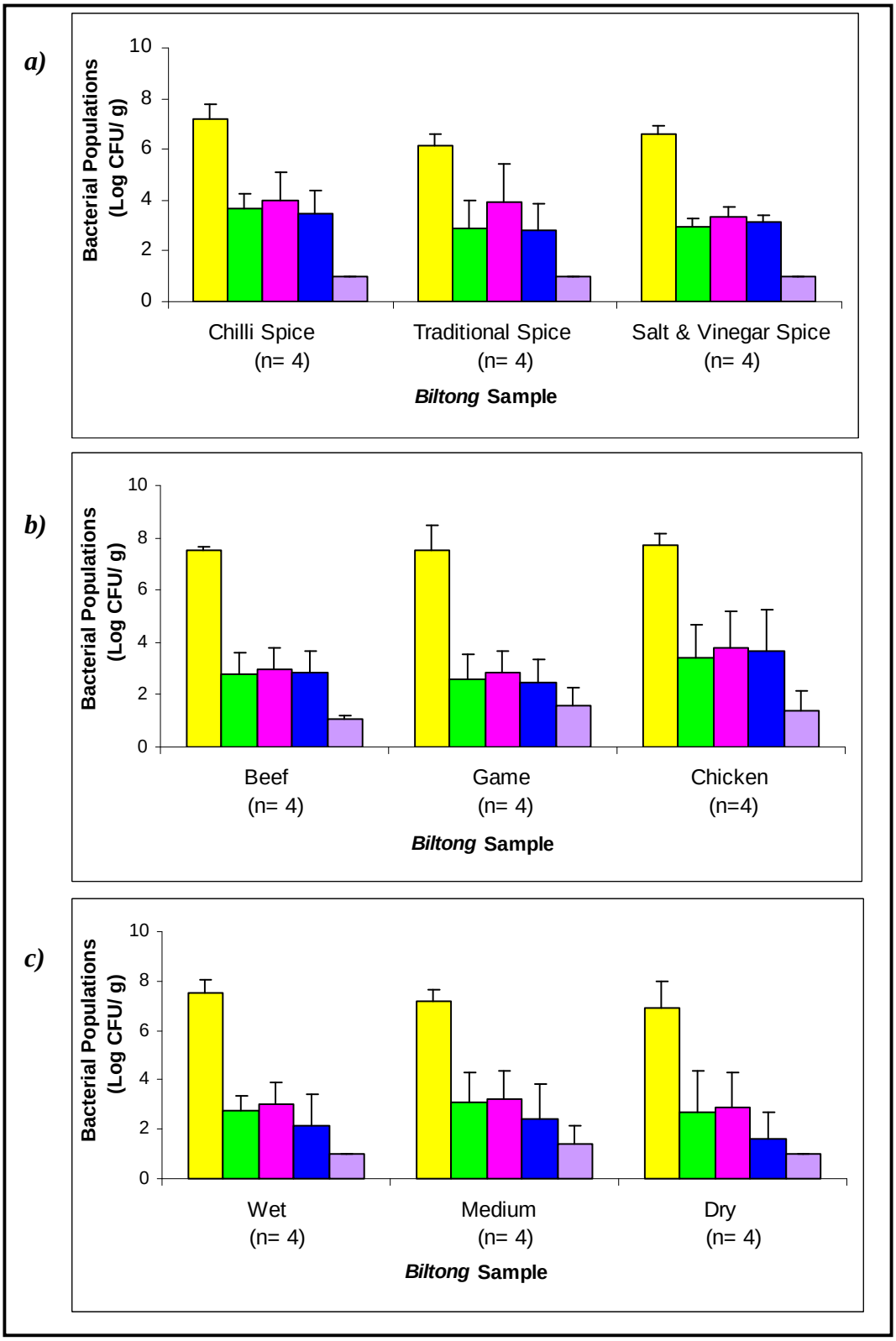


Figure 2.5: Bacterial populations including aerobic plate (yellow bar), Gram-negative (green bar), Enterobacteriaceae (pink bar), Coliform (blue bar) and *E. coli* (lilac bar) counts obtained from *biltong* retailers A (*a*), B (*b*) and C (*c*). (Lower detection limit = 1 Log CFU/ g). Standard deviation (τ).

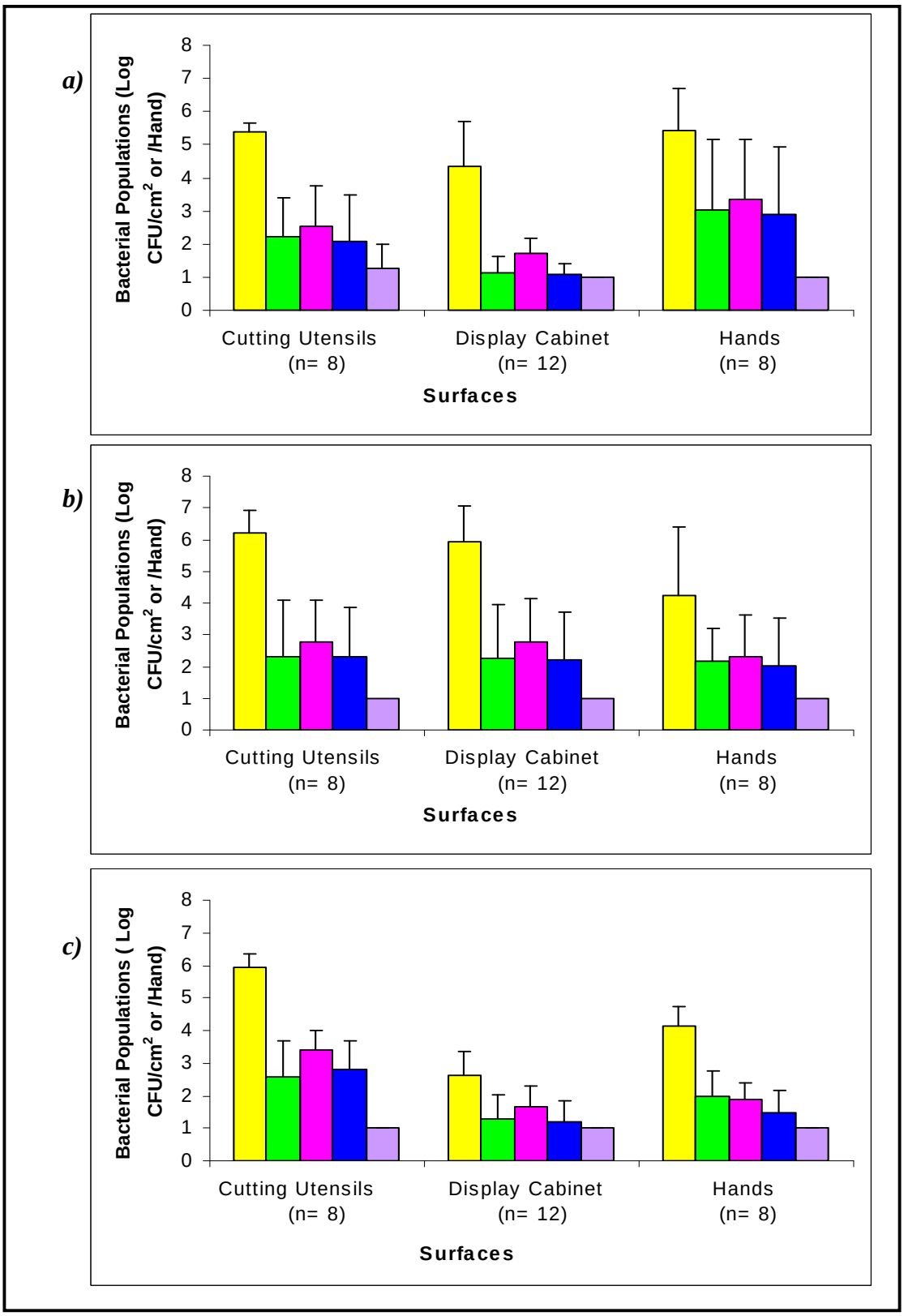


Figure 2.6: Aerobic plate (yellow bar), Gram-negative (green bar), Enterobacteriaceae (pink bar), Coliform (blue bar) and *E. coli* (lilac bar) counts obtained from swab samples of various *biltong* contact surfaces at point-of-sale from retailers A (*a*), B (*b*) and C (*c*). (Lower detection limit= 1 Log CFU/ cm^2 or /Hand). Standard deviation (τ).

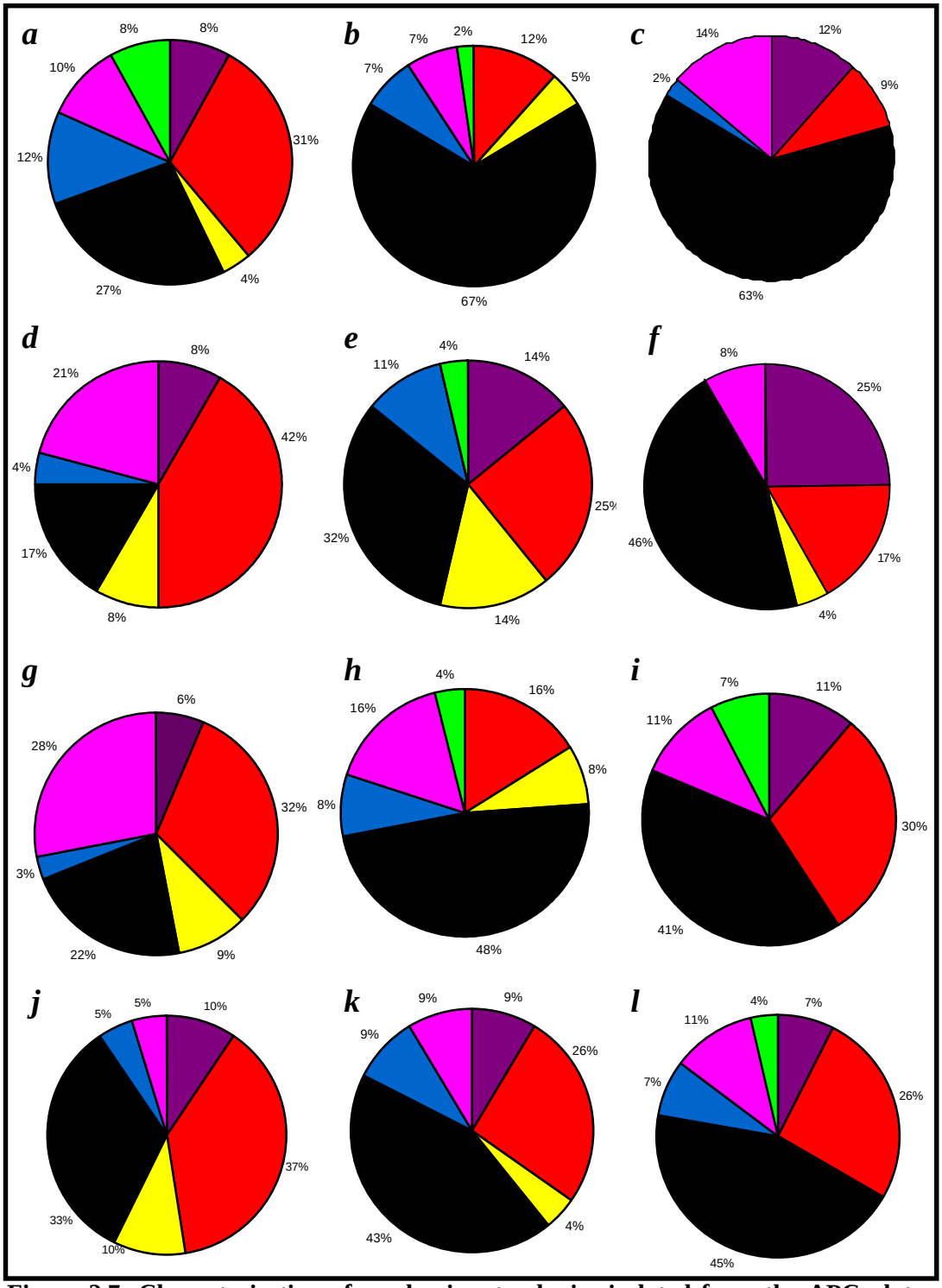


Figure 2.7: Characterisation of predominant colonies isolated from the APC plates of the *biltong* product (*a, b, c*), display cabinet (*d, e, f*), hands of *biltong* handler (*g, h, i*) and cutting utensil (*j, k, l*) samples from retailer A (*a, d, g, j*), B (*b, e, h, k*) and C (*c, f, i, l*). ■ *Staphylococcus,* ■ *Bacillus,* ■ Micrococcaceae, ■ Yeast, ■ Gram-Negative rods, ■ Enterococcaceae and ■ *Lactobacillus.* (n=359).

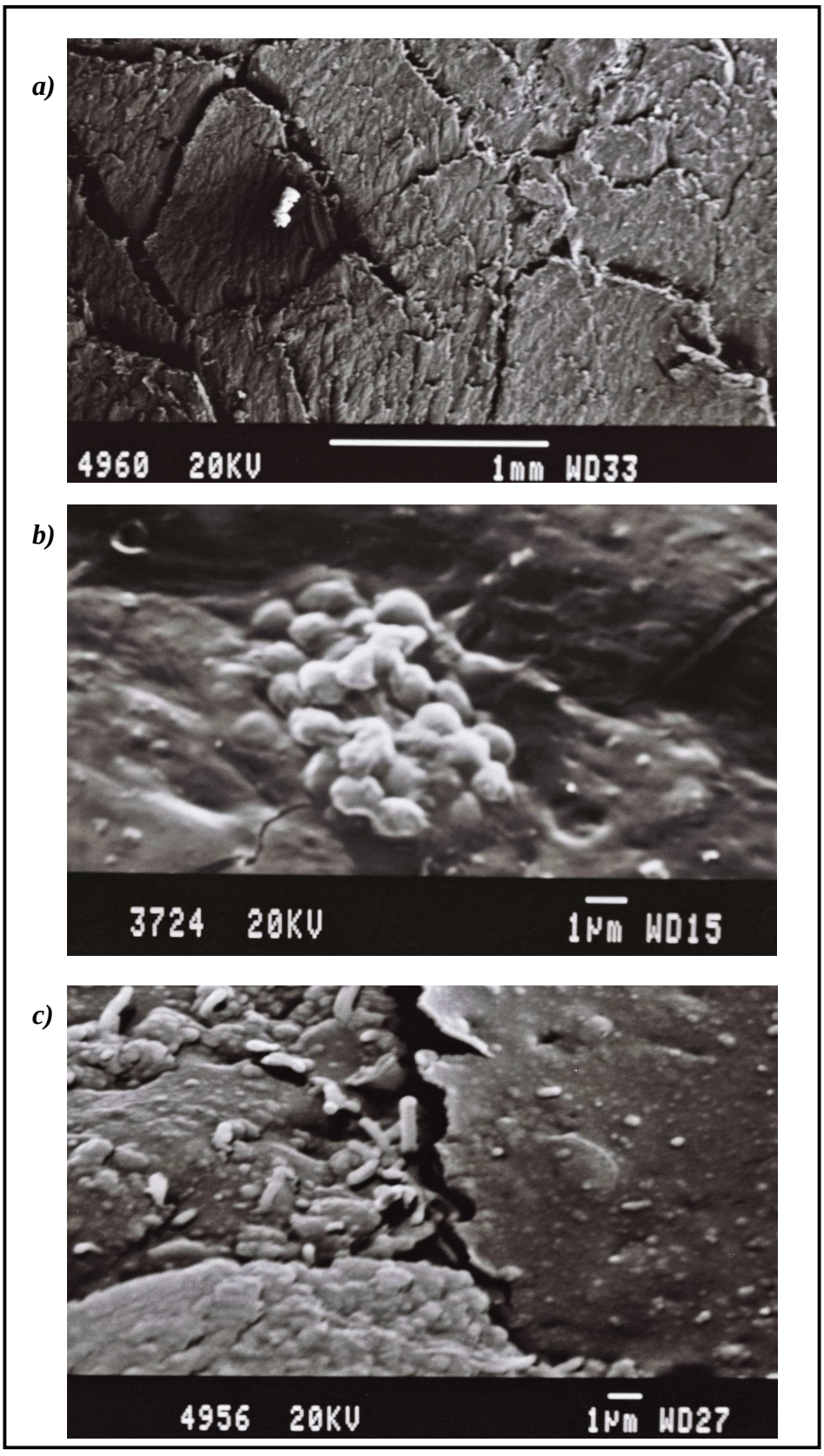


Figure 2.8: Scanning electron micrographs indicating the attachment of bacterial cells to *biltong* product surface (*a)*, the presence of coccoid- (*b)* and rod-shaped bacterial cells (*c*).

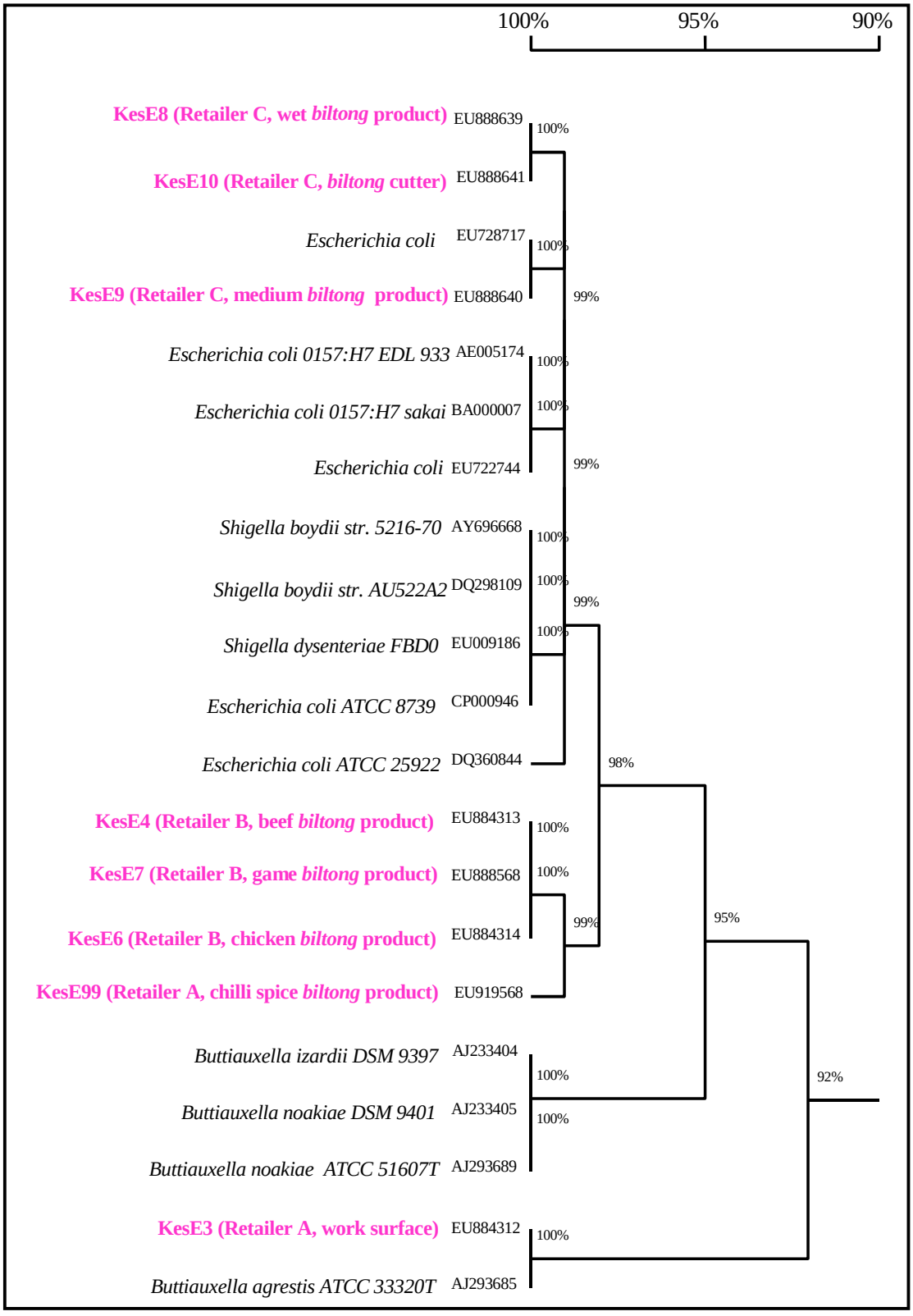


Figure 2.9: A rooted homology tree highlighting the clustering of presumptive *E. coli* strains (bolded pink text) constructed using DNAMAN version 4, Lynnon Biosoft.

CHAPTER 3:

LISTERIA MONOCYTOGENES *AND* *ENTEROTOXIN-PRODUCING* STAPHYLOCOCCUS AUREUS *ASSOCIATED WITH SOUTH AFRICAN* BILTONG *IN THE GAUTENG PROVINCE.*

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ABSTRACT

In this study, 150 samples of the South African dried and ready-to-eat meat product, *biltong*, were collected from various geographical locations in the Gauteng province. Samples were analysed for aerobic, Enterobacteriaceae, coliform, *Escherichia coli* and presumptive *Staphylococcus aureus* counts. *Salmonella* spp. and *Listeria monocytogenes* were also detected using standard detection methods. Presumptive pathogens were selected and further identified using 16S rDNA molecular sequencing methods. Aerobic plate, were the highest (*ca.* 7 Log CFU/ g), followed by Enterobacteriaceae (*ca.* 4 Log CFU/ g), coliforms (*ca.* 3 Log CFU/ g), presumptive *Staphylococcus* (*ca.* 3 Log CFU/ g) and *Escherichia coli* (*ca.* 1 Log CFU/ g) counts in descending order. Results from 16S rDNA sequence analysis showed that *Salmonella* spp. were absent in all samples tested, while 2 samples tested positive for *Listeria monocytogenes* and 3 samples for enterotoxin-producing *Staphylococcus* strains. Results from this study highlighted *biltong*, an increasingly popular dried meat commodity worldwide, as a reservoir of potential foodborne pathogens, which may hold emetic and foodborne illness implications.

1. INTRODUCTION

Foodborne disease outbreaks have become an increasingly global problem (de Souza, 2008). Ready-to-eat (RTE) foods are commodities that require no further culinary processing prior to consumption, and have recently been implicated in several foodborne disease outbreaks (Gilbert *et al*., 2000; Gillespie *et al*., 2000; CDC, 2002; Christison *et al*., 2008; Pradhan *et al*., 2009; Ross *et al*., 2009; Valero *et al*., 2009). Of these RTE commodities, low and intermediate moisture meat and meat products have frequently been vectors in human foodborne disease outbreaks, such as listeriosis, salmonellosis and shigellosis (Jay *et al*., 2005; Jemmi and Stephan, 2006; Siriken *et al*., 2006; Norrung and Buncic, 2008). The RTE meat products implicated in these outbreaks included wieners (hot dogs), bologna, dry and semi-dried sausages, Genoa salami, jerky and *biltong*, and were often prepared by either curing, smoking, fermenting or drying of raw meats (CDC, 1995; Keene *et al*., 1997; Nummer *et al*., 2004; Jay *et al*., 2005; Bohaychuk *et al*., 2006; Kathariou *et al*., 2006; Berzins *et al*., 2007; Montet *et al*., 2009).

Historically cured, fermented and dried meat products are regarded as relatively microbially safe RTE foods due to the low water activity (a_w), low pH and the presence of curing salts associated with these products (Montet *et al*., 2009). However, the survival and prevalence of shiga-toxin producing *Escherichia coli* 0157:H7, *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus*, have previously been detected in these meat products (Siriken *et al*., 2006; Ferreira *et al*. 2007; Giovannini *et al*., 2007; Norrung and Buncic, 2008; Montet *et al*., 2009).

Indeed, several outbreaks linked with *Escherichia coli* 0157:H7 in RTE meat products have been recorded, including in dry (Genoa salami, pepperoni) and semi-dry (Lebanon bologna, cervelat) sausages (Williams *et al*., 2000; Moore, 2004; Hwang *et al*. 2009; Montet *et al*., 2009). In addition, 5 outbreaks of salmonellosis were also recorded due to the consumption of contaminated fermented sausages (Moore, 2004; Jay *et al*., 2005; Siriken *et al*., 2006; Hwang *et al*., 2009). Although beef jerky, the American dried RTE meat which has some similarities to South African *biltong,* is a low moisture RTE meat snack, and is considered shelf-stable, there have been 9 recorded foodborne illness outbreaks, and 5 recalls associated with this commodity to date (CDC, 1995; Keene *et al*., 1997; Nummer *et al*., 2004; Smelser, 2004; Allen *et al*., 2007; Porto-Fett *et al*., 2009). Several pathogens associated with such outbreaks and recalls have included *Salmonella* spp., *Staphylococcus aureus* and *E. coli* 0157:H7 (CDC, 1995; Keene *et al*., 1997; Nummer *et al*., 2004; Smelser, 2004; Borowski *et al*., 2009; Porto-Fett *et al*., 2009). Although no documented outbreaks of listeriosis linked to jerky exist, Allen and associates (2007) reported a 0.52 % prevalence of *L. monocytogenes* in beef jerky.

Biltong is a traditional South African RTE, dried and spiced meat product (Nortje *et al*., 2006; Dzimba *et al*., 2007). It has become a popular RTE meat snack both locally and internationally, as it is relatively easy to produce. All that is required is a selection of either beef, game (Kudu, Springbok, Impala), chicken or ostrich meat, which is then cured at low temperatures with several basic spices (salt, black pepper, dried and roasted coriander and brown sugar) (Dzimba *et al*., 2007), and vinegars (apple cider, brown spirit, wines), and is dried, at ambient temperatures, for several days (Burnham

et al., 2008). As a result, *biltong* production is often a home industry in South Africa (Mothershaw *et al*., 2003), and the safety of this commodity is of concern (Sweeney, 2005). Further to this, *biltong* has been linked to several foodborne disease outbreaks (Neser *et al*., 1957; Bokkenheuser, 1963; Van den Heever, 1970; Tshekiso, 2002). However, the most recent studies that appear in the literature regarding the microbiological safety of traditional South African *biltong* were reported in 1963, 1970 and 1972 (Bokkenheuser, 1963; Van den Heever, 1970; Taylor, 1976). As such, the aim of this study was to update the currently held knowledge on the prevalence of bacterial foodborne pathogens associated with *biltong*.

2. MATERIALS AND METHODS

2.1 Sample collection

One hundred and fifty *biltong* samples were obtained from various geographical locations around the Gauteng province in South Africa. Suppliers included butcheries, *biltong* bars (small outlets usually selling *biltong* as the main source of income, often found in shopping malls), convenience stores (supermarkets), *biltong* shacks (a *biltong* bar that sells both *biltong* and raw meat in the same establishment on a smaller scale), home-made industries and sweet (confectionary) shops during July - October 2008.

2.2 Sample processing

For each *biltong* sample, 20 g of the *biltong* product was transferred into a Whirl-Pak bag (Nasco, USA), combined with 180 ml diluent [0.1 % Bacteriological Peptone

(BioLab, Midrand, South Africa) + 0.85 % sodium chloride (Saarchem-Merck Chemicals, South Africa)] and homogenised for 2 min using a Colworth 400 Stomacher (Georgnaras and von Holy, 2000; Mosupye and von Holy, 2000; Christison *et al*., 2008). The homogenised *biltong* samples were serially diluted in diluent and plated in duplicate using standard plating methods onto several types of growth media (Table 3.1) and incubated aerobically for 48 h at 37 ºC. The pH of homogenised samples was also recorded using a laboratory pH meter (Metrohm 744).

2.3 Enumeration

Duplicate plates containing between 30 to 300 colonies, or the highest number if below 30 were enumerated for aerobic plate (APC), Enterobacteriaceae (EBC) (Fig 3.1), coliform (CC) (Fig 3.1), *E. coli* (EC) (Fig 3.1) and presumptive *Staphylococcus aureus* (SAC) (Fig 3.2) counts (Kunene *et al*., 1999; Christison *et al*., 2007; Christison *et al*., 2008) and expressed as Log colony forming unit (Log CFU) per gram. In addition, presumptive *E. coli* colonies (purple colonies) on REC (Fig 3.1) were isolated, streak plated onto TSA plates and incubated at 37 ºC for 48 h. Selected isolates were further identified using 16S rDNA sequencing.

2.4 Pathogen detection and confirmation

Staphylococcus aureus

In order to confirm the presence of *Staphylococcus aureus*, 4 shiny black colonies with halos (Burnham *et al*., 2007) were selected from duplicate Baird-Parker plates of each *biltong* sample showing growth on the highest dilution (Fig 3.2). Isolates were

re-streaked onto TSA and incubated for 48 h at 37 ºC. From these TSA plates, isolates were re-streaked onto DNAse agar (Scharlau, Spain) as well as Rapid *Staph'* agar (RSA) (Bio-Rad, France) supplemented with egg yolk tellurite (0.5 % w/v) (Scharlau, Spain) and incubated for 24 h at 37 ºC. Isolates that grew as black colonies surrounded by a halo on RSA (Fig 3.3) and showed a zone of clearing on DNase agar after the addition of 1 ml hydrochloric acid (1 M) (Christison *et al*., 2008), were selected for further molecular identification (Christison *et al*., 2008). These same isolates were also tested for enterotoxin production using SET-RPLA TD900 kits (Oxoid, London) (Bergdoll, 1990; Normanno *et al*., 2005; Mhlambi *et al*., In press).

***Salmonella* spp.**

From each *biltong* sample, 25 g was combined with 225 ml of Buffered Peptone Water (Scharlau, Spain) (Hein *et al*., 2006) and placed in a stomacher for 2 min (Colworth 400 Stomacher). Homogenised samples were incubated for 18 h at 37 ºC after which 1 ml was extracted and inoculated into 10 ml Muller-Kauffmann broth (MKB) (Scharlau, Spain) supplemented with Brilliant Green and Novobiocin (Scharlau, Spain) and 200 µl Gram's Iodine and incubated for 24 h at 30 ºC (Chen *et al*., 2008; Christison *et al*., 2008). An additional 0.1 ml of homogenised sample was added to 9.9 ml of Rappaport Vassiliadis broth (RVB) (Scharlau, Spain) and incubated at 41.5 ºC for 24 h (Ray *et al*., 1989; Rijpens *et al*., 1999). A loopful of each broth (MKB and RVB) was streak plated onto both Xylose Lysine Deoxycholate agar (XLD) (Scharlau, Spain) and Brilliant Green Modified agar (BGMA) (Scharlau, Spain). Isolates that grew both as red colonies with black edges on XLD and red to pink on BGMA (Fig 3.4) (South African Bureau of Standards 6579:1993, 1993) after

48 h were Gram-stained and further selected for molecular identification (Christison *et al*., 2008).

Listeria monocytogenes

Each *biltong* sample was pre-enriched by combining 25 g with 225 ml Fraser ½ broth (Bio-Rad, France) and homogenised in a Colworth 400 Stomacher for 2 min. Pre-enriched samples were incubated for 24 h at 30 ºC after which 0.1 ml was extracted and inoculated into 10 ml Fraser 1 broth (Bio-Rad) and incubated at 37 ºC for 48 h serving as a secondary enrichment step. Samples which resulted in the blackening of Fraser 1 broth (Fig 3.5) were streaked onto Rapid '*L. Mono* agar (Bio-Rad, France), incubated at 37 ºC for 48 h and blue colonies without a yellow halo (Fig 3.6) were Gram-stained and selected for further molecular analysis (Christison *et al*., 2008).

2.5 Molecular Identification of presumptive bacterial pathogens

Presumptive pathogens were further identified using 16S rDNA sequencing. Polymerase chain reaction (PCR) amplification was carried out as previously described using the primer sets U1392R (5'- ACG GGCGGT GTG TRC-3') and Bac27F (5'-AGA GTT TGA TCM TGG CTC AG-3') in combination with Fermentas 2x PCR Master Mix (Fermentas Life Science). The purified PCR product was sequenced and analysed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against the 16S rDNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) (Christison *et al*., 2007). Phylogenetic trees highlighting the clustering of the presumptive bacterial pathogens were then

constructed using the Observed Divergency Method in DNAMAN version 6, Lynnon Biosoft (Iniguez-Palomares *et al*., 2007).

3. RESULTS AND DISCUSSION

3.1 Overall observations of counts from *biltong* samples linked to points-of-sale

Overall, *biltong* samples obtained from *biltong* bars in this study had the highest associated APC counts (*ca.* 7.01 Log CFU/ g) (Fig 3.7), which may be attributed to the constant and excessive handling of the product at point-of-sale (Mothershaw *et al*., 2003). In contrast pre-packaged samples had the lowest associated APC counts (*ca.* 6.14 Log CFU/ g) (Fig 3.7), this may be attributed to the lack of direct human contact with the actual product after production as a result of the barrier provided by packaging (Huong *et al*., 2010). *Biltong*, an RTE dried meat product is produced under several microbial growth-limiting intrinsic factors such as curing (salts, spices and vinegars) (Mothershaw *et al*., 2003), refrigeration and drying (Notermans *et al*., 1995; Wolter *et al*., 2000). Traditional *biltong* reportedly has a water activity (a_w) of 0.74 - 0.77 and pH of 5.5 - 5.8 associated with the final product (Van den Heever, 1970; Dzimba *et al*., 2007). Although these factors reduce the presence of several microbial populations, *biltong* favours the prevalence of heat and salt tolerant microorganisms (Wolter *et al*., 2000).

There are currently no standards for this RTE dried meat in South African legislation (Van den Heever, 1970). Historically it was suggested that the survival and composition of microbial populations varies too greatly between different *biltong*

pieces and their places of origin (Van den Heever, 1965). Previous studies have indicated that *biltong* carries APC loads of between 5 - 7.5 Log CFU/ g (Taylor, 1976; Prior, 1984) and has a pH of *ca.* 5.5 - 5.8 (Van den Heever, 1970; Dzimba *et al*., 2007). When comparing the APC counts observed in this study, it was evident that irrespective of the location the counts associated with *biltong* at point-of-sale (Fig 3.7) were within the previously published ranges. When comparing the pH values observed in this study (Table 3.2) it was evident that all *biltong* samples were often slightly below the previously published pH ranges by 0.12 - 0.45 (Table 3.2).

EBC, CC and EC were used in this study to assess the overall product hygiene as high EBC and ECs are indicative of enteric pathogens (Barros *et al*., 2007). From results obtained in this study, it was evident that *biltong* samples obtained from convenience stores showed the highest associated EBC, CC and EC counts (3.94, 3.03 and 1.57 Log CFU/ g) (Fig 3.7) while, in contrast, pre-packed samples had the lowest associated EBC, CC (2.21 and 1.73 CFU/ g) (Fig 3.7) and were the only samples with EC counts below the lower detection limit (Fig 3.7). The presence of *E. coli*, an index organism, further highlighted the suspected presence of pathogens such as *Salmonella*, *Shigella* and *E. coli* 0157:H7 in these *biltong* samples, as they are all capable of contaminating and surviving in the same environmental niches (Moore and Griffith, 2002; Li *et al*., 2005; Lues and Van Tonder, 2007).

From the SAC counts obtained in this study, it was evident that samples obtained from convenience stores followed closely by butcheries and *biltong* bars were associated with comparatively higher SAC counts than the other point-of-sale origins

(Fig 3.7). Indeed, all SAC counts observed were below 3 Log CFU/ g (Fig 3.7) and although conformed to Japanese acceptance levels for unheated meat products that are either smoked or salted and dried (Todd, 2004), samples from *biltong* bars, butcheries and convenience stores were deemed unsatisfactory by standards proposed by Gilbert and associates (2000). Since these populations are native to the human nose, throat and skin, high levels of *Staphylococcus* counts are often indicative of poor human handling practices (Gillespie *et al*., 2000; Mothershaw *et al*., 2003; Christison *et al*., 2008; Todd *et al*., 2008; Valero *et al*., 2009).

3.2 Prevalence of bacterial pathogens in *biltong.*

Staphylococcus aureus

After screening 159 presumptive *Staphylococcus* isolates obtained from *biltong* samples, 15 isolates were singled out as presumptive *S. aureus* strains and further identified using 16S rDNA sequencing. Results from molecular analysis showed that of the 15 isolates, only 3 strains were confirmed as *S. aureus* (Shia-St64.1, Shia-St122.1 and Shia-St122.4) (Fig 3.8). All 15 isolates were also tested for enterotoxin production. Results showed that 3 isolates produced enterotoxin B including 2 strains identified as *S. aureus* (Shia-St64.1 and Shia-St122.1) (Fig 3.8). Enterotoxin B-producing strains are reportedly the serotypes that are 3rd most commonly associated with food poisoning events, after enterotoxins A and D (Balaban and Rasooly, 2000). Interestingly, a further strain (Shia-St108.3), which also produced a positive reaction for the production of enterotoxin B, was identified as a strain of *S. pasteuri* (Fig 3.8). Although enterotoxin production is often characteristic of coagulase positive

Staphylococcus strains such as *S. aureus*, it is not limited to this species (Portocarrero *et al*., 2002b; Mhlambi *et al*., In press). Although *S. pasteuri* strains are often associated with food commodities (Morita *et al*., 2007), there is no record of this species previously implicated in foodborne illness outbreaks.

The presence of enterotoxin-producing *S. aureus* in *biltong* could potentially be attributed to the high concentration of salts, acidic pH (Portocarrero *et al*., 2002b) and increased a_w of a moister *biltong* product, especially since strains of *S. aureus* are capable of growth and enterotoxin production at a_ws of 0.85 (Portocarrero *et al*., 2002b; Nortje *et al*., 2006). It is reported that the modern consumer markets favour more moist *biltong*, which is considered more appealing to the palate (Dzimba *et al*., 2007). Traditionally, dried *biltong* has a water activity (a_w) of 0.77 (Dzimba *et al*., 2007), however, the favoured *biltong* has 40 % more moisture than traditional *biltong* and has an a_w of between 0.85 and 0.93 (Nortje *et al*., 2006; Dzimba *et al*., 2007) which supports the growth of several pathogens. Enterotoxin production by *S. aureus* reportedly occurs optimally during the middle and end of the exponential growth phase as a result of high cell numbers (10^5 CFU) (Balaban and Rasooly, 2000; Portocarrero *et al*., 2002b; Soriano *et al*., 2002). Further to this, literature suggests that a minimum toxin dose of as little as 1 ng/ g is capable of causing foodborne illness (Bergdoll, 1990). As *biltong* samples were not directly tested for the presence of enterotoxin, it is possible that several samples could potentially have harboured several enterotoxins, even though viable organisms were not isolated (Hudson, 2004). As *S. aureus* has previously been implicated as the causal agent of 2 separate outbreaks associated with jerky (Eidson *et al*., 2000; Nummer *et al*., 2004), and

kaddid (Mothershaw *et al*., 2003), which are similar products to *biltong*, the presence of enterotoxin-producing strains of *Staphylococcus* in *biltong* may potentially hold foodborne illness implications.

Listeria monocytogenes

The findings of this study showed that 2 of the 150 (1.33 %) *biltong* samples tested were positive for *L. monocytogenes* (Shia-L1.1 accession number FJ160766 and Shia-L2.1 accession number FJ160767) (Fig 3.9). In comparison, the prevalence of *L. monocytogenes* observed in this study was 0.81 % higher than the cumulative prevalence that had been observed in a study associated with jerky (Allen *et al*., 2007).

L. monocytogenes is a Gram-positive rod shaped bacterium ubiquitously found in the food industry and is the causal agent of listeriosis in humans and more than 40 animals (Buncic and Avery, 2004; Nelson *et al*., 2004; Pal *et al*., 2008). Outbreaks of this disease have often been associated with the consumption of contaminated RTE foods, whereby symptoms observed included abortion in pregnant women, stillbirths, septicaemia, meningitis, encephalitis and several fatalities in some instances (Buncic and Avery, 2004; Nelson *et al*., 2004; Pal *et al*., 2008). Although the minimum dose of *L. monocytogenes* cells required to cause foodborne illness is variable, foodborne illness has often been coupled with elevated levels of this pathogen (Gilbert *et al*., 2000; Norrung, 2000).

Biltong would generally be considered as potentially unfavourable environment for this pathogen due to its low a_w and high salt concentrations (Buncic and Avery, 2004), and would therefore be less likely to harbour and support its growth (Lake *et al*., 2002). The Codex Alimentarius Commission and the European Commission (EU Regulation 2073/2005) have recently concluded that RTE foods, which do not support the growth of this pathogen, may contain no more than 100 CFU (2 Log CFU/ g) (Gerner-Smidt and Whichard, 2008). As the CFU/ g of *L. monocytogenes* was not evaluated in this study, it cannot be deduced if the *biltong* samples were in accordance with this accepted tolerance level (Vitas and Garcia-Jalon, 2004) at point-of-sale. In addition, chicken meats are known to generally promote better growth of *L. monocytogenes* than other meats (Buncic and Avery, 2004). In this study, this pathogen was isolated from chicken *biltong* and included strains Shia-L1.1 (FJ160766) and Shia-L2.1 (FJ160767) (Fig 3.9). The presence of these strains could be attributed to the contamination of *biltong* prior to and at production, distribution and within the retail environments of this commodity due to the ubiquitous prevalence of this foodborne pathogen (Pal *et al*., 2008).

***Salmonella* spp.**

Although results from this study showed high incidences of indicator organisms, only 2 samples had tested positive for presumptive *Salmonella* spp. However, subsequent 16S rDNA molecular analysis indicated that these isolates were 99 % genetically similar to *Proteus mirabilis* [(Shia-Sal3 accession number FJ178299 and Shia-Sal5 accession number FJ178300)], thus once more highlighting the need for confirmation of presumptive positive isolates from selective growth media. The prevalence of

Proteus mirabilis in *biltong* at point-of-sale is a cause for concern, as several strains of this species have recently been implicated as the causal agents of several outbreaks of foodborne illness (Cooper *et al*., 2005; Wang *et al*., 2010).

Escherichia coli

Molecular analysis of presumptive *E. coli* isolates (purple colonies on REC) indicated that 1 strain, Shia-ES114.3 (accession number FJ392807), was 99 % genetic similar to *Shigella* (*Sh.*) *dysenteriae* and further highlighted the potential prevalence of *E. coli* 0157:H7 in this sample. It has recently been suggested that *E. coli* 0157:H7 strains are merely *Shigella* in a cloak of *E. coli* antigens (Johnson, 2000). The occurrence of *E. coli* 0157:H7 in dried RTE meat products was first documented in the 1995 outbreak whereby contaminated deer jerky had sickened several people (Keene *et al*., 1997), as a result of this pathogen's tolerance to desiccation (Shaw *et al*., 2003). *Biltong* produced in another province of South Africa (the Eastern Cape) has also previously been shown to harbour strains of *E. coli* 0157:H7 (Abong'o and Momba, 2008; Abong'o and Momba, 2009). It is suggested that for future evaluations of *biltong* product, detection for specifically *E. coli* 0157:H7 should include pre-enrichment procedures (Keene *et al*., 1997; Abong'o and Momba, 2008; Abong'o and Momba, 2009).

Even though this study showed the presence of foodborne pathogens, including enterotoxin-producing *S. aureus*, *L. monocytogenes* and *Sh. dysenteriae*, associated with *biltong* sold in the Gauteng province of South Africa, the overall prevalence of these pathogens in product at point-of-sale was low, ranging from 0.5 - 2 %. The

presence of these pathogens potentially suggested that contamination of *biltong* samples occurred either during the production of *biltong* or as a result of post-production cross-contamination at point-of-sale.

4. CONCLUSION

This study highlighted the potential of *biltong* at point-of-sale as a potential vehicle for foodborne pathogens. The survival of foodborne pathogens throughout the *biltong* manufacturing process was questioned and forms the basis of Chapter 4.

Table 3.1: Culture methods, media and the aerobic incubation time and temperatures utilised in the analysis of the 150 *biltong* samples surveyed in this study.

Population Tested For	Time (h)	Temp (°C)	Plating Method	Bacterial Growth Media
Aerobic Plate Counts (APC)	48	37	Pour (10^1), Spread	Tryptone Soy agar (TSA) (BioLab, Midrand South Africa).
Enterobacteriaceae Counts (EBC) (purple, green and white colonies)	48	37	Spread	Rapid *E. coli* 2 agar™ (REC) (Bio-Rad, France) (Fig 3.1)
Coliform Count (CC) (blue, green colonies)	48	37	Spread	Rapid *E. coli* 2 agar™ (REC) (Bio-Rad, France) (Fig 3.1)
***E. coli* Counts (EC) (purple, indigo colonies)**	48	37	Spread	Rapid *E. coli* 2 agar™ (REC) (Bio-Rad, France) (Fig 3.1)
Presumptive *Staphylococcus aureus* Counts (SAC) (shiny black colonies surrounded by a halo in the media)	48	37	Spread	Baird Parker agar supplemented with egg's yolk tellurite sterile emulsion (0.5 % w/v) (Scharlau, Spain) (Fig 3.2)

Table 3.2: The observed pH ranges associated with *biltong* samples obtained from various origins of point-of-sale.

Source of *biltong* samples	**Associated pH ranges**
Butcheries	5.05 - 5.66
Pre-Packaged Samples	5.25 - 5.45
Biltong Bars	5.15 - 5.68
Convenience Stores and Supermarkets	5.11 - 5.56
Biltong Shacks	5.10 - 5.69
Home-made Industries	5.18 - 5.66
Sweet Shops	5.11 - 5.40

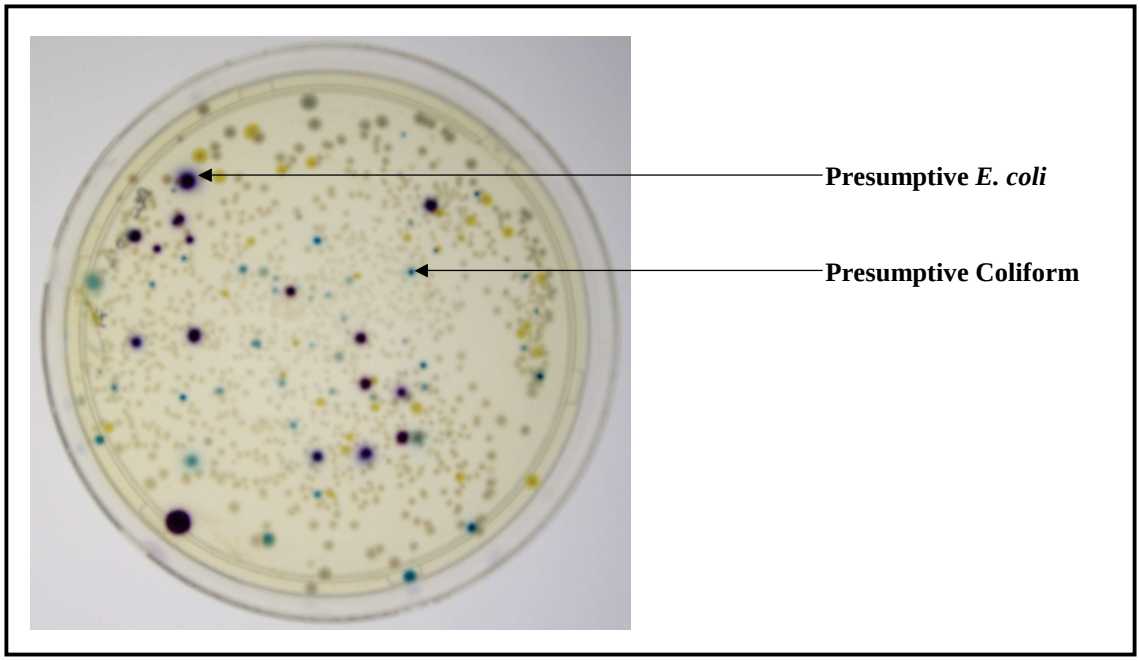


Figure 3.1: A microbial analysis of *biltong* indicating the presence of presumptive Enterobacteriaceae (all colonies), coliforms (green, blue and teal colonies) and *E. coli* (purple, violet and indigo colonies) isolates observed on Rapid *E. coli* 2 agar™.

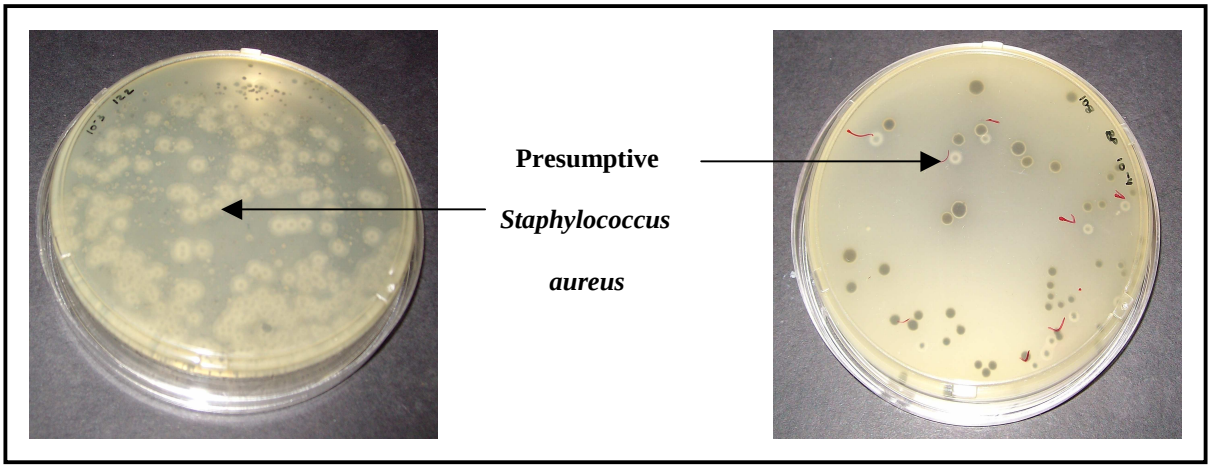


Figure 3.2: Microbial analysis of *biltong* highlighting the presence of presumptive *Staphylococcus aureus* (black colonies surrounded by a halo in the media) isolates on Baird-Parker agar supplemented with egg's yolk tellurite sterile emulsion.

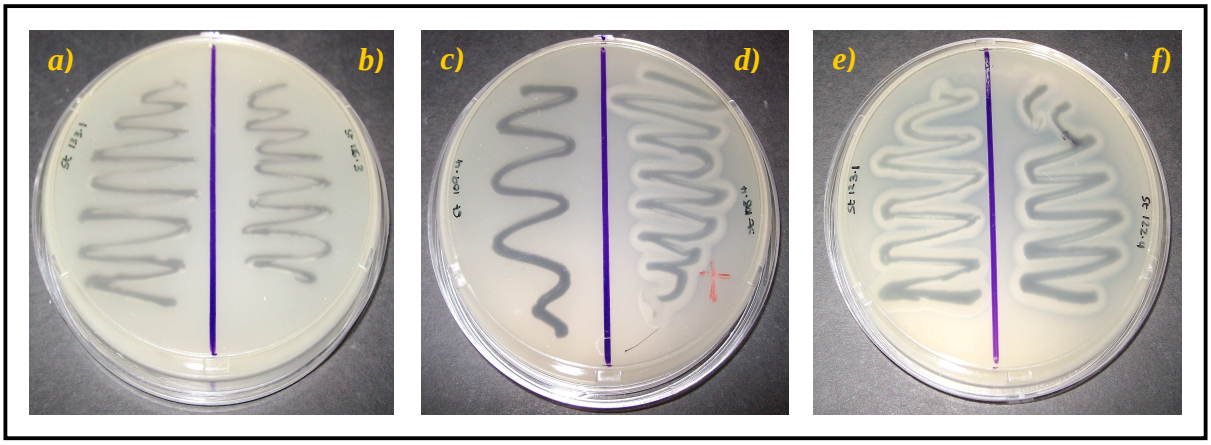


Figure 3.3: Presumptive *Staphylococcus aureus* isolates streaked onto Rapid *Staph'* agar supplemented with egg's yolk tellurite sterile emulsion. Isolates lacking a black colour and halo in the media (*a, b, c*) were dismissed as *S. aureus*, while isolates showing a black colouring in conjunction to producing a halo in the media (*d, e, f*) were regarded as presumptive *S. aureus* and selected for further molecular identification.

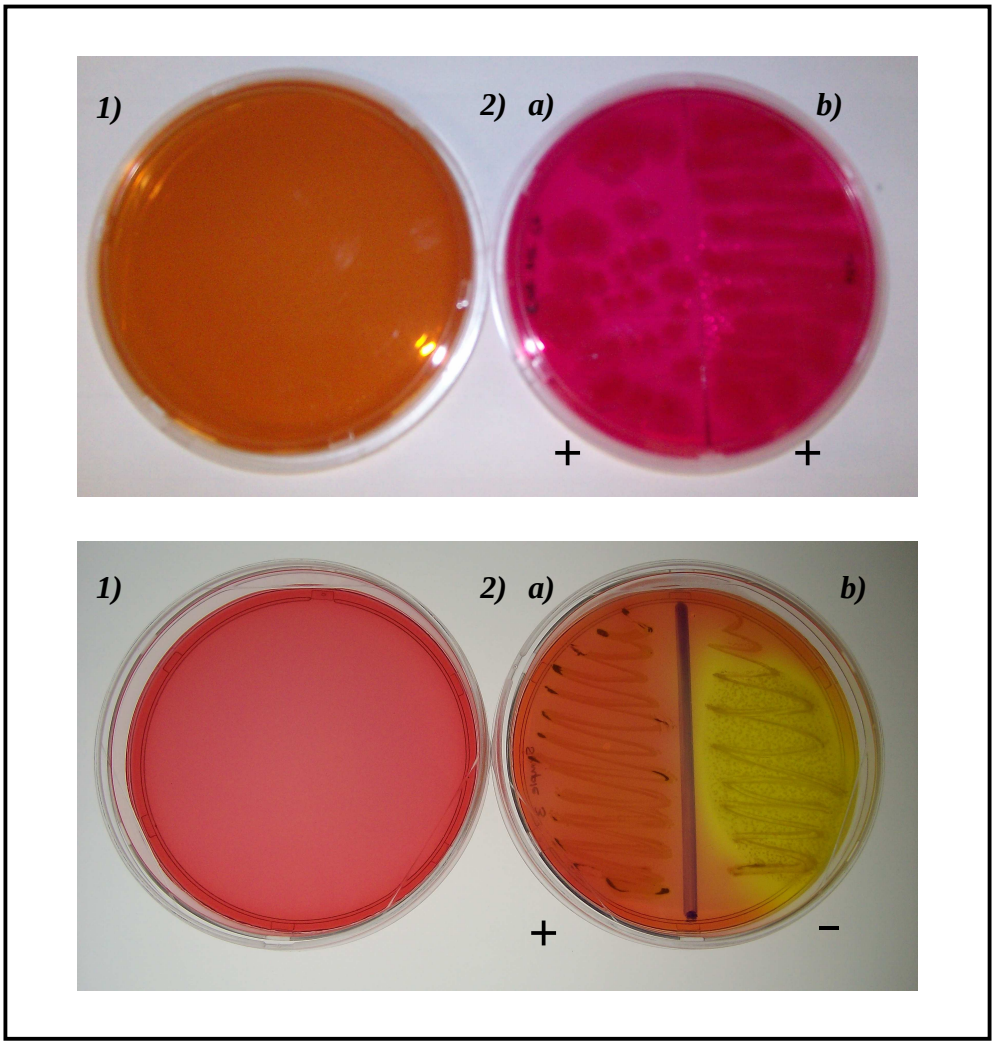


Figure 3.4: Microbial analysis highlighting the presence of presumptive *Salmonella* spp. associated with *biltong* samples. 1) Un-inoculated control plates of Brilliant Green Modified agar (BGMA- brown media plate) and Xylose Lysine Deoxycholate agar (XLD- red media plate). *2a*) Isolates regarded as presumptive *Salmonella* spp. on both types of media, *2b*) Isolates dismissed as presumptive *Salmonella* spp. (+) expected growth and (-) non-expected growth for *Salmonella* on particular selective medium.

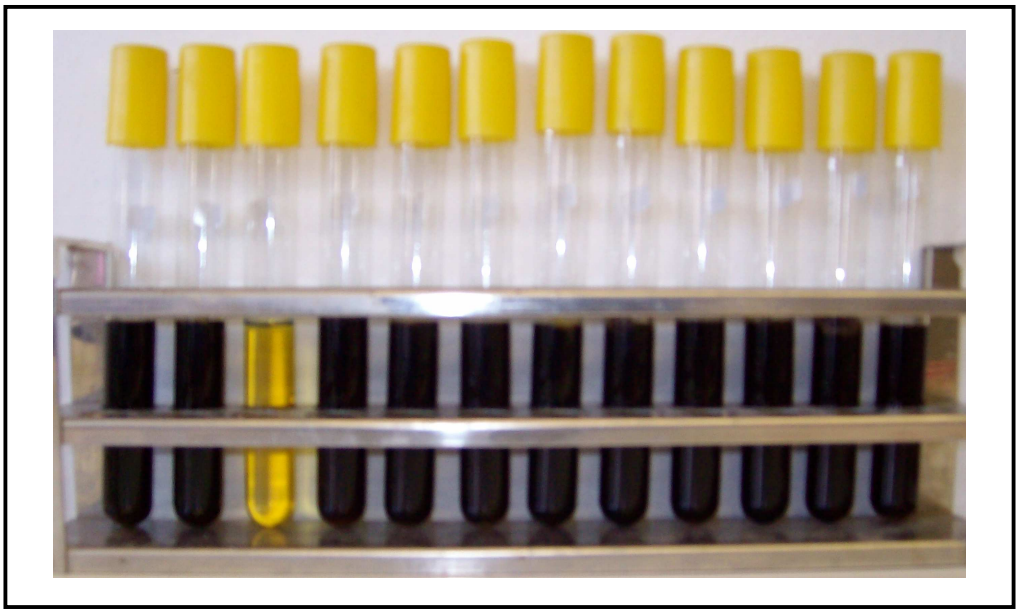


Figure 3.5: The presence of presumptive *L. monocytogenes* in *biltong* samples highlighted in the secondary enrichment step (indicated by the blackening of Fraser 1 broth).

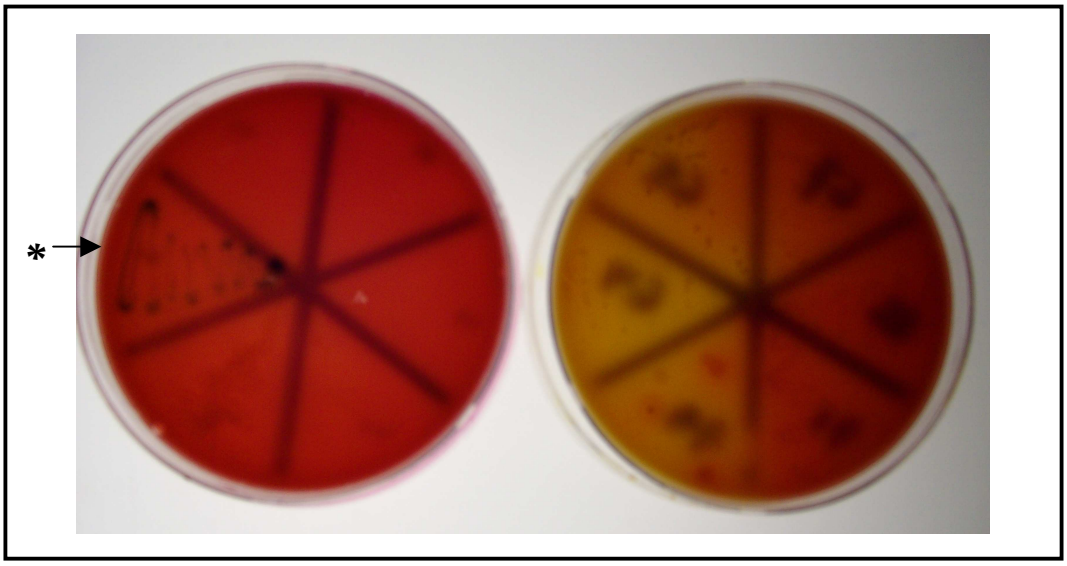


Figure 3.6: Isolation of presumptive *L. monocytogenes* isolates observed from Rapid '*L. Mono* agar. Isolates that grew as blue colonies lacking a halo and changing of media colour (*) were selected and identity confirmed using molecular methods.

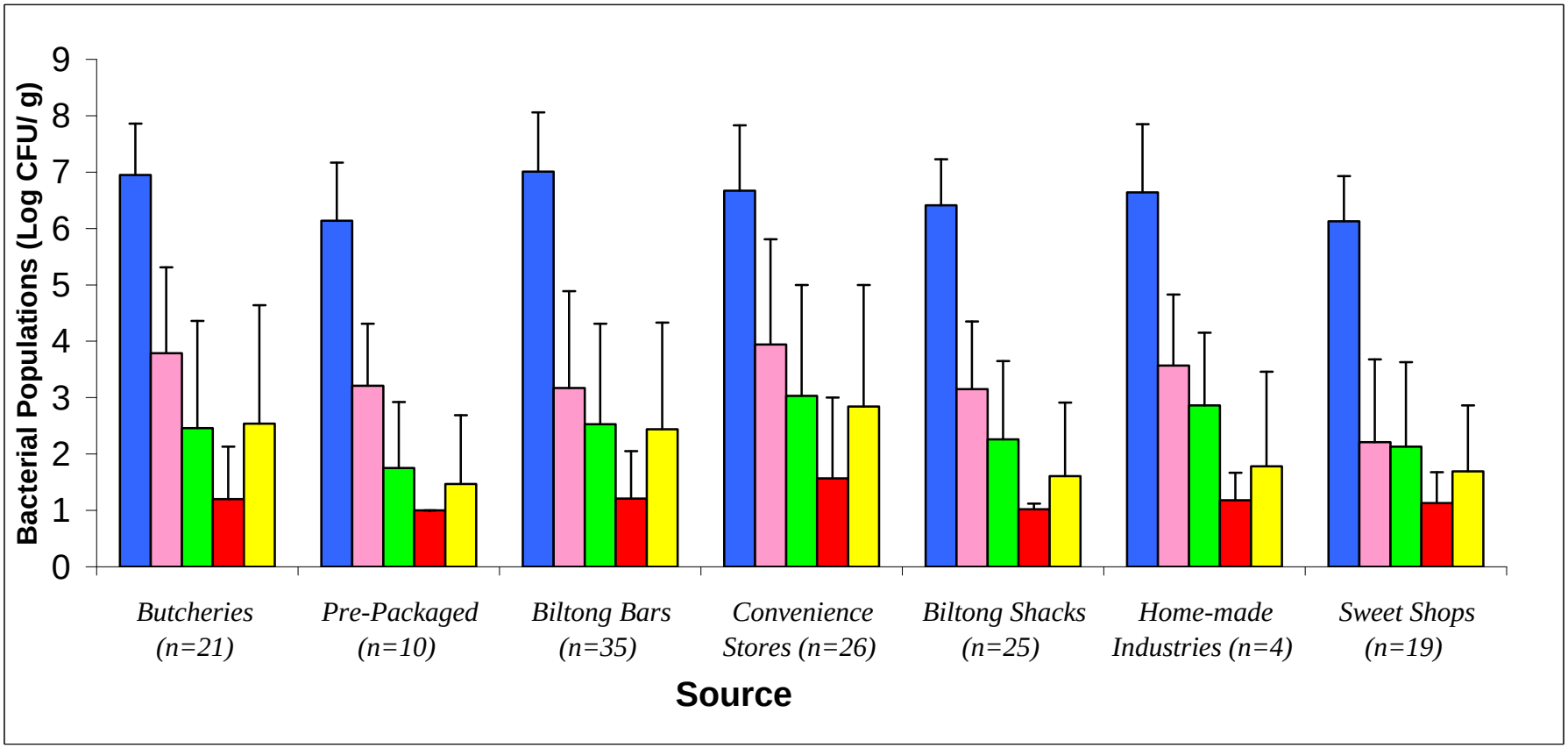


Figure 3.7: Bacterial populations including aerobic plate (blue bar), total Enterobacteriaceae (pale pink bar), Coliform (green bar), *E. coli* (red bar) and presumptive *Staphylococcus aureus* (yellow bar) counts obtained from 150 *biltong* samples. (Lower detection limit = 1 Log CFU/ g). Standard deviation (τ).

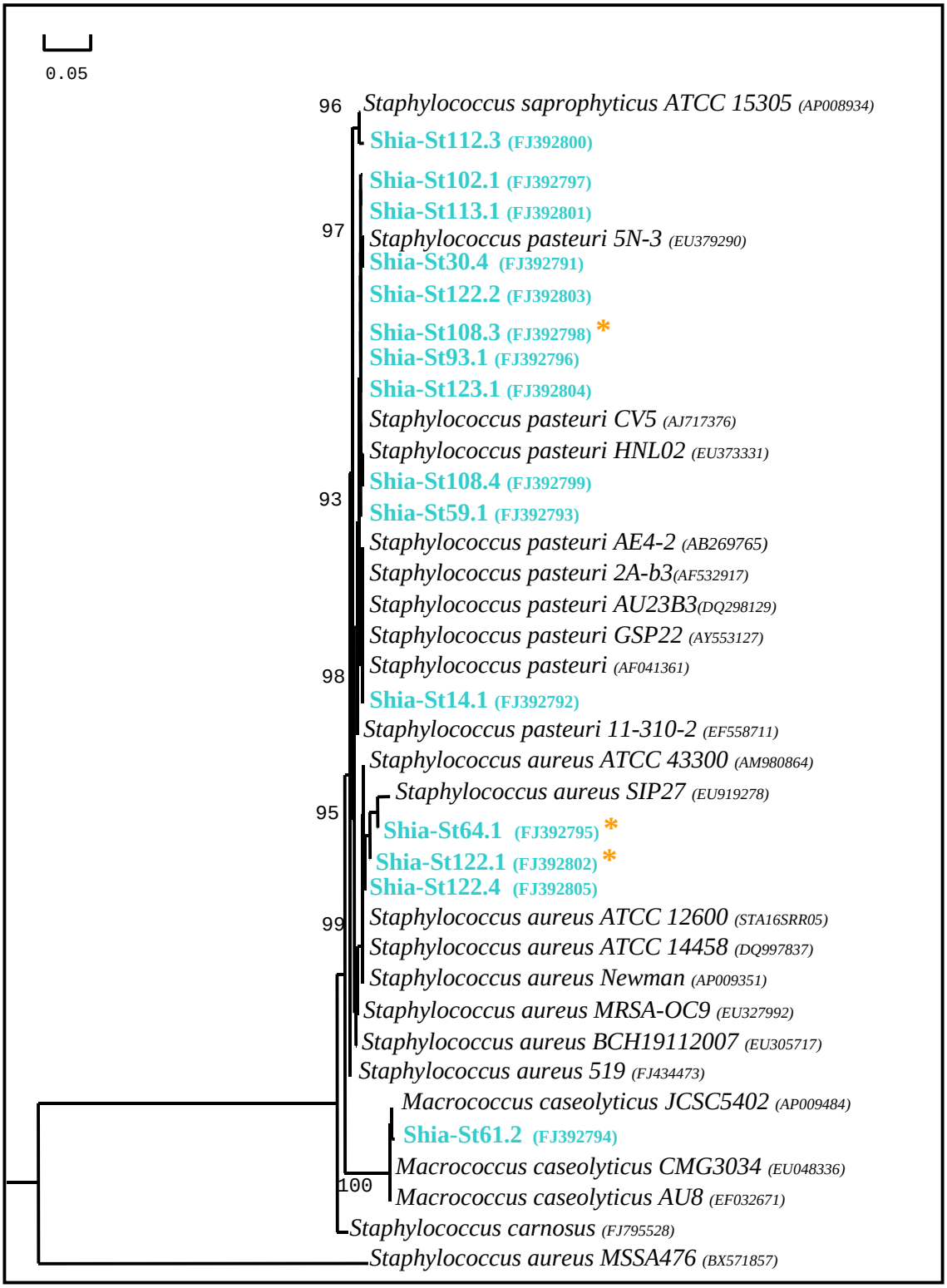


Figure 3.8: A rooted observed divergency phylogenetic tree highlighting the clustering of presumptive *Staphylococcus aureus* strains (blue bolded text) had been created using the DNAMAN version 6, Lynnon Biosoft. Strains that produced enterotoxin B indicated by an *.

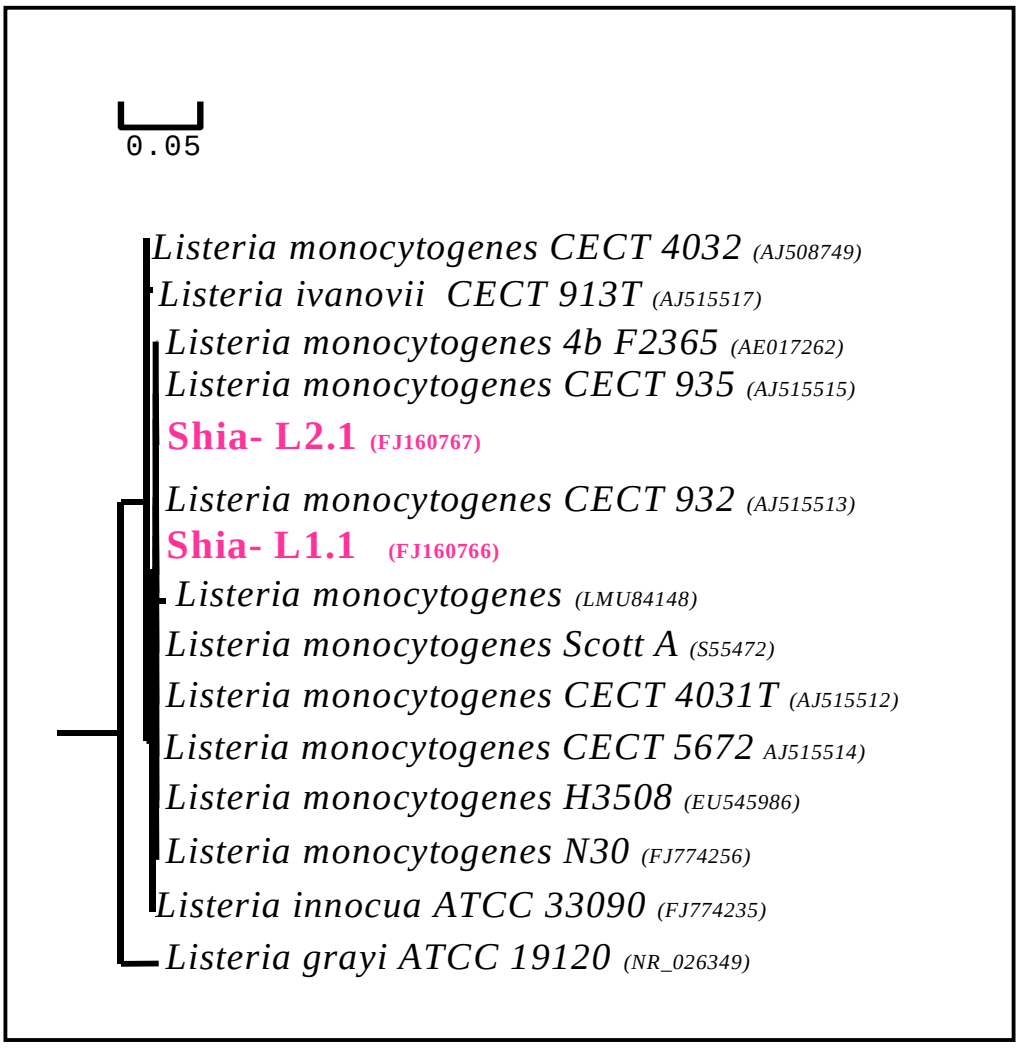


Figure 3.9: A rooted observed divergency phylogenetic tree highlighting the clustering of 2 presumptive *Listeria monocytogenes* strains (Pink bolded text) had been created using the DNAMAN version 6, Lynnon Biosoft.

CHAPTER 4:

SURVIVAL OF POTENTIAL FOODBORNE PATHOGENS DURING THE BILTONG *MANUFACTURING PROCESS.*

CHAPTER 4.1:

IN VITRO *RESPONSE OF POTENTIAL FOODBORNE PATHOGENS TO THE CONDIMENTS AND CONDITIONS USED DURING THE* BILTONG *MANUFACTURING PROCESS.*

This Chapter has been accepted for publication to the *Journal of Food Protection Trends*.

ABSTRACT

Biltong, a ready-to-eat (RTE) dried meat product, is produced under several microbial growth-limiting intrinsic factors, such as curing (salts, spices and vinegars), refrigeration and drying. This study aimed to evaluate the *in vitro* antimicrobial properties of each component in this process against several bacterial isolates previously isolated from *biltong* product at point-of-sale. Bacterial isolates were tested for growth at various temperatures and in the presence of varying salt concentrations, vinegar and spice. From the results observed in this study, 90 % of the isolates showed un-altered growth patterns in the presence of 10 % NaCl concentrations while 70 % grew in the presence of 15 % NaCl. Apple cider and brown spirit vinegars inhibited the growth of 60 and 40 % of the isolates, respectively, when compared to complete inhibition by glacial acetic acid (control). Furthermore, all 10 isolates showed the same growth patterns in the presence and absence of spices traditionally used to manufacture *biltong*. Optimal growth temperature ranges of all isolates were between 25 - 37 ºC. Overall, *Listeria monocytogenes* Shia-L1.1, *Staphylococcus aureus* Shia-St64.1 and *Staphylococcus pasteuri* Shia-St108.3 showed the most growth in all assays conducted. The findings of this study highlighted that although the *biltong* manufacturing process is associated with several hurdles, each hurdle, individually, was inadequate at completely eradicating potential bacterial pathogens. The latter questioned if there was a synergistic effect of the hurdles during the *biltong* manufacturing process on bacterial pathogens.

1. INTRODUCTION

The presence of Staphylococcal enterotoxins and foodborne pathogens, such as *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* 0157:H7, in food commodities has become a major concern of the Food Safety and Inspection Services (FSIS) especially in ready-to-eat (RTE) foods which require no further processing prior to consumption (Levine *et al*., 2001). The elevated demand for enhanced food safety has initiated modifications in the processing and production of meat products (Jensen *et al*., 1998). As such the steps in production and processing of food commodities (critical control points) should ideally eradicate the presence of pathogenic and spoilage microorganisms (Jay *et al*., 2005; Tiganitas *et al*., 2009). These antimicrobial-processing steps may include salting, marinating, drying, cooking, smoking and refrigeration (Collignan *et al*., 2001).

Biltong, an RTE dried meat product is produced under several microbial growth-limiting intrinsic factors such as curing (marinating), refrigeration and drying (Notermans *et al*., 1995; Wolter *et al*., 2000; Collignan *et al*., 2001; Mothershaw *et al*., 2003). There are 4 steps associated with the preparation of *biltong*, the first being the selection of the meat to be processed into *biltong*, such as beef, game (Kudu, Springbok, Impala), chicken or ostrich meat. Meat portions (Fig 4.1.1) are marinated in spice mixtures (consisting of brown sugar, salt, black pepper and dried roasted coriander) (Dzimba *et al*., 2007) and vinegar (apple cider, brown spirit or wines) at refrigeration temperatures for 18 - 24 h and thereafter dried at ambient temperatures for several days (Dzimba *et al*., 2007). Traditional *biltong* reportedly has a water

activity (a_w) of 0.74 - 0.77 and pH of 5.5 - 5.8 associated with the final product (Van den Heever, 1970; Dzimba *et al*., 2007). Although these factors reduce the presence of several microbial populations, *biltong* (Fig 4.1.2) favours the prevalence of heat and salt tolerant microorganisms (Wolter *et al*., 2000). This study aimed to evaluate the *in vitro* antimicrobial properties of each component of this process against several bacterial isolates previously isolated from *biltong* product at point-of-sale.

2. MATERIALS AND METHODS

As previously mentioned several potential foodborne pathogenic strains were isolated from *biltong* (Chapter 3). In order to establish tolerance of selected strains to the hurdles introduced by the *biltong* manufacturing process, such as curing (spices and vinegar), refrigeration and drying, several growth assays were conducted.

2.1 Isolates selected for growth/ tolerance assays

Several potential foodborne pathogens previously isolated from *biltong* (Chapter 3) were selected including: *Listeria* (*L.*) *monocytogenes* strains Shia-L1.1 (accession number FJ160766) and Shia-L2.1 (accession number FJ160767), *Staphylococcus* (*S.*) *aureus* strains Shia-St64.1 (accession number FJ392795), Shia-St122.1 (accession number FJ3922802) and Shia-St122.4 (accession number FJ392805), and *Staphylococcus* (*S.*) *pasteuri* strains Shia-St 108.3 (accession number FJ392798), Shia-St123.1 (accession number FJ392804) and Shia-St112.3 (FJ392800). Control strains of *Salmonella* spp. and *S. aureus* (non-food derived isolates) were included (Table 4.1.1).

In order to generate working inocula, each isolate was successively cultured twice (from previously frozen stock cultures) for 24 h at 37 °C in Tryptone Soy Broth (TSB) (BioLab, Midrand South Africa), streaked onto Tryptone Soy agar plates (TSA) (BioLab, Midrand South Africa) and incubated for 48 h at 37 °C. Colony morphology as well as Gram-stain reactions were examined to ensure pure cultures of each isolate and plates were stored at 4 °C (Buege *et al*., 2006). Frozen stock cultures of each isolate were also prepared in sterile 2 ml Ependorf centrifuge tubes by aseptically combining 850 µl of liquid cultures with 150 µl of sterile glycerol and stored at –20 ºC (Burnham *et al*., 2008).

2.2 Growth and tolerance assays

Prior to tolerance assays (*ca.* 48 h), a single colony of each bacterial isolate, from stored working inocula plates, was successively streaked twice onto TSA plates and incubated at 37 ºC for 24 h. Isolates were Gram-stained and used as cultures for subsequent inoculation for all assays described below (Buege *et al*., 2006).

Growth in high salt concentrations

In order to evaluate the salt tolerance (Table 4.1.1), each isolate was plated by the streak plate technique, in triplicate and on four separate occasions, onto TSA plates supplemented with varying concentrations (5, 10, 15, 20, 25 %) of sodium chloride (NaCl) (Saarchem, Merck Chemicals- South Africa) (Chesneau *et al*., 1993). Inoculated plates were incubated at 37 ºC and qualitatively viewed every 24 h for 7 days for signs of bacterial growth.

Growth at various temperatures

A loopful of each isolate (Table 4.1.1) was inoculated into 20 ml of TSB, as well as plated by the streak plate technique onto TSA plates, in triplicate and on four separate occasions, and incubated at 4, 25, 30, 37 and 45 ºC for 7 days. At 24 h intervals, TSA plates were observed for bacterial growth. In addition, a loopful from each inoculated TSB broth was streak plated onto TSA plates and incubated for 24 h at the appropriate temperature. For example, TSB-grown cultures incubated at 4 °C were plated and incubated again at 4 °C. These plates were also observed to confirm any bacterial growth.

Growth in the presence of organic acids

In order to determine if bacterial strains (Table 4.1.1) were tolerant to the organic acids used in the *biltong* manufacturing process, spot-on-lawn assays (Bae *et al*., 2004) were conducted in triplicate and on four separate occasions. A colony for each isolate was selected and inoculated into 50 ml of TSB, followed by incubation at 37 ºC for 18 - 20 h. Bacterial lawns and indicator plates were prepared by pour and spread plating 1 ml of this overnight bacterial culture together with TSA to a final colony count of *ca*. 10^5 - 10^6 CFU/ ml (Sagdic and Ozcan, 2003; Bae *et al*., 2004; Hechard *et al*., 2005). Plates were allowed to stand for 5 h at ambient temperature to allow drying of the surface. Indicator plates were divided into sections and 50 µl each of sterile distilled water (negative control) or undiluted apple cider vinegar (ACV) (commercial product, South Africa) or brown spirit vinegar (BSV) (commercial product, South Africa), or 99.7 % glacial acetic acid (GAC) (Associated Chemical Enterprises, South Africa) (positive control), were spotted into each section. Plates

were incubated at 4, 25 and 37 ºC and observed every 24 h for 7 days for zones of clearing indicating inhibition of bacterial growth (Bae *et al*., 2004).

Growth in the presence of* biltong *spice

In order to determine the growth of bacterial isolates in the presence of traditional *biltong* spice (commercially available product, www.*biltong*makers.com), 8 different agar combinations were prepared as follows:

- TSA was supplemented (prior to autoclaving) with traditional *biltong* spice (40 g/ L) and was referred to as TSAB
- Bacteriological agar (13 g/ L) (Merck, South Africa) was supplemented with a combination of beef extract (10 g/ L) (BioLab, South Africa), brown sugar (3 g/ L) (Selati, South Africa), sodium chloride (5 g/ L) and traditional *biltong* spice (40 g/ L) and was referred to as mock *biltong* agar (MBA 1)
- Six variations of MBA were also created to highlight growth or inhibition of the various components, as shown in Table 4.1.2.

Bacterial isolates were streak plated, in triplicate and on four separate occasions, onto TSAB and all variations of MBA (Table 4.1.2), incubated at 25 ºC and observed every 24 h for 7 days for bacterial growth.

3. RESULTS AND DISCUSSION

3.1 Overall growth/ tolerance of bacterial isolates in various *in vitro* assays

Overall, *L. monocytogenes* Shia-L1.1, *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 (Table 4.1.3) showed growth in most of the assays conducted.

3.2 Growth in the presence of varying salt concentrations

From the results observed in this study, 90 % of the isolates (Table 4.1.3) showed un-altered growth patterns in the presence of 10 % NaCl concentration while 70 % (Table 4.1.3) grew in the presence of 15 % NaCl concentrations. In addition it was evident that isolates belonging to the *Staphylococcus* genus (Table 4.1.3), in particular isolates *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 (Table 4.1.3), were the only isolates that showed growth at ≥ 15 % NaCl concentrations. This was not unexpected, as several strains of *Staphylococcus* have previously been shown to survive in environments containing high salt concentrations (Chesneau *et al*., 1993; Ingham *et al*., 2005). Previous studies have suggested that although several enterotoxin-producing strains of *Staphylococcus* may grow in the presence of elevated NaCl concentrations (> 10 %), enterotoxin production is drastically reduced in the presence of ≥ 3 % NaCl (McLean *et al*., 1968). Thus, although high salt concentrations might support the growth of enterotoxin-producing strains, it may not favour the production of enterotoxins, in particular enterotoxin B (McLean *et al*., 1968). As *biltong* is a commodity associated with elevated salt concentrations, the latter is of particular importance with regard to ensuring commodity safety and preventing potential foodborne illness.

3.3 Growth at various temperatures

Both the plate and broth methods that were used in this study qualitatively depicted the same growth patterns. Results (Table 4.1.3) showed that only *L. monocytogenes* Shia-L1.1 and Shia-L2.1 had shown slight growth at 4 ºC, while all isolates grew optimally between the temperature ranges of 25 - 37 ºC (Table 4.1.3). In addition, none of the 10 isolates tested showed growth at 45 ºC (Table 4.1.3).

It is important to note that during the *biltong* manufacturing process, meat slices are often marinated at refrigeration temperatures of *ca.* 4 ºC. While this temperature does not favour the growth of 80 % of the isolates evaluated in this study or pathogens in other studies (Kinsella *et al*., 2007; Valero *et al*., 2009), it did however, support the growth of strains of *L. monocytogenes*. These findings are not uncommon, as strains of *L. monocytogenes* are known to proliferate at refrigeration temperatures (Mellefont *et al*., 2008; Cunningham *et al*., 2009; Zhang *et al*., 2009). The latter findings thus highlighted that marination at refrigeration temperatures may potentially favour the proliferation of *L. monocytogenes*. In addition, several strains of *L. monocytogenes* have shown proliferation at refrigeration temperatures, even under high salt and low pH conditions (Mellefont *et al*., 2008; Cunningham *et al*., 2009; Zhang *et al*., 2009).

3.4 Growth in the presence of organic acids

In order to determine isolate tolerance to organic acids, spot-on-lawn assays were conducted and plates incubated at 4, 25 and 37 ºC. Results for the assays conducted at 4 ºC were excluded, as 80 % of the isolates tested did not produce a lawn at this temperature. Assays conducted at both 25 and 37 ºC had yielded the same growth or inhibition patterns and therefore results for both temperatures had been consolidated (Table 4.1.3). Results from this study showed that all isolates were inhibited by undiluted glacial acetic acid (positive control) (Fig 4.1.3), while the apple cider and brown spirit vinegars only inhibited the growth of 60 and 40 % of the isolates, respectively.

It has previously been reported that acetic acids, such as vinegars and wines, retain bacteriostatic and bacteriocidal properties (Entani *et al*. 1998; Steinkraus, 2002;

Stivarius *et al*., 2002; Marshall, 2003; Johnston and Gaas, 2006). For example, a study conducted by Entani and associates (1998) showed that even at minimal concentrations, vinegar had bacteriocidal properties against *E. coli* 0157:H7. Efficacy of bacteriostatic or bacteriocidal properties of vinegar *in vitro*, is known to be associated with the concentration of acetic acid present, as well as sodium chloride, temperature, duration of incubation and the number of viable cells present (Entani *et al*., 1998; Marshall, 2003). Results from this study showed that in comparison the brown spirit vinegar, apple cider vinegar exhibited better antimicrobial action against the bacterial strains tested here. Enhanced efficacy of apple cider vinegar may be related to the production of additional compounds as the fermentation of apple juice may potentially yield end products that harbour antimicrobial properties (Vijayakumar and Wolf-Hall, 2002). Although apple cider vinegar exhibited enhanced antimicrobial properties in comparison to brown spirit vinegar, both vinegars were inadequate at complete growth inhibition of foodborne pathogenic and enterotoxin-producing strains, including *L. monocytogenes* Shia-L1.1, *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 (Table 4.1.3). Vinegar marination is an important component of the *biltong* manufacturing process and survival of potential foodborne pathogens during this process is cause for concern.

3.5 Growth in the presence of *biltong* spice

Several spices have previously been associated with antimicrobial, in particular antibacterial properties (Arora and Kaur, 1999; Delaquis *et al*., 2002; Sagdic *et al*., 2002; Sagdic and Ozcan, 2003). In order to determine the growth of bacterial isolates in the presence of traditional *biltong* spice, different types of media were created (Table 4.1.2). Upon preparation of media (Table 4.1.2) it was expected that the

antimicrobial compounds associated with spices would be released and incorporated into the agar, and hence inhibit the bacterial isolates (Shelef *et al*., 1980). Results showed that all 10 isolates exhibited the same growth patterns (Table 4.1.3) in the presence and absence of traditionally used *biltong* spice. This suggests that either: 1) the created media was insufficient at depicting the expected antibacterial properties of traditional safari *biltong* spice, or 2) the antimicrobial properties associated with the traditional *biltong* spice were too low to show a distinctive visual growth inhibition of bacterial strains on created media.

Furthermore, spices such as black pepper and coriander, which are the predominant spices in traditional *biltong* spice mix, are known to possess weak to mild antimicrobial properties in general (Zaika, 1988; Billing and Sherman, 1998; De *et al*., 1999; Burdock and Carabin, 2009). In addition, sugar, another constituent of traditional *biltong* spice, which is known to exhibit antimicrobial properties at high enough concentrations and enhance the diffusion of antimicrobial properties of other spices (Briozzo *et al*., 1989; Billing and Sherman, 1998), did not enhance the antimicrobial properties of the *biltong* spice in this study. For example, 90 % of the bacterial strains evaluated in this study showed the same growth patterns (Table 4.1.3) on MBA with or without sugar (MBA 1 and 2) (Table 4.1.3).

Although the constituent spices of the traditional *biltong* spice mixture may encompass antimicrobial properties, the findings of this study suggested that these properties could potentially have been inconsequential, or were poorly soluble in the media (Delaquis *et al*., 2002), or the antimicrobial effect destroyed during the high temperature associated with autoclaving (Billing and Sherman, 1998) and thus

showed no definitive growth reductions or changes in growth patterns on created media. It is therefore suggested that in order to determine the true antimicrobial properties associated with traditional *biltong* spice used in the *biltong* manufacturing process, one could use several other diffusion methods and scanning electron microscopy (Sagdic *et al*., 2002; Panuwat *et al*., 2003; Burt, 2004; Zhang *et al*., 2009).

In addition to depicting antimicrobial properties, the created media MBA 1 (Table 4.1.2) was prepared in order to mimic the conditions (ambient temperature 25 ºC) and factors (meat extract, spices) associated with the *biltong* manufacturing process. From the results observed it was evident that all isolates showed prolific growth on this media implying that these strains might harbour the potential to survive throughout the *biltong* manufacturing process.

Evidently, potential pathogens including *L. monocytogenes* Shia-L1.1, *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 showed growth in all or most of the assays conducted in this study, implying that these isolates are capable of surviving the individual hurdles imposed by the *biltong* manufacturing process. In the processing of several shelf-stable foods, temperature and intrinsic properties such as pH, water activity, and salt concentrations are synergistically responsible for the limitation and eradication of growth in populations of bacterial pathogens and spoilage organisms (Whiting *et al*., 1996; Delaquis *et al*., 2002; Burt, 2004). For example, the use of salt and/ or several spices in conjunction with citric or acetic acid at slightly elevated temperatures produces potent antimicrobial effects during the production of food commodities (Billing and Sherman, 1998; Entani *et al*., 1998). The latter questions the

possibility that each hurdle in the *biltong* manufacturing process may work synergistically and result in the eradication of bacterial pathogens and spoilage organisms.

4. CONCLUSION

Findings of this study have highlighted the potential of pathogens such as *L. monocytogenes* Shia-L1.1, *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 to survive the hurdles imposed by the *biltong* manufacturing process. The latter questions if there is a possibility that each hurdle in the *biltong* manufacturing process may work synergistically and as such reduce bacterial populations. Such a study forms the basis of Chapter 4.2.

Table 4.1.1: Identities and origins of selected bacterial isolates used in the survival assays conducted in this study.

STRAIN	*Genebank Accession Number*	Origin of Isolation
Listeria monocytogenes Shia-L1.1	(FJ160766)	Chicken *biltong*
Listeria monocytogenes Shia-L2.1	(FJ160767)	Chicken *biltong*
Staphylococcus aureus Shia-St64.1	(FJ392795)	Beef *biltong*
Staphylococcus aureus Shia-St122.1	(FJ392802)	Beef *biltong*
Staphylococcus aureus Shia-St122.4	(FJ392805)	Beef *biltong*
Staphylococcus pasteuri Shia-St108.3	(FJ392798)	Beef *biltong*
Staphylococcus pasteuri Shia-St112.3	(FJ392800)	Kudu *biltong*
Staphylococcus pasteuri Shia-St123.1	(FJ392804)	Beef *biltong*
Staphylococcus aureus	N/A	(Non food isolate, University of the Witwatersrand, School of Molecular and Cell Biology culture collection laboratory working strain)
Salmonella Typhimurium	N/A	(Non food isolate, University of the Witwatersrand, School of Molecular and Cell Biology culture collection laboratory working strain)

Table 4.1.2: Amendments made to the composition of mock *biltong* agar (MBA), in order to create variations that highlight growth susceptibilities to each component.

Variation of MBA	Modification of components
MBA 1	No modifications made to original media
MBA 2	Exclusion of brown sugar
MBA 3	Exclusion of beef extract
MBA 4	Exclusion of sodium chloride
MBA 5	Exclusion of both beef extract and *biltong* spice
MBA 6	Exclusion of both *biltong* spice and brown sugar
MBA 7	Exclusion of *biltong* spice

Table 4.1.3: Growth patterns of bacterial isolates observed in the presence of various *in vitro* conditions associated with the *biltong* manufacturing process.

ISOLATE	Growth at varying concentrations of NaCl (%)					Growth at varying Temperatures (ºC)					Growth in the presence of organic acids				Growth in the presence of *biltong* spice							
	5	*10*	*15*	*20*	*25*	*4*	*25*	*30*	*37*	*45*	*ACV*	*BSV*	**(CONTROLS)** GAC	dH_2o	*TSAB*	*MBA 1*	*MBA 2*	*MBA 3*	*MBA 4*	*MBA 5*	*MBA 6*	*MBA 7*
Listeria monocytogenes Shia-L1.1*	+	+	+	-	-	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+
Listeria monocytogenes Shia-L2.1	+	+	+	-	-	+	+	+	+	-	-	-	-	+	+	+	-	-	+	+	+	+
Staphylococcus aureus Shia-St64.1*	+	+	+	+	-	-	+	+	+	-	+[a]	+[a]	-	+	+	+	+	-	+	+	+	+
Staphylococcus aureus Shia-St122.1	+	+	+	-	-	-	+	+	+	-	-	+[a]	-	+	+	+	+	-	+	+	+	+
Staphylococcus aureus Shia-St122.4	+	+	+	-	-	-	+	+	+	-	-	+[a]	-	+	+	+	+	-	+	+	+	+
Staphylococcus pasteuri Shia-St108.3*	+	+	+	+	-	-	+	+	+	-	+[a]	+[a]	-	+	+	+	+	-	+	+	+	+
Staphylococcus pasteuri Shia-St112.3	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+
Staphylococcus pasteuri Shia-St123.1	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+
Staphylococcus aureus (Non-Food)	+	+	-	-	-	-	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+
Salmonella Typhimurium (Non-Food)	-	-	-	-	-	-	+	+	+	-	+[a]	+[a]	-	+	+	+	+	-	+	+	+	+

+ Denotes bacterial growth was observed, - denotes no bacterial growth was observed, *a* denotes growth observed towards the end of assays on days 6 or 7. Isolates selected for *biltong* fabrication assays (*).



Figure 4.1.1: An example of whole muscle meat portions used to produce *biltong*.



Figure 4.1.2: *Biltong* strips displayed in cabinets at point-of-sale prior to being sliced or sold.

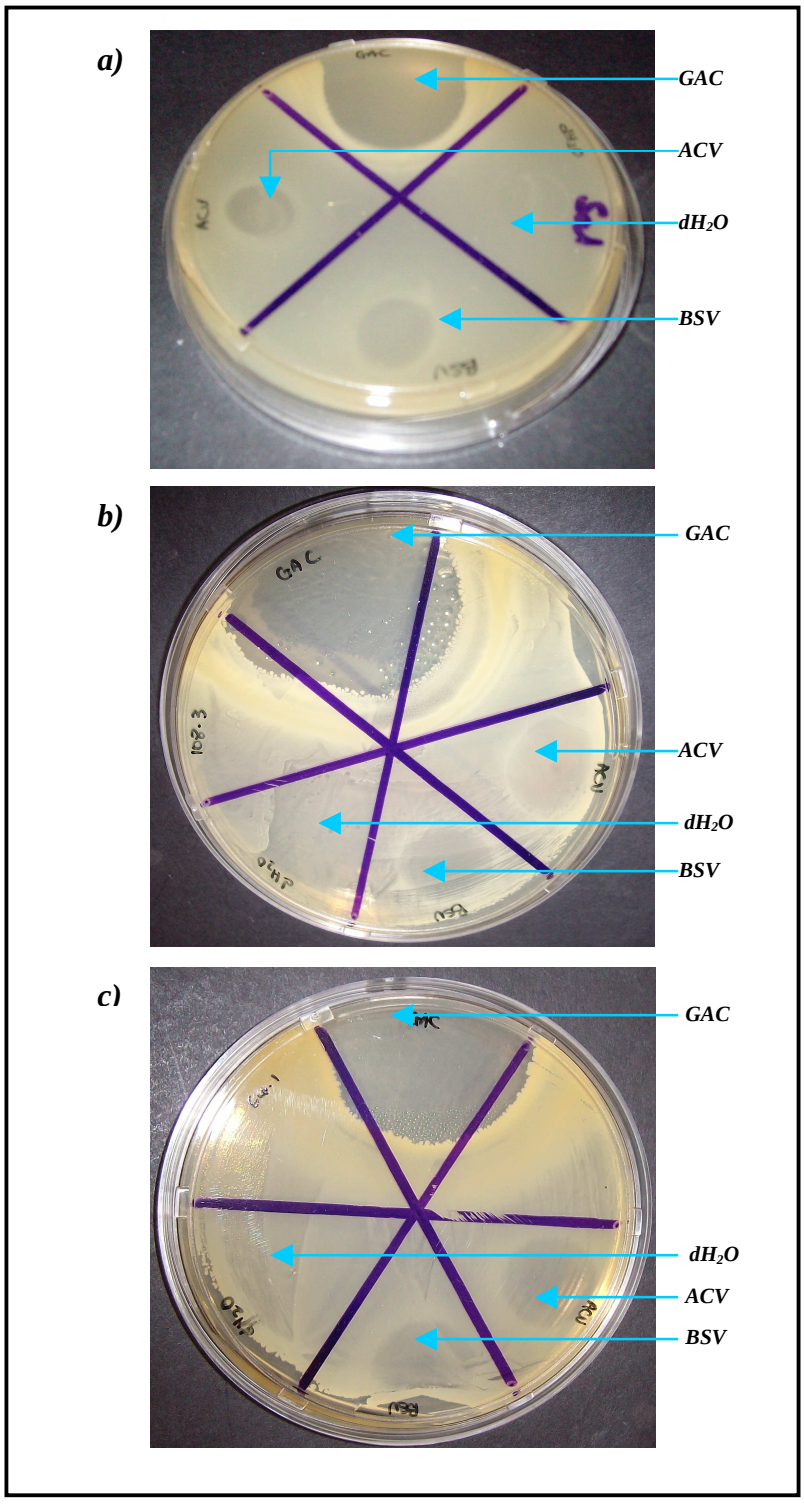


Figure 4.1.3: Inhibition of *a) Salmonella* Typhimurium, *b) Staphylococcus pasteuri* Shia-St108.3 and *c) Staphylococcus aureus* Shia-St64.1 in the presence of Glacial acetic acid (GAC) (positive control), Apple Cider vinegar (ACV), Brown Spirit vinegar (BSV) and distilled water (dH_2O) (negative control) as observed on day 7 of assays.

CHAPTER 4.2:

SURVIVAL OF LISTERIA MONOCYTOGENES, *AND ENTEROTOXIN-PRODUCING* STAPHYLOCOCCUS AUREUS *AND* STAPHYLOCOCCUS PASTEURI, *DURING TWO TYPES OF* BILTONG *MANUFACTURING PROCESSES.*

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ABSTRACT

South African *biltong* is a ready-to-eat (RTE) meat product, and is produced from raw muscle meat that is marinated or cured and dried without cooking. This commodity is gaining international popularity as a snack food and has found markets in Australia, Portugal, the USA and the UK. As the survival of potential foodborne pathogens in RTE meat products is of worldwide concern, the aim of this study was to determine the survival of 3 representative bacterial pathogens, *Listeria monocytogenes*, and enterotoxin-producing *Staphylococcus aureus* and *Staphylococcus pasteuri*, throughout the manufacturing process of South African *biltong* prepared using two different methods. Beef portions were surface inoculated with selected isolates, previously isolated from *biltong* product, and prepared using either a traditional, home-style or modern manufacturing method prior to drying at 25 ºC for 96 h. These two methods differed by the way in which beef pieces were marinated before drying. Samples were taken at 12 h intervals and bacteria enumerated by plate counts. In general, *biltong* produced using the modern method was associated with lower counts compared to product produced using the traditional technique. Overall, population decreases associated with *L. monocytogenes* were generally significantly ($p < 0.05$) greater (4.6 Log reductions) and more rapid than those associated with *S. aureus* (2 - 3 Log reductions) or *S. pasteuri* (1 - 2 Log reductions) during drying. At the end of the drying process, < 1 Log CFU/ g of *L. monocytogenes* cells were detected, while *ca.* 2 - 4 Log CFU/ g of cells for both strains of *Staphylococcus* were detected. The latter finding may hold future foodborne illness implications, as these strains of *Staphylococcus* were enterotoxin-producers.

1. INTRODUCTION

The consumption of meat and meat products are an integral component of the human diet (Reddy *et al*., 2006), especially since these commodities not only prove to be appealing to the palate, but are also high sources of amino acids and B-complex vitamins and minerals (Friedman, 1996; Jimenez-Colmenero *et al*., 2001; Rahman *et al*., 2005). Historically, fresh meats have gained a reputation for being highly perishable commodities (Lee and Fung, 1986; Mothershaw *et al*., 2003; Poligne *et al*., 2005). In addition, the relatively high water activity, partial acidity and presence of proteins, as well as carbohydrates, such as glycogen, associated with these commodities often provide optimal niches for the growth of spoilage and pathogenic organisms (Mothershaw *et al*., 2003; Jay *et al*., 2005; Rahman *et al*., 2005; Zhang *et al*., 2009).

For several centuries, dry-curing (Toldra, 2006) has been the oldest method employed for the traditional preservation of meat products against the growth of microbial populations and subsequent spoilage (Mothershaw *et al*., 2003; Trystram, 2004; Jay *et al*., 2005). Preservation by drying is achieved by the extraction or binding of moisture, resulting in the reduction of water activity (a_w) within the commodity (Dzimba *et al*., 2007). The reduction of a_w ultimately results in the inhibition of the microbial enzymes that are responsible for growth, degradation of food and general metabolism (Mothershaw *et al*., 2003; Jay *et al*., 2005; Rahman *et al*., 2005). The efficacy of drying preservation varies greatly due to the temperature used, relative

humidity during the process, rate of air movement and the desired characteristics of the final products (Burnham *et al*., 2008; Choi *et al*., 2008).

To date several dry-cured meat commodities are known for their distinctly unique tastes (Arnau *et al*., 2007), such as jerky (USA) (Burnham *et al*., 2008) and *biltong* (South Africa) (Burnham *et al*., 2008), and are produced using several hurdle technologies including the dry-curing preservation method (Mothershaw *et al*., 2003; Trystram, 2004; Jay *et al*., 2005; Choi *et al*., 2008). In addition to a prolonged shelf life, these ready-to-eat (RTE) dried meat products are generally considered microbially stable due to the presence of curing salts, organic acids, low pH and a_w values $\leq$ 0.77 (Faith *et al*., 1998; Calicioglu *et al*., 2003; Lara *et al*., 2003; Mothershaw *et al*., 2005; Montet *et al*., 2009). Although deemed microbially stable, the prevalence of several foodborne pathogens, such as *Staphylococcus aureus* (Lara *et al*., 2003), *Escherichia coli* 0157:H7 (Faith *et al*., 1998), *Salmonella* (Calicioglu *et al*., 2003) and *Listeria monocytogenes* (Calicioglu *et al*., 2002), have been observed in jerky and were subsequently linked to several outbreaks of foodborne illness (Keene *et al*., 1997; Nummer *et al*., 2004). Although there are no documented outbreaks of listeriosis associated with jerky, a 0.52 % prevalence of *L. monocytogenes* at point-of-sale has been reported for this commodity (Levine *et al*., 2001). The latter initiated research into the lethality associated with the utilisation of ground and whole muscle portions of beef and poultry meats, various marinating and processing techniques and drying methods implemented in production of this commodity (Nummer *et al*., 2004; Yoon *et al*., 2005; Konieczny *et al*., 2007; Choi *et al*., 2008).

Results from these studies showed, drying jerky meats at the FSIS/ USDA stipulated temperatures (≥ 65 °C) had significantly reduced bacterial pathogens by 2 - 5 Log CFU/ g in 4 - 8 h (Calicioglu *et al*., 2002; Yoon *et al*., 2005). However, several studies have shown that sub-lethal drying conditions not only promoted pathogen survival and favoured the development of resistance to the drying process (Portocarrero *et al*., 2002b; Calicioglu *et al*., 2002; Calicioglu *et al*., 2003; Buege *et al*., 2006; Allen *et al*., 2007; Porto-Fett *et al*., 2009), but was also a contributing factor in cases of foodborne illness (Keene *et al*., 1997; Nummer *et al*., 2004). Indeed, these studies have highlighted the inadequacy of lower drying temperatures at eradicating pathogenic populations (Nummer *et al*., 2004).

RTE dried meats, such as *biltong*, are similar to jerky but are usually produced at ambient drying temperatures for extended periods (Nummer *et al*., 2004; Burnham *et al*., 2008). This commodity is gaining international popularity as a snack food and has found markets in Australia, Portugal, the USA and the UK (Attwell, 2003). In addition to the recent prevalence of several pathogens such as *E. coli* 0157:H7 (Abong'o and Momba, 2008; Abong'o and Momba, 2009), *L. monocytogenes* (Chapter 3), *Shigella dysenteriae* (Chapter 2; Chapter 3; Naidoo and Lindsay, 2010) and *S. aureus* (Chapter 3; Mhlambi *et al*., In press) in South African *biltong*, there is limited data within the literature describing the survival of pathogens in *biltong*. The aim of this study was to determine the survival of selected potential foodborne bacterial pathogens throughout the manufacturing process of South African *biltong* prepared using two different methods.

2. MATERIALS AND METHODS

2.1. Inoculum preparation

Three isolates namely *Listeria* (*L.*) *monocytogenes* Shia-L1.1, *Staphylococcus* (*S.*) *aureus* Shia-St64.1 and *Staphylococcus pasteuri* Shia-St108.3, that showed growth in all or most of the growth and tolerance assays previously conducted (Chapter 4.1), were selected in order to evaluate the lethality of the *biltong* production process.

Prior to inoculum preparation (*ca.* 48 h), a single colony of each of the 3 bacterial isolates (from plates stored at 4 °C) were successively streak plated twice onto TSA and incubated at 37 ºC for 24 h. Isolates were examined for colony morphology and Gram-stained to confirm culture purity (Buege *et al*., 2006; Borowski *et al*., 2009). A single colony was selected from these plates, inoculated into 50 ml of Tryptone Soy Broth (TSB) (BioLab, Midrand South Africa) and incubated with shaking (120 rpm) for 24 h at 37 ºC (Ingham *et al*., 2006a; Leverentz *et al*., 2006; Burnham *et al*., 2008; Porto-Fett *et al*., 2009). A volume (1 ml) of an overnight culture of each isolate was harvested by centrifugation at 10 000 rpm for 10 min (Eppendorf minispin, bench top centrifuge). The bacterial pellet was washed and re-suspended in 1 ml of sterile saline diluent (0.85 % sodium chloride), and diluted in the same diluent to produce a final cell concentration of *ca.* 7 Log CFU/ ml (Yoon *et al*., 2005; Buege *et al*., 2006; Ingham *et al*., 2006a; Burnham *et al*., 2008; Borowski *et al*., 2009 Porto-Fett *et al*., 2009). Cell concentrations were determined by spread plating in duplicate 1 ml of either *L. monocytogenes* Shia-L1.1 prepared inoculum onto Rapid '*L. Mono* agar

(Bio-Rad, France), or *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 prepared inocula onto Rapid *Staph*' agar (RSA) (Bio-Rad, France) supplemented with egg yolk tellurite (0.5 % w/v) (Scharlau, Spain) (Table 4.2.1). Agar plates were incubated for 48 h at 37 ºC and bacterial counts recorded (Porto-Fett *et al*., 2009; Valero *et al*., 2009).

2.2. *Biltong* fabrication and inoculation

a. Meat preparation

For each of the 6 trials (3 replicates for each method), 4 fresh vacuum-sealed meat pieces (beef steak portions) individually packaged, each weighing *ca.* 400 g (*ca.* 30 cm length x 15 cm width and 2.5 cm in thickness), were obtained from a local butchery 24 h prior to inoculation and kept at 4 ºC (Ingham *et al*., 2006a; Burnham *et al*., 2008).

b. Inoculation of meat

Foil cooking trays (Hullet, South Africa) had been surface sterilised by exposure to UV light for 30 min followed by an ethanol spray (70 % v/ v). Meat pieces were individually rinsed twice with sterile distilled water (Fig 4.2.1) transferred to individual trays (Fig 4.2.2b) and placed in a bio-safety cabinet. Each meat piece was surface inoculated (Fig 4.2.1) by aliquotting 1 ml of either, sterile saline (un-inoculated control), *L. monocytogenes* Shia-L1.1, *S. aureus* Shia-St64.1 or *S. pasteuri* Shia-St108.3 prepared inoculum onto the exposed upper surface, followed by spreading using a sterile bent glass rod (Buege *et al*., 2006). Foil trays were sealed (Fig 4.2.2c) and meat pieces left to stand at ambient temperature for 20 min to ensure

the attachment of respective bacterial cells onto meat surfaces. Meat pieces were then surface inoculated on the reverse side in a similar manner (Yoon *et al*., 2005; Burnham *et al*., 2008; Porto-Fett *et al*., 2009).

c. Biltong processing

Two of the most commonly used meat preparation and marination methods for *biltong* production were tested in this study on three separate occasions (Fig 4.2.1).

Traditional method: The traditional preparation method is often used during home-style *biltong* preparation. Inoculated meat pieces were individually placed into sterile trays containing 100 ml of undiluted apple cider vinegar for 30 sec per side. Excess vinegar was allowed to drip off and each meat piece transferred to another sterile foil tray (Fig 4.2.2b). Sixteen grams of traditional *biltong* spice (predominantly consisting of black pepper, coriander, salt and brown sugar) (commercially available at www.*biltong*makers.com) was aseptically spread onto each side of the meat piece and trays sealed.

Modern method: The modern method is more often used in larger scale *biltong* manufacturing factories. Inoculated meat pieces were individually placed into sterile trays (Fig 4.2.2b). Sixteen grams of *biltong* spice was combined with 16 ml of apple cider vinegar (1:1 g/ ml ratio), and this mixture was aseptically spread onto each side of the meat pieces. Trays were sealed and shaken for 1 min in order to ensure meat pieces were fully coated in marinade.

Sealed trays (Fig 4.2.2c) were then placed into zip lock freezer plastic bags (Fig 4.2.2d) and refrigerated (4 °C) for 18 - 20 h (Fig 4.2.1) (Buege *et al*., 2006). Following refrigeration, 20 g of each meat piece was aseptically excised for microbial analysis (0 h), while the remaining 380 g meat pieces were suspended in *biltong*-drying units [2 kg *Biltong* buddy home-dryer, fitted with a 40 Watt light bulb that generated a constant temperature of 25 ºC (www.*biltong*makers.com)] that were housed in a bio-safety cabinet (Fig 4.2.3). Once *biltong*-drying units were sealed, the window of the bio-safety cabinet was closed and UV conditions applied (Fig 4.2.3). Meat pieces were sampled every 12 h for 96 h.

2.3. Microbiological analysis

For each sampling time interval, 20 g of each meat piece was excised, transferred into a sterile Whirl-Pak bag (Nasco, USA), combined with 180 ml peptone-saline diluent [0.1 % Bacteriological Peptone (BioLab, Midrand, South Africa) + 0.85 % sodium chloride (Saarchem-Merck Chemicals, South Africa)] and homogenised for 2 min using a Colworth 400 Stomacher (Geornaras and von Holy, 2000; Mosupye and von Holy, 2000; Christison *et al*., 2008). The homogenised samples were serially diluted in peptone-saline diluent, plated in duplicate using standard plating methods (Lindsay and von Holy, 1999) onto respective growth media (Table 4.2.1) and incubated aerobically for 48 h at 37 ºC. Duplicate plates were enumerated (Kunene *et al*., 1999; Christison *et al*., 2007; Christison *et al*., 2008) and expressed as Log colony forming units (Log CFU) per gram.

For *L. monocytogenes*: Surviving populations of *L. monocytogenes* Shia-L1.1 were determined by standard plating methodology (Lindsay and von Holy, 1999) (Table 4.2.1). When counts were below the lower detection limit of 1 Log CFU/ g, enrichment was also employed to confirm detection or non-detection. In this case, samples taken from meat inoculated with *L. monocytogenes* were pre-enriched by inoculating 10 ml of Fraser ½ broth (Bio-Rad, France) with 1 ml of homogenised sample and incubating for 24 h at 30 ºC. Thereafter, 0.1 ml was extracted and inoculated into Fraser 1 broth (Bio-Rad, France) for 48 h at 37 ºC (Calicioglu *et al*., 2002), after such time broth was streak plated onto Rapid '*L. Mono* agar (Bio-Rad, France), incubated at 37 °C for 48 h and examined for blue colonies lacking a yellow halo (Christison *et al*., 2008). At each sampling time, colonies of *L. monocytogenes* Shia-L1.1 were selected, Gram-stained and observed for catalase and oxidase reactions in order to confirm the presence of the inoculated strains (Ingham *et al*., 2006b).

For *Staphylococcus* strains: Surviving populations of *S. aureus* Shia-St64.1 and *S. pasteuri* Shia-St108.3 were determined by standard plating methods (Lindsay and von Holy, 1999) (Table 4.2.1). At each sampling time, colonies of *Staphylococcus* were selected, Gram-stained and observed for catalase and oxidase reactions in order to confirm the presence of the inoculated strains (Ingham *et al*., 2006b).

For un-inoculated controls: Changes in the background microbial populations were evaluated by selecting predominant populations from Tryptone Soy agar (TSA) (BioLab, Midrand South Africa) plates of control samples (von Holy and Holzapfel,

1988) and further characterising these isolates with the aid of dichotomous keys (Faller and Schleifer, 1981; Fischer, *et al*., 1986) (Fig 4.2.4).

2.4. Statistical analysis

To determine statistically significant differences between the traditional and modern *biltong* production methods, counts obtained for inoculated and un-inoculated beef cuts during the drying process were compared using the general linear ANOVA model at 95 % confidence levels in Minitab (version 15) and graphical outputs, including main effect plots and two-way interaction plots, were generated.

3. RESULTS

Overall lethality associated with both methods of producing *biltong*

Two of the most commonly used preparation methods for producing *biltong* were tested in this study, namely the traditional and modern methods. Cell populations *ca.* 7 Log CFU/ g of presumptive pathogens were initially inoculated onto each beef portion before marination. After the marination process used in both methods (i.e. 0 h and before drying), an overall 2 Log reduction in pathogen counts was observed (Fig 4.2.5). Statistical analysis indicated that overall, during drying, *biltong* produced in the traditional way exhibited a generally slower die-off of bacteria over time than *biltong* product produced in the modern way (Fig 4.2.5) and produced a product associated with significantly higher population counts ($p < 0.05$, 95 % confidence levels).

3.1 The effect of both *biltong* manufacturing processes on microbial populations

Un-inoculated control

Counts associated with un-inoculated control samples prepared using either the traditional or modern method, were similar after marination and before the drying process (0 h) (*ca.* 4.67 - 4.70 Log CFU/ g) (Fig 4.2.5). At the end of *biltong* production, counts from un-inoculated controls prepared using the traditional method showed a marginal increase of 0.97 Log CFU/ g (Table 4.2.2), while counts of corresponding controls prepared using the modern method decreased slightly by 0.96 Log CFU/ g (Table 4.2.2). The changes in microbial numbers observed step-wise throughout the production method and using both production techniques were not statistically significantly different ($p > 0.05$) (Fig 4.2.5).

Predominant populations associated with un-inoculated control samples showed a succession of microbial populations. Initially populations of Gram-positive rods, and yeasts (Fig 4.2.6) were prevalent in samples associated with both the traditional and modern methods of production, while Gram-positive cocci and Gram-negative rods (Fig 4.2.6) were only identified from samples associated with the traditional method (Fig 4.2.6). Although a more diverse composition of microbial populations was associated with the traditional method (Fig 4.2.6), both methods showed the succession of yeasts as the predominant populations throughout and at the end of the *biltong* manufacturing process (Fig 4.2.6).

Listeria monocytogenes

Although counts of *L. monocytogenes* Shia-L1.1 after marination were reduced by *ca.* 2 Log CFU/ g, it was the drying step that ultimately achieved significant ($p < 0.05$) Log reductions of 4.63 and 4.68 respectively, in comparison to un-inoculated controls, for the modern and traditional preparation methods of *biltong* (Table 4.2.2). Counts of *L. monocytogenes* steadily decreased during both methods of production (Fig 4.2.5). The complete eradication of *L. monocytogenes* populations was observed at 84 h (traditional methods) and 96 h (modern methods) of drying, respectively. In addition, pre-enrichment procedures further confirmed that when counts were below the lower detection limit (< 1 Log CFU/ g), *L. monocytogenes* was not detected. Evidently, both methods of producing *biltong* achieved an overall *ca.* 7 Log reduction, however, the traditional method attained this reduction 12 h sooner than the modern method (Fig 4.2.5). In addition, the reduction of *L. monocytogenes* populations was not significantly different ($p > 0.05$) between each step of the drying process of the traditional and modern methods of producing *biltong* (Fig 4.2.5).

Enterotoxin-producing* Staphylococcus *strains

Overall, population decreases associated with *S. aureus* were generally greater (*ca.* 1 Log CFU/ g) than decreases observed with *S. pasteuri* (Table 4.2.2). Decreases associated with the populations of both strains were higher (Table 4.2.2) when using the modern method of producing *biltong*. However, there were no statistically significant differences ($p > 0.05$) between the decreases obtained by the traditional and modern methods (Fig 4.2.5). In addition, neither of the production methods had completely eradicated (Table 4.2.2) or reduced populations of *S. aureus* and *S.*

pasteuri below the lower detection limit (Fig 4.2.5). Results showed that the drying step of the *biltong* manufacturing process achieved *ca.* 2 and 4 Log reductions while in totality the entire process achieved *ca.* 4 and 6 Log reductions in populations of *S. aureus*, when prepared in the traditional and modern methods, respectively. In addition, in comparison to *L. monocytogenes* and *S. aureus* (Fig 4.2.5), the populations of *S. pasteuri* showed greater resistance not only to the drying step (Fig 4.2.5) but also to the entire production process as the lowest reductions in counts were achieved during 1) the drying step (*ca.* 1 - 2 Log reductions) (Table 4.2.2), and consequently 2) the entire *biltong* production process (*ca.* 3 - 4 Log reductions).

4. DISCUSSION

Biltong is produced under several hurdle conditions including marination (curing with spices and organic acids) (Mothershaw *et al*., 2003), refrigeration and drying which results in this commodity being associated with a low a_w [(0.74 - 0.77) (Dzimba *et al*., 2007] and pH values ranging from 5.4 - 5.8 (Chapter 3). As such, it is expected that these conditions are responsible for controlling and limiting the proliferation of microorganisms (Albright *et al*., 2003).

4.1 Effect of the *biltong* production process on potential foodborne pathogens

Un-inoculated control

From the reduction values observed in this study, it could be suggested that the type of marination method used in the production of *biltong* may affect the lethality of the

manufacturing process against microbial populations, as noted in the jerky manufacturing process (Calicioglu *et al*., 2003; Nummer *et al*., 2004). It may be suggested that the efficacy of the modern method of *biltong* preparation was potentially attributed to the enhanced antimicrobial properties of vinegar in the presence of the spices and in particular salt (Entani *et al*., 1998). The type of preparation method had a significant influence on natural meat microorganisms or background microorganisms in this study.

Changes in background microbial populations for both preparation methods were observed. Although, a more diverse composition of predominant populations (Gram-positive rods, Gram-positive cocci and yeast) had been initially associated with the traditional preparation method, both preparation methods allowed the proliferation of Gram-positive rods, and ultimately yeasts as predominant populations. Previous studies evaluating the changes in microbial populations during the production of *biltong* have reported yeasts and Gram-positive populations, such as *Micrococcus, Staphylococcus*, *Lactobacillus* and *Bacillus*, often replacing the native Gram-negative populations that are predominantly associated with fresh meat (Taylor, 1976; Leistner, 1987). The prevalence of yeasts as predominant populations as observed in this study, has also previously been associated with the processing of several other dry-cured and intermediate moisture meats (Untermann and Muller, 1992; Garcia *et al*., 1995; Coppola *et al*., 2000; Wolter *et al*., 2000; Andrade *et al*., 2006; Nortje *et al*., 2006), and may be attributed to these organisms being more resilient to desiccation (Wolter *et al*., 2000; Jay *et al*., 2005).

Listeria monocytogenes

Although *L. monocytogenes* Shia-L1.1 survived the marination process of both preparation methods, drying was the step of the process that ultimately achieved a safe and *L. monocytogenes*-free end product. Even though both preparation methods resulted in the complete eradication of this pathogen, the traditional method ultimately achieved this result sooner than the modern method, however, there was no significant ($p > 0.05$) difference between the reductions observed.

Although pathogens such as *L. monocytogenes* are capable of surviving and acquiring acid-tolerance in acidic environments, such as those associated with marination, acid tolerance does not solely provide an enhanced resistance to the hurdles imposed by drying (Calicioglu *et al*., 2002; Calicioglu *et al*., 2003). Since the only difference associated with the 2 preparation methods in this study was the marination procedure, it may be suggested that the type of marination step applied was not as critical a factor as drying was in achieving complete lethality towards *L. monocytogenes* throughout *biltong* production (Buege *et al*., 2006). The latter was further confirmed when no *L. monocytogenes* was detected after the pre-enrichment of samples taken at 84 h (traditional) and 96 h (modern). These results also highlighted the differences between *biltong* and jerky products with respect to *L. monocytogenes*. A study by Porto-Fett and associates (2009) showed *L. monocytogenes* was found in dried beef jerky products after enrichment, when jerky had been prepared by marinating, cooking and drying meats at *ca.* 82.22 ºC for 1.5 - 3.5 h.

A recent study by Burnham and associates (2008) also evaluated survival of foodborne pathogens in various dried meats, including *biltong*. However, the method used to manufacture this product was at a much lower drying temperature and for a longer duration (*ca.* 22.2 ºC for 17 - 26 days). The latter study showed that these conditions achieved 2 - 4 Log reductions in *L. monocytogenes* populations however, this pathogen was not completely eradicated at the end of the drying step. In contrast, our study has evaluated two actual preparation techniques used during *biltong* manufacture where marinated meat is dried at higher ambient temperatures (25 ºC). Both methods tested in this study resulted in a safe *biltong* product with respect to *L. monocytogenes* within 84 h (prepared in the traditional manner) or 96 h (prepared in the modern manner) by achieving a 99.998 % reduction in the population of *L. monocytogenes*, and no detectable cells even after pre-enrichment procedures. Currently FSIS compliance guidelines, have stipulated a zero tolerance policy for *L. monocytogenes* in red meats as well as in RTE meat products such as jerky (Calicioglu *et al*., 2002; Calicioglu *et al*., 2003; Porto-Fett *et al*., 2009). It is evident from the results obtained in this study that the *biltong* manufacturing processes tested here resulted in product that conformed to the compliance guidelines proposed by the FSIS for *L. monocytogenes*.

However, a cause for concern is the popularity for a moister *biltong* product demanded by consumer markets (Dzimba *et al*., 2007). Moist *biltong* would only be dried for *ca.* 60 - 72 h, and is thought to have a better flavour and taste (Dzimba *et al*., 2007). Results from our study showed that irrespective of the preparation method used, drying at ambient temperatures for the length of time required to produce a

moister product would be insufficient for the complete eradication of *L. monocytogenes*.

Enterotoxin-producing *Staphylococcus*

Staphylococcus species are pervasive inhabitants of the skin and nasal cavities of humans and animals, often enough, these species have found their way into most food commodities (Jay *et al*., 2005).

Staphylococcus aureus is a common cross contaminant of food commodities during processing and production (Whiting *et al*., 1996; Jensen *et al*., 1998). Previous inoculation studies associated with the production of jerky and related products have shown that growth and proliferation of *S. aureus* ceased during the drying steps of producing these commodities (Ingham *et al*., 2006b; Gormley *et al*., 2009). Survival of *S. aureus* during the production of dry-cured meat products as noted in this study, is not uncommon and may potentially be attributed to this organism's competence in inaugurating, proliferating and sustaining growth under sub-optimal conditions such as high salt concentrations, low a_w and acidic pH as such (Portocarrero *et al*., 2002b; Lara *et al*., 2003; Rahman *et al*., 2005).

This study showed that the drying step of the *biltong* manufacturing process achieved *ca.* 2 and 4 Log reductions while the entire process achieved *ca.* 4 and 6 Log reductions in populations of *S. aureus* when prepared in the traditional and modern methods respectively. Although populations of *S. aureus* were reduced, neither of the 2 methods had produced *biltong* product free of *S. aureus*. It was evident however,

that producing *biltong* in the modern way is slightly more lethal and produced a product associated with lower populations of *S. aureus*. The latter highlights, irrespective of the method employed to produce *biltong*, that the *biltong* manufacturing process was inadequate at achieving the complete eradication of enterotoxin-producing *S. aureus*. In addition, the *biltong* manufacturing process was not the only process inadequate at completely eradicating *S. aureus* populations (Nummer *et al*., 2004). The production of jerky in a home-style dehydrator whereby whole muscle beef was dried for 8 h at 68.38 ºC had shown a 15 % survival of the initial *S. aureus* population (Nummer *et al*., 2004). It was suggested that even though populations of *S. aureus* were observed at the end of the jerky manufacturing process, jerky was considered safe. However, 2 foodborne outbreaks associated with the consumption of jerky contaminated with *S. aureus* have been recorded (Nummer *et al*., 2004). As such, the prevalence of *S. aureus* throughout and at the end of the *biltong* manufacturing process as seen in this study, may potentially hold similar foodborne illness implications as that observed with jerky, especially if enterotoxins are produced during the processing and production of *biltong*. Results from Chapter 3 showed that the strains used here produced enterotoxin B in pure culture. Future studies may include an evaluation of the production of enterotoxins throughout the *biltong* and jerky manufacturing processes in addition to the final RTE meat product itself.

To date there have been no documented studies evaluating the survival of *S. pasteuri* during the production of food commodities, although this organism has been prevalent in several dried RTE meat products (Morot-Bizot *et al*., 2003). Results from this study

have highlighted that in comparison to *L. monocytogenes* and *S. aureus*, the populations of *S. pasteuri* showed greater resistance, not only to the drying step but also the entire production process, as it was the population least reduced during 1) the drying step (*ca.* 1 - 2 Log reductions), and consequently 2) the entire *biltong* production process (*ca.* 3 - 4 Log reductions). Evidently, the modern method was shown to be the better method for the reduction of *S. pasteuri* populations as a 98.7 % reduction had been achieved in comparison to the 85 % obtained by the traditional method. Several strains of *Staphylococcus* including *S. pasteuri* (Iacumin *et al*., 2006) are known to contribute to the texture, aroma, flavour and final characteristics of food commodities (Morot-Bizot *et al*., 2003; Iacumin *et al*., 2006). The presence of *S. pasteuri* in *biltong* production however, has not been previously explored and as such it is not fully understood if this species may potentially attribute to the distinctive flavour of *biltong*. A cause for concern is that the *S. pasteuri* strain evaluated in this study was previously identified as an enterotoxin B-producer (Chapter 3). Therefore, the survival of this strain during production of *biltong* may potentially hold future illness implications if enterotoxins are produced.

Even though the *biltong* manufacturing process is associated with several hurdles, the survival of *S. aureus* and *S. pasteuri* was evident in both methods, thus deeming the *biltong* process inadequate at eradicating these enterotoxin-producing strains. Survival of these strains may hold potential foodborne illness implications, especially if enterotoxins are produced during processing and production. It suggested that future studies should evaluate the production and presence of enterotoxins during the *biltong* manufacturing process.

5. CONCLUSION

This study showed that the type of *biltong* preparation method might influence survival of potential foodborne pathogens on the final product. Both preparation methods were lethal to *L. monocytogenes*, while the modern preparation method was more effective than the traditional method at reducing populations of enterotoxin-producing *S. aureus* and *S. pasteuri*. However, populations of enterotoxin-producing *Staphylococcus* strains were only reduced by 85 – 99.9 %. The latter may hold future foodborne illness implications especially if enterotoxins are produced on meat during the preparation and production of *biltong*.

Table 4.2.1: Culture media utilised for the enumeration of inoculum organisms.

Inoculum Organism	**Culture Medium Utilised**
Control	Tryptone Soy agar (TSA), (BioLab, Midrand South Africa)
***Listeria monocytogenes* Shia-L1.1**	Rapid '*L. Mono* agar (Bio-Rad, France)
***Staphylococcus aureus* Shia-St64.1**	Rapid Staph' agar (RSA)(Bio-Rad, France) supplemented with egg yolk tellurite (0.5 % w/ v) (Scharlau, Spain)
***Staphylococcus pasteuri* Shia-St108.3**	Rapid Staph' agar (RSA)(Bio-Rad, France) supplemented with egg yolk tellurite (0.5 % w/ v) (Scharlau, Spain)

Table 4.2.2: Reduction of *Listeria monocytogenes, Staphylococcus aureus* and *Staphylococcus pasteuri* populations during the drying step of the *biltong* manufacturing processes.

Sampling Time	Control (Un-inoculated)		*L. monocytogenes* Shia-L1.1		*S. aureus* Shia-St64.1		*S. pasteuri* Shia-St108.3	
	Reduction		Reduction		Reduction		Reduction	
	Log	%	Log	%	Log	%	Log	%
***T0* (0 h)**								
Method 1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Method 2	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
***T1* (12 h)**								
Method 1	**0.26**	**48**	**0.92**	**88**	**0.56**	**72**	**0.37**	**58**
Method 2	**0.57**	**73**	**0.95**	**89**	**0.71**	**81**	**0.72**	**80**
***T2* (24 h)**								
Method 1	**0.69**	**81**	**0.96**	**89**	**0.90**	**87**	**0.41**	**62**
Method 2	**-0.07**	**-23**	**0.76**	**83**	**0.94**	**88**	**1.05**	**91.09**
***T3* (36 h)**								
Method 1	**0.62**	**77**	**1.54**	**97.12**	**1.13**	**92.59**	**0.01**	**4.50**
Method 2	**-0.47**	**-202**	**1.23**	**94.11**	**0.93**	**88**	**0.91**	**87.70**
***T4* (48 h)**								
Method 1	**-0.53**	**-231**	**1.44**	**96.37**	**0.97**	**89**	**0.36**	**57**
Method 2	**-0.53**	**-255**	**1.65**	**97.76**	**1.33**	**95.32**	**0.87**	**98.7**
***T5* (60 h)**								
Method 1	**-0.80**	**-503**	**1.62**	**97.60**	**1.14**	**97.91**	**0.44**	**65**
Method 2	**-0.36**	**-129**	**3.27**	**99.95**	**1.21**	**93.83**	**1.23**	**94.11**
***T6* (72 h)**								
Method 1	**-0.74**	**-561**	**2.72**	**99.81**	**0.97**	**89**	**0.40**	**61**
Method 2	**-0.34**	**-99**	**2.78**	**99.83**	**1.51**	**96.91**	**1.59**	**97.43**
***T7* (84 h)**								
Method 1	**-1.12**	**-1158**	**4.68**	**99.998**	**1.70**	**97.96**	**0.74**	**82**
Method 2	**0.84**	**-86**	**3.76**	**99.98**	**2.62**	**99.76**	**1.60**	**97.49**
***T8* (96 h)**								
Method 1	**-0.97**	**-771**	**4.68**	**99.998**	**1.62**	**97.60**	**0.81**	**85**
Method 2	**0.96**	**89**	**4.63**	**99.998**	**3.07**	**99.91**	**1.88**	**98.68**

Method represents the 1) traditional and 2) modern method of producing *biltong*. Each value is the reduction value at a particular time and was calculated by subtracting the Log CFU/ g at each sampling time from the Log CFU/ g of 0 h from Fig 4.2.5. Negative reduction values indicate an increase in the Log CFU/ g, highlighting an increase in the growth of microorganisms.

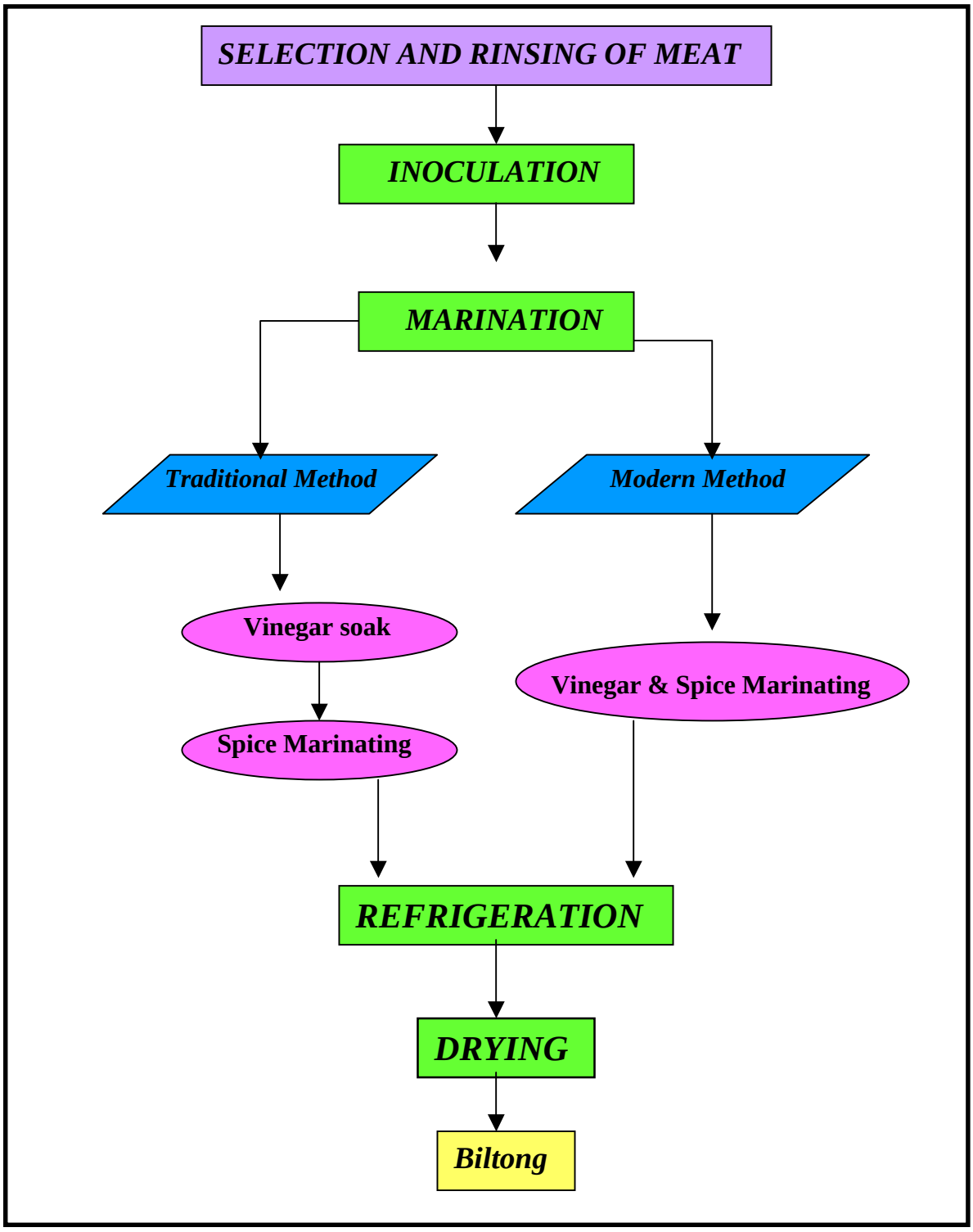


Figure 4.2.1: A schematic representation of the *biltong* fabrication process, showing the variations in meat preparation of both the Traditional and Modern methods of producing *biltong* post inoculation. Green blocks represent the hurdles steps associated with the *biltong* manufacturing process.



Figure 4.2.2: Hullet foil freezer packs that were used as inoculation trays in the *biltong* fabrication process. *a)* Lid of tray, *b)* foil tray, *c)* sealed foil tray *d)* closed foil tray sealed within Ziploc bag, ready for refrigeration.

a)



b)



Figure 4.2.3: *Biltong* drying units housed within bio-safety cabinets. Under *a)* non-UV conditions for sampling and *b)* UV conditions.

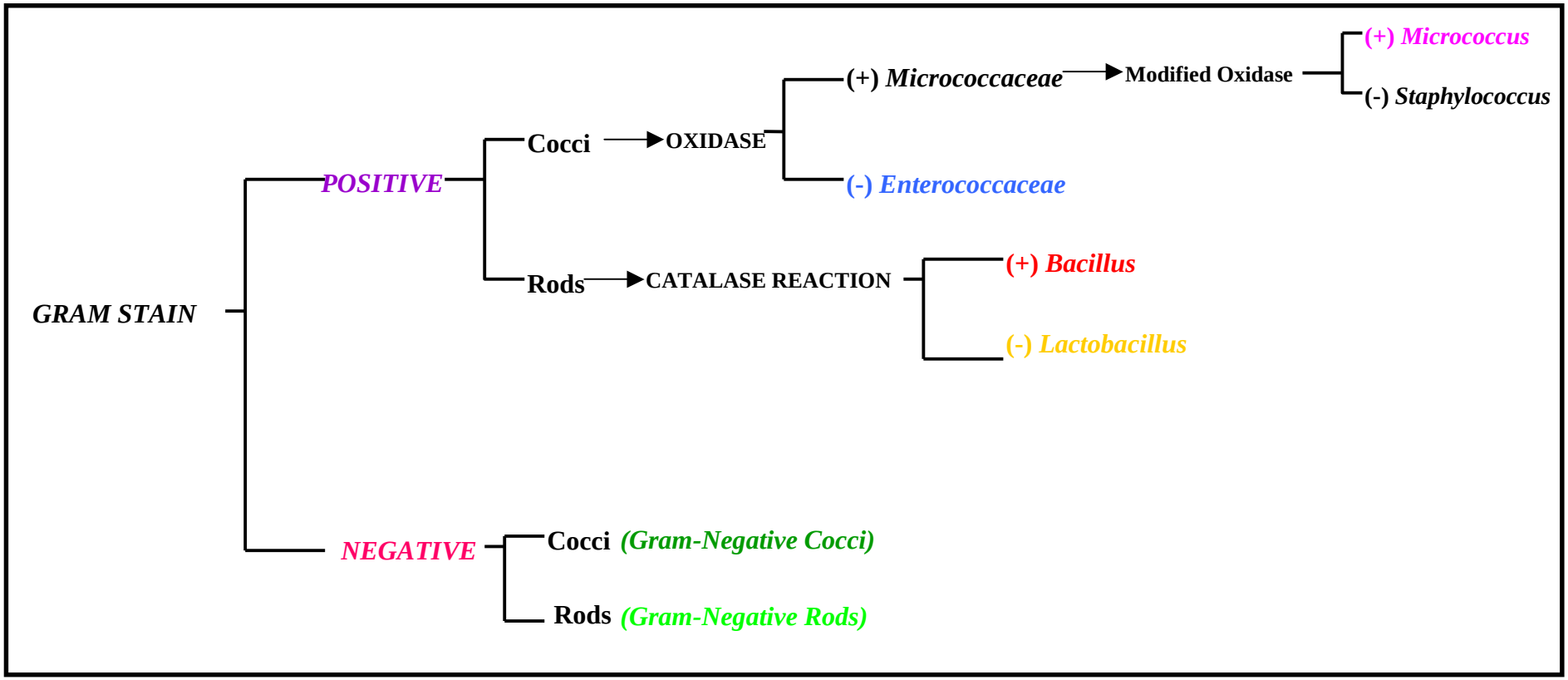


Figure 4.2.4: Reconstruction and consolidation of the Faller and Schleifer (1981) and Fischer *et al.* (1986) dichotomous keys that were used in this study in order to characterise predominant populations isolated from control plates at each sampling time of both methods.

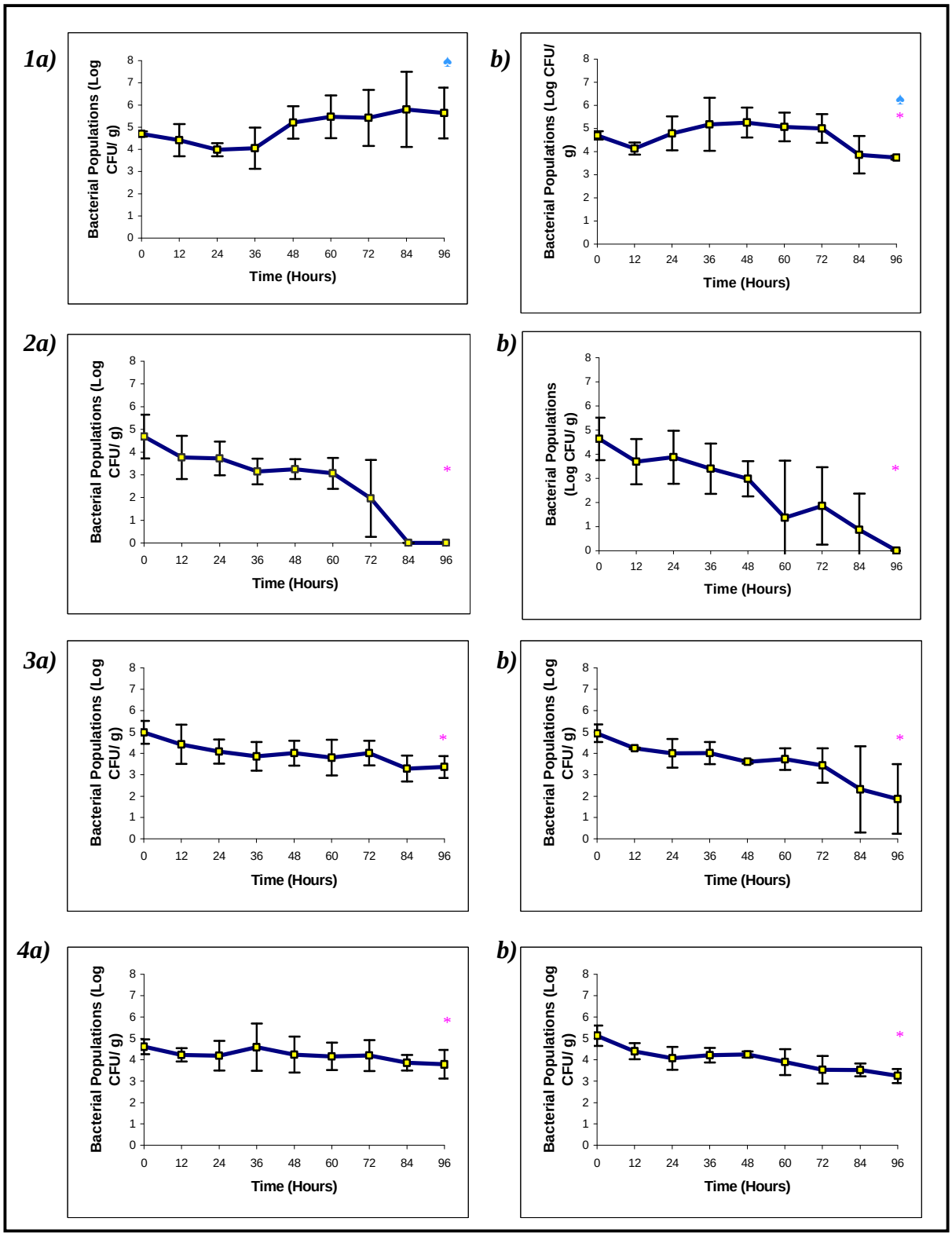


Figure 4.2.5: Growth and survival trends observed for 1) Un-inoculate control (representing natural meat microbiota), 2*) Listeria monocytogenes,* 3*) Staphylococcus aureus* and 4*) Staphylococcus pasteuri* inoculated meat during the drying step of the *biltong* manufacturing process after *a)* traditional and *b)* modern marination methods. (Lower detection limit =1 Log CFU/ g). * Significantly ($p < 0.05$) lower counts associated with samples at the end of the drying step. ♠ Significant difference ($p < 0.05$) associated with the counts observed for a particular strain during both methods of production. I represents standard deviation.

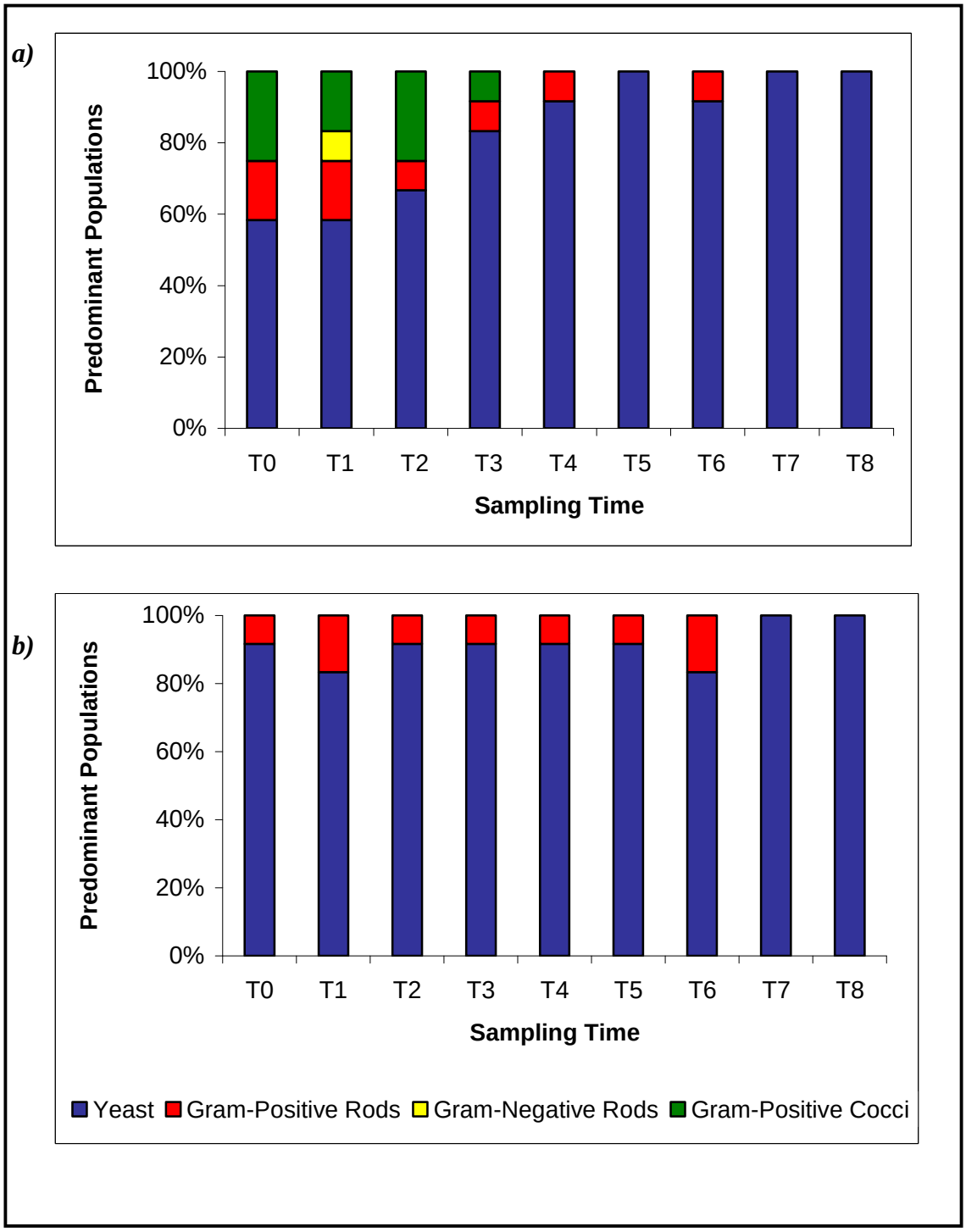


Figure 4.2.6: Changes in the predominant (background) populations during the drying step of the *biltong* manufacturing process after the *a)* traditional and *b)* modern marination of meat.

CHAPTER 5:

SUMMARISING DISCUSSION

AND

CONCLUSIONS

Biltong is considered to be the pride of South African snack commodities. This ready-to-eat (RTE) dried meat product is ubiquitously found at almost all supermarkets and convenience stores, flea markets, sporting events, bars and pubs, shopping malls, butcheries and garages or petrol stations in addition to specialist stores (*biltong* bars and shacks) which sell *biltong* on a large scale. Due to the increased international events hosted in South Africa, in particular sporting events, this commodity has not only gained international recognition and popularity but has also become a tremendously popular tourist attraction (Royer, 2009). Additionally, the emigration of South African citizens worldwide has led to the introduction and production of "traditional" South African *biltong* in several countries such as New Zealand, Australia, United States of America and United Kingdom (Read, 2004; Sweeney, 2005; Berry, 2007; del Grosso, 2009).

Producing *biltong* is a fairly easy process and as a result has lead to an increase in the home-production and small scale-production of this commodity on farms and in butcheries in South Africa. The safety of *biltong* represents a particular cause for concern, not only has this commodity been implicated in several outbreaks of foodborne illness, but there appears to be limited knowledge associated with the production of this commodity in terms of hygiene, good manufacturing practices and ensuring microbial safety by the individuals producing *biltong* on the home front and within small establishments (Nortje *et al*., 2006).

Furthermore, there appears to be limited knowledge within the literature regarding 1) the changes in microbial populations during the process of producing *biltong,* 2)

microbial populations associated with *biltong* product at point-of-sale and 3) the surfaces that come into direct contact with *biltong* product as potential points of cross-contamination of product.

The potential cross-contamination of *biltong* by contact surfaces at point-of-sale (Chapter 2)

Cross-contamination has previously been identified as a major factor contributing to several cases of foodborne illness (Tebbutt *et al*., 2007). It has been documented that these outbreaks occurred as a result of the cross-contamination of food commodities by direct contact with hands of food handlers or via contaminated food surfaces and equipment (Tebbutt *et al*., 2007).

Whole muscle meat portions (Fig 4.1.1) are generally used to produce *biltong*. After processing, the final *biltong* product is usually a strip of dried whole muscle (Fig 4.1.2). These *biltong* strips are often hung within display cabinets in retail establishments (Fig 2.3, 4.1.2) until selected by consumers, where it is then sliced or cubed using mechanical slicing devices (Fig 2.2) by store assistants and dispensed to the consumer (Fig 2.4). As consumer markets favour sliced *biltong* over whole muscle strips, several *biltong* retailers often pre-slice strips for their convenience. In this scenario, both the slices and strips of *biltong* were often displayed and slices often sold as first preference to consumers, unless the consumer had requested a particular strip to be sliced. Furthermore, *biltong* slices that were not sold at the end of business trading hours, were left in display containers overnight and mixed with freshly sliced *biltong* at the start of next day of trading.

As the latter procedure was observed as a common practice in several *biltong* establishments, the aim of this study was therefore to determine if the surfaces that come into direct contact with *biltong* product at point-of-sale, such as the walls of the display cabinet, blades of cutter and associated utensils, and the hands of the *biltong* handler (Fig 2.4) were potential sources of cross-contamination of *biltong* product at point-of-sale in 3 *biltong* retailers. Aerobic mesophilic, Gram-Negative, Enterobacteriaceae (EBC), coliform (CC) and *E. coli* (EC) counts were determined for all swab samples from contact surfaces and *biltong* product samples obtained from the 3 retailers participating in this study. In addition, predominant populations were also identified.

Results showed that *biltong* product carried counts ranging from *ca.* 6 - 8 Log CFU/ g for aerobic mesophilic populations, 2 - 4 Log CFU/ g for Gram-negative and EBC populations, 1 - 2 Log CFU/ g for CC populations and < 1 Log CFU/ g for EC populations. In addition, it was apparent that moisture content of *biltong* product influenced counts ($p < 0.05$) of aerobic mesophilic populations, with moister *biltong* displaying generally higher counts compared with dry and medium moisture *biltong* product samples. By contrast, the type of meat used to produce the *biltong* product did not significantly ($p > 0.05$) influence counts obtained (beef, game or chicken), and different seasonings also did not significantly ($p > 0.05$) affect the associated populations. The findings from this chapter were novel in that, there has been no previously documented comparative studies evaluating the bacterial counts associated with different meat, moisture and spice variants of *biltong* at point-of-sale.

In this chapter, it was further noted that several presumptive *E. coli* isolates were obtained from *biltong* product produced from different types of meats, which indicated the potential presence of other foodborne pathogens in *biltong* product. Presumptive *E. coli* isolates were further analysed using 16s rDNA molecular methods and it was evident that 3 of the isolates from the same retailer, obtained from the different products, were 100 % genetically similar to each other and 99 % similar to *Shigella dysenteriae*/ *boydii* (Fig 2.9). The latter result had strongly suggested a potential product to product cross-contamination. It was suspected that this cross-contamination could have occurred during production, transportation or handling of the various *biltong* products as *biltong* was not produced on site. Furthermore, it was observed within this retailer that *biltong* strips were often hung within display cabinets prior to store trading hours and were removed and refrigerated (Fig 2.1) after trading hours. The latter practice might have contributed to dissemination of cross-contaminants between different *biltong* products. It is recommended that retailers avoid such practices in order to reduce all unnecessary handling and cross-contamination of *biltong* product.

Upon analysis of surface samples it was evident that all swab samples from contact surfaces exceeded the South African National Health Regulations of 2 Log CFU/ cm^2 (Lues and Van Tonder, 2007) and highlighted the inadequacy of cleaning regimes employed within all 3 retailers. Although all the swab samples of contact surfaces exceeded the stipulated regulation, samples from cutting utensils consistently exceeded this regulation the most and display cabinets the least. Thus, based on associated bacterial counts, it may be speculated that cutting utensils hold a

potentially higher risk in contributing to cross-contamination of *biltong* product than the hands of *biltong* handler or display cabinets.

Previous studies have shown that the prevalence of EBC populations, in particular CC and EC populations in the food processing environment can been used as an indicator of hygiene and food safety (Jay *et al*., 2005). In addition, the detection of indicator organisms might also highlight the potential prevalence of bacterial pathogens such as *Shigella*, *Salmonella* and *E. coli* 0157:H7 (Jay *et al*., 2005; Tebbutt *et al*., 2007; Lues and Van Tonder, 2007). Thus the detection and prevalence of these populations on contact surfaces at point-of-sale as seen in this study may insinuate that 1) the current cleaning regimes are inadequate and new improved cleaning regimes need to be implemented and 2) surfaces in direct contact with *biltong* product at point-of-sale may harbour potential pathogens.

Surface swab samples taken from display cabinets were associated with lower EBC, CC and EC and suggested that the cleaning regimes associated with display cabinets were relatively more efficient. Although display cabinets were associated with fairly adequate cleanliness, *biltong* strips hanging within display cabinets (Fig 2.3, 4.1.2) were open to contamination by the patrons of the retail establishment. It is suggested that *biltong* retailers should introduce a barrier between the *biltong* product hanging in the *biltong* cabinets (Fig 2.3, 4.1.2) and customers, in order to reduce excessive handling and the potential contamination, especially since it had been observed that several patrons were handling the hanging strips of *biltong* without the use of gloves.

During this study it was noted that the personnel dispensing *biltong* product in 2 of the 3 retailers consistently wore disposable gloves while personnel in the other retailer did not (Fig 2.6). Results from this study showed (Fig 2.6), exposed hands (retailer A) were associated with higher bacterial counts than gloved hands. It is therefore recommended that the management of *biltong* retail establishments not only enforce the strict use of new, clean, undamaged disposable gloves when handling and dispensing *biltong* product, but also practice frequent and proper hand-washing techniques to fully ensure a reduced risk associated with product cross-contamination.

The *biltong* product obtained from all three retailers was sliced on site, using mechanical slicers (Fig 2.2). Often the same cutting utensils were used to slice different variants of *biltong*. Slicing devices have previously gained a reputation of being tedious and problematic to clean (Aarnisalo *et al*., 2006). As cutting devices used to slice *biltong* were constantly and continuously utilised, it was assumed from both visual observations (Fig 2.2) and the counts associated with indicator bacterial populations (Fig 2.6) that these devices were either seldomly cleaned or associated with inadequate cleaning regimes in all 3 retailers. Aside from being associated with inadequate cleaning regimes, swab samples from cutting utensils often harboured *E. coli*. It was therefore suspected that these devices potentially favoured the attachment, proliferation and dissemination of microbial contaminants onto and from the *biltong* product as it passed through the slicers.

Bacterial cross-contamination between *biltong* product and the associated surfaces at point-of-sale was further confirmed when predominant populations from TSA plates, were characterised (Fig 2.7). It was evident that although, Gram-positive isolates,

particularly members of the *Bacillus* and *Staphylococcus* genera, predominated in all samples, the types and frequencies of other populations associated with *biltong* product, and in particular swabs from cutting utensils, were fairly similar (Fig 2.7). The latter thus provided indirect evidence relating to the possibility of cross-contamination of *biltong* product by the cutting utensils or *visa versa*. Molecular identification of presumptive *E. coli* isolates from 2 samples of *biltong* product (wet and medium) and the associated cutting utensil surface showed a 100 % genetic similarity between all isolates. The latter findings provided substantial evidence supporting the possibility that the cutting utensils acted as a potential reservoir for the contamination of *biltong* product, as both variants of *biltong* product were sliced using the same cutting utensils.

To date, several studies have implicated cutting utensils as the sources of contamination of RTE products (Tebbutt, 1986; Little and de Louvois, 1998; Lunden *et al*., 2002; Christison *et al*., 2007; Tebutt *et al*., 2007; Christison *et al*., 2008; Pal *et al*., 2008). In some instances these devices have aided in the dissemination of bacterial pathogens (Lunden *et al*., 2002; Pal *et al*., 2008), which have recently lead to severe foodborne illnesses (Bouzane and Wylie, 2008). It has been postulated that, of all the surfaces that come into direct contact with *biltong* product at point-of-sale, the cutting utensils might act as the most important reservoir aiding in the contamination and dissemination of pathogenic microorganisms to *biltong* product.

As 16S rDNA sequence analysis only provides a genetic similarity of isolated strains, to provide definitive evidence for the latter findings, future work could include plasmid profiling, randomly amplified polymorphic DNA analysis (RAPD), pulse-

field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) to further fingerprint strains from product and environmental surfaces (Dykes *et al*., 1994; Giovannacci *et al*., 1999; Levin, 2003; Larrassa *et al*., 2004; Vogel *et al*., 2004; Senczek *et al*., 2000; Lim *et al*., 2005; Thevenot *et al*., 2006; Tominaga *et al*., 2008).

Although, several documented studies associated with other RTE products have shown that the hands of food handlers, utensils, processing equipment and work surfaces are all potential vectors aiding in the contamination of RTE food products, there is a gap in knowledge pertaining to *biltong*. To our knowledge, this is the first documented study associated with *biltong* product and the surfaces that come into direct contact with this commodity at point-of-sale. Results obtained from this study are of a particular importance as it was similarly shown that surfaces, in particular cutting devices and associated utensils in direct contact with *biltong* product at point-of-sale are potential reservoirs aiding in the cross-contamination of the *biltong* product.

Microbiological quality and prevalence of potential foodborne pathogens associated with *biltong* at point-of-sale (Chapter 3)

This chapter evaluated the presence of potential foodborne pathogens in *biltong* product at point-of-sale.

Results from this study showed that aerobic mesophilic populations associated *biltong* at point-of-sale irrespective of the supplier, were within the previously published

(Prior, 1984) and observed ranges (Chapter 2). Furthermore, aerobic mesophilic counts were the highest (*ca.* 6 - 7 Log CFU/ g), followed by Enterobacteriaceae (*ca.* 2.5 - 4 Log CFU/ g), coliforms (*ca.* 1.5 - 3 Log CFU/ g), presumptive *Staphylococcus* (*ca.* 1 - 3 Log CFU/ g) and *E. coli* (*ca.* 1 Log CFU/ g) counts in descending order. Currently there are no standards in South Africa or any other country relating to the associated bacterial populations or the acceptable levels of these bacterial populations on *biltong* product at point-of-sale. Thus, the findings of this study were a snapshot of the prevalent bacterial populations and highlighted the presence of potential foodborne pathogens in *biltong* product at point-of-sale from different retailers.

The latest documented study evaluating the microbial populations and hygiene associated with *biltong* at point-of-sale in Johannesburg was conducted in 1963 (Bokkenheuser, 1963) and had reported irrespective of the source *biltong* product was unsatisfactory. In contrast, the findings of the our study showed that *biltong* obtained from *biltong* bars, butcheries and convenience stores were associated with the worst hygiene (Fig 3.7). Upon sample collection from these establishments, it was observed that 1) *biltong* was predominantly sold unpackaged and 2) the personnel who dispensed *biltong* within these establishments did not solely dispense *biltong* nor did they use gloves when dispensing all commodities. It is suspected that the latter practices may have potentially attributed to the high counts and poor hygiene associated with *biltong*.

Furthermore, *biltong* and raw meats within butcheries were often positioned together and personnel interchangeably handled raw meat and *biltong* without gloves. Such practices, although not tested in this study, highlighted a potentially higher risk

associated with the cross-contamination of *biltong* product by potential foodborne pathogens. It is recommended that *biltong* retailers who also supply raw meats should introduce separate sections for dispensing each commodity in order to prevent the cross-contamination and the dissemination of potential foodborne pathogens from raw meats to *biltong*.

Pre-packaged *biltong* was associated with the lowest bacterial counts, particularly in presumptive *Staphylococcus* populations (Fig 3.7). This result was attributed to packaging serving as protective barriers preventing post-production cross-contamination. A previous report has revealed that vacuum packaging not only provides a protective barrier, but also creates a modified environment that is known to reduce bacterial populations on *biltong* product (Burnham *et al*., 2008). However, although it may be ideal for *biltong* to be pre-packaged in vacuum packaging after production, packaging costs result in a higher priced product that is conceivably uneconomical and may in turn be less favourable to the consumer.

The prevalence of foodborne pathogens such as *Salmonella* spp., enterotoxin-producing *Staphylococcus* (*S.*) and *E. coli* 0157:H7 in *biltong* at point-of-sale has previously been reported (Bokkenheuser, 1963; Abong'o and Momba, 2008; Abong'o and Momba, 2009; Mhlambi *et al*., In press). Evidently, results from the present study indicated that overall, *biltong* at point-of-sale was generally associated with a low prevalence (0.5 - 2 %) of bacterial pathogens such as *L. monocytogenes*, *Shigella*, *Proteus mirabilis* and enterotoxin-producing *S. aureus* and *S. pasteuri* and an absence of *Salmonella* spp. These findings indicated that although *biltong* has previously been regarded as a microbially safe and stable commodity, it might still be a vehicle for

foodborne illnesses such as listeriosis, shigellosis or staphylococcal enteritis. The presence of foodborne pathogens in *biltong* may potentially be attributed to cross-contamination during manufacture and at point-of-sale, as *Listeria* spp. are known to be ubiquitous to and often contaminants in processing environments.

Results from this study are the first to report the presence of *L. monocytogenes, Shigella* (Chapter 2), *Proteus mirabilis* and enterotoxin-producing *S. pasteuri* on *biltong* at point-of-sale. If enterotoxin-producing strains were to produce enterotoxins on *biltong*, it may potentially lead to cases of food poisoning. In order to predict the potential and extent of foodborne illness implications associated with *biltong*, further studies should 1) evaluate the sero-types associated with isolated strains of *L. monocytogenes* and 2) directly test *biltong* at point-of-sale for the presence of enterotoxins. As contamination of food commodities with *S. aureus* is often linked to human handling, it is suggested that *biltong* retailers should establish and maintain better hygiene practices to reduce the contamination of *biltong* product with enterotoxin-producing *Staphylococcus* populations.

The presence of *Shigella*, as noted in this study and the previous study (Chapter 2), highlighted the potential of *biltong* as a vector for shigellosis, and suggested the presence of *E. coli* 0157:H7 as it has recently been suggested that *E. coli* 0157:H7 strains are *Shigella* in a cloak of *E. coli* antigens (Johnson, 2000). A shortcoming of this study was the lack of pre-enrichment steps for the detection of *E. coli* 0157: H7. It is therefore suggested that future studies should include specific pre-enrichment steps, as used by Keene and associates (1997) or Abong'o and Momba (2008), for the detection of *E. coli* 0157:H7 in *biltong* product.

The *in vitro* response of selected foodborne pathogens to the condiments and conditions used during the *biltong* manufacturing process.

Biltong has previously been implicated in several outbreaks of salmonellosis, and it was suggested that the foodborne illnesses had arisen due to the survival of *Salmonella* spp. throughout the *biltong* manufacturing process. The prevalence of several bacterial foodborne pathogens on *biltong* at point-of-sale (Chapter 3), questioned the potential survival of these strains throughout the *biltong* manufacturing process.

In order to establish the tolerance of selected strains to the hurdles introduced by the *biltong* manufacturing process, such as curing (spices and vinegar), refrigeration and drying, several growth assays were conducted. The findings of this study highlighted that isolates obtained from *biltong*, in particular potential pathogens, were generally capable of growth in the presence of high salt concentrations (15 % NaCl), suggesting that *biltong* favours the prevalence and proliferation of salt tolerant foodborne pathogens. Commercially available vinegars, such as Apple Cider and Brown Spirit vinegars, did not exhibit adequate bactericidal properties against *L. monocytogenes* or enterotoxin-producing *Staphylococcus* strains. Additionally, traditional *biltong* spices did not show any visual inhibition suggesting that the spices were associated with no or very weak inhibitory properties. Furthermore, this study showed that although the *biltong* manufacturing process was associated with several perceived hurdles, individually each hurdle was inadequate at eradicating potential bacterial pathogens. *Biltong* has a long history of being a relatively safe RTE meat snack product, with a

few exceptions. Therefore, the conclusions drawn from this chapter gave rise to the following questions:

- Do the hurdles imposed during the *biltong* manufacturing process work synergistically to reduce foodborne pathogens?
- And if so, were the pathogens isolated from *biltong* product at point-of-sale in Chapter 3 accidental contaminants?
- Or do selected pathogens indeed survive the *biltong* production procedure?

Survival of potential foodborne pathogens during the *biltong* manufacturing process (Chapter 4)

Literature on the survival of foodborne pathogens during the jerky production process is quite extensive, however, there appears to be limited research investigating the microbial changes and survival of potential foodborne pathogens during the *biltong* manufacturing process.

Currently, there are several recipes and methods of producing *biltong*. Many traditional recipes are passed down from one generation to the next. Although methods may differ in spices and organic acids used (for example different vinegars) the basic principles of the *biltong* manufacturing process remains the same. Two of the more popularly used methods (Fig 4.2.1) namely the traditional and the modern methods were evaluated in this study. The findings of this study have highlighted that overall, during drying, *biltong* produced in the traditional way exhibited a generally slower die-off of bacterial populations over time than *biltong* product produced in the modern way (Fig 4.2.5) and produced a product associated with significantly higher

population counts ($p < 0.05$). Furthermore, results showed that the type of *biltong* preparation method might influence survival of potential foodborne pathogens on the final product. Both preparation methods were lethal to *L. monocytogenes*, while the modern preparation method was more effective than the traditional method at reducing populations of enterotoxin-producing *S. aureus* and *S. pasteuri*. However, populations of enterotoxin-producing *Staphylococcus* strains were only reduced by 85– 99.9 % in total. It may therefore be deduced that the modern method, currently utilised by many bulk manufacturers, was the microbiologically safer method of producing *biltong*.

Findings of this study further showed that members of the *Staphylococcus* genera are resistant to the *biltong* manufacturing process irrespective of the preparation method. It is therefore essential for *biltong* producers to prevent cross-contamination from associated personnel and equipment during the processing and production of *biltong*, as the hurdles imposed by the manufacturing process are not sufficiently lethal to these populations. The latter may hold future foodborne illness implications especially if enterotoxins are produced on meat during the preparation, production and processing of *biltong*. Several dry-cured meat products have previously been implicated in outbreaks of staphylococcal foodborne illness (Martin *et al*., 1992; Borneman *et al*., 2009), it is suspected that these outbreaks had resulted from the production of staphylococcal enterotoxins on meat during the processing and production of these dried meat commodities as shown by several studies (Niskanen and Nurmi, 1976; Portocarrero *et al*., 2002b; Simon and Sanjeev, 2007; Bang *et al*., 2008). In order to fully evaluate the true extent of foodborne illness associated with *biltong*, future studies should evaluate the production and presence of enterotoxins

during the *biltong* manufacturing process and determine which point in the process favours the production of such enterotoxins, in addition to monitoring the survival of enterotoxin-producing *Staphylococcus* populations.

An additional cause for concern is the popularity of moister *biltong* by consumer markets, as it is considered to have a better flavour and taste. It is expected that under the conditions observed in this study, moist *biltong* would only be dried for *ca.* 60 - 72 h. Results from this study showed that irrespective of the preparation method used, drying at ambient temperatures for the length of time required to produce a moister product would be insufficient for the complete eradication of *L. monocytogenes*. The survival of *L. monocytogenes* may in turn highlight the potential survival of other foodborne pathogens. It suggested that further work should determine the potential link between the prevalence of foodborne pathogens and *biltong* with different moisture contents.

From a practical point of view, it is suspected that several *biltong* manufactures may often re-use marinades. In the event that such marinades become contaminated with foodborne pathogens or the toxins they produce, it is likely that the *biltong* subsequently produced, may potentially hold future foodborne illness implications especially if sub-lethal drying conditions are applied. Furthermore, as findings of this study showed that drying at ambient temperatures was not lethal to enterotoxin-producing *Staphylococcus* strains, it might be suggested that if contamination from marination had to occur, drying at ambient temperatures will not be sufficiently lethal to eradicate these contaminants and may lead to subsequent foodborne illness implications.

To our knowledge this study presents the first report of it is kind. As methods and recipes for producing *biltong* are constantly changing the findings of this study are of particular importance especially since it highlights that marination and preparation methods have an effect on the survival and eradication of selected foodborne pathogens. This work only evaluated traditionally used beef pieces. However, other types of *biltong* are also common (Chapter 2). It is suggested that future studies evaluate the effect of different meats (game, chicken, ostrich), portions at point-of-sale (slices, chucks or sticks), spices and organic acids (vinegars and wines) may have on the survival, eradication and prevalence of selected foodborne pathogens when producing other *biltong* variants.

Conclusions

The fundamental contributions to the literature made by this work can be summarised as follows:

a) Sources of point-of-sale, display conditions, distribution, handling, processing and methods used to produce *biltong* all have an effect on the hygiene and safety associated with this commodity.

b) Surfaces at point-of-sale that are in direct contact with *biltong*, in particular cutting utensils, are contributing agents in the cross-contamination of *biltong* product at point-of-sale.

c) *Biltong* at point-of-sale is a potential reservoir for the dissemination of foodborne pathogens, such as *L. monocytogenes*, *Shigella* spp., *Proteus mirabilis* and enterotoxin-producing *Staphylococcus*.

d) *Biltong* retail establishments are associated with poor hygiene practices, which may in turn have a direct effect on the bacterial populations and counts associated with *biltong* at point-of-sale.

e) Evidently the methods used to produce *biltong* influences the survival of potential foodborne pathogens on the final product.

f) Although, both methods used in the *biltong* manufacturing process were lethal to *L. monocytogenes*, it was shown that the period of drying was the most important preservative factor for complete eradication of this pathogen.

g) Enterotoxin-producing *Staphylococcus* strains were resistant to the *biltong* manufacturing process irrespective of the preparation method employed. A major concern is that, if enterotoxins are produced on *biltong* during production and at point-of-sale, it may potentially lead to cases of staphylococcal enteritis.

h) In addition, moister *biltong* product, which is currently favoured by consumers, may be associated with a higher risk for illness due to survival of potential foodborne pathogens.

Significance of present work

a) It is the most recent and detailed work into the evaluation of *biltong* at point-of-sale since the 1960's, and since this commodity is the national snack its safety is of utmost importance.

b) It is important to note that there are currently no standards or acceptable levels for bacterial populations associated with *biltong* in South African legislation or anywhere else in the world. Although there are limits associated with jerky, the product somewhat most similar to *biltong*, these cannot be used for *biltong*, as they are two separate commodities that differ greatly in terms of preparation methods. As these acceptance levels are lacking for *biltong*, this study aids in providing some guidelines into establishing safety standards for *biltong* product during production and at point-of-sale.

c) Furthermore, results from this study may provide a starting point towards developing a risk assessment model for South African *biltong*.

CHAPTER 6:

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