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Effect of food safety systems on the microbiological quality of beef

by

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Thesis submitted in partial fulfilment of the requirements for the degree

Doctor of Philosophy (PhD)

in the

Department of Food Science

Faculty of Natural and Agricultural Science

University of Pretoria

Republic of South Africa

January 2011

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Declaration of independent work

I, PAPISO ARIETTE TSHABALALA declare that the thesis herewith submitted for the degree of PhD (Food Science) at the University of Pretoria, is my own independent work and has not been previously submitted by me for a degree at any other institution of higher education.

Papiso Ariette Tshabalala

Signature of student Date

Dedication

My husband, mother, children, siblings, nephews, nieces, and friends: you showed me everlasting love and supported me throughout, mostly by putting me under tremendous pressure. For all of you, the younger generation, a life strategy and formula: always strive to see the end to everything that you start. For the older generation: everything is possible.

Acknowledgements

- Professor Elna Buys and Dr Henriette de Kock for their priceless leadership and support during the study;
- Dr Duodu for just that smile and few words of encouragement;
- The Department of Agriculture for creating an enabling environment for me to even consider furthering my studies;
- Mr Dries Venter, of the Gauteng Veterinary Services, for being a source of valuable information;
- Mr Johan Du Preez for allowing me to use his pictures;
- Mandla, my husband, for always allowing me to fly as high as I possibly can;
- My mother, for words of encouragement and for always reminding me that being the elder child means one always needs to set a standard;
- All my friends for showing love and giving me immeasurable support and love, nothing has gone unnoticed;
- Mrs Francina Makhoane, a friend and a sister for all the help and immense support;
- My fellow study mates for helping me get started, helping with IT challenges and just keeping me going.

Above all, I have grown in faith and know that the strength, the perseverance and the blessing of all the wonderful people in my life are a gift from God the Almighty.

Abstract

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Degree: PhD Food Science

Contamination of meat with microorganisms during slaughter is inevitable. Hygiene management systems (HMSs) such as the Hygiene Assessment System (HAS) and Hazard Analysis Critical Control Point (HACCP) are used to prevent the contamination of beef with both spoilage and pathogenic microorganisms during slaughter. This study compared the effect of the HAS alone and a combination of HAS + HACCP on the microbiological quality of beef and investigated the survival of *Escherichia coli* O157:H7 co-cultured with different levels of *Pseudomonas fluorescens* and *Lactobacillus plantarum* on fresh beef.

HAS alone and HAS combined with HACCP systems were each represented by two abattoirs. Sponge swab samples were collected from chilled beef carcasses for indicator organisms: Aerobic Plate Counts (APC), Enterobacteriaceae, *Pseudomonas* spp., and lactic acid bacteria. Swabs were also collected for pathogenic bacteria: *E. coli* O157:H7, *Staphylococcus aureus* and *Salmonella* spp. There was no significant difference between the microbiological quality of beef carcasses processed in the abattoirs with the HAS and that of beef carcasses processed in abattoirs with combined HAS + HACCP. *E. coli* O157:H7 was isolated from carcasses processed in an abattoir with the combined HAS + HACCP system. Moreover, although overall *S. aureus* counts at all abattoirs were comparable, a higher incidence (47% of carcasses) was obtained from an abattoir with combined HAS + HACCP. *Salmonella* spp. was not detected during the study. The microbiological quality of beef at HAS abattoirs is not significantly different to that of beef processed at HAS + HACCP abattoirs. The combined HAS + HACCP did not prevent contamination of beef carcasses with *E.*

coli O157:H7 and *S. aureus*. Effective implementation of HAS can reduce contamination of beef with spoilage and pathogenic microorganisms.

The effect of different levels of *P. fluorescens* (10^2 and 10^6 $\log_{10}$ cfu/ml) and *L. plantarum (*10^2 and 10^4 $\log_{10}$ cfu/ml) on the survival of *E. coli* O157:H7 on beef loins was investigated. Sterile beef loins inoculated with *E. coli* O157:H7 and *P. fluorescens* were aerobically stored for 7 days at 4 °C, while those inoculated with *E. coli* O157:H7 and *L. plantarum* were vacuum-packaged and stored for 8 weeks at 4 °C. APC, *E. coli* O157:H7 and either *P. fluorescens* or *L. plantarum* counts were determined at different storage intervals. For the aerobically packaged beef loins, *E. coli* O157:H7 was detected throughout the 7-day storage period regardless of the *P. fluorescens* level in the inoculum. For the vacuum packaged beef loins, similar inoculum levels of *E. coli* O157:H7 and *L. plantarum* allowed *E. coli* O157:H7 to survive until week 5 of storage, while a higher inoculum level of *L. plantarum* inhibited *E. coli* O157:H7 from week 3. Once fresh beef has been contaminated with *E. coli* O157:H7 the level of *P. fluorescens* in the background flora does not inhibit its survival and growth. However, under vacuum storage, the application of *L. plantarum* as a biopreservative inhibits the survival of *E. coli* O157:H7 on beef. Comprehensive strengthening of preventive strategies is required to eliminate contamination of beef carcasses with *E. coli* O157:H7.

Bacterial contamination of carcasses during slaughter is inevitable. Effective implementation of HAS at abattoirs produces beef carcasses of microbiological quality comparable to that produced through the use of combined HAS and HACCP. While the level of *P. fluorescens* on beef does not inhibit the survival of *E. coli* O157:H7 on aerobically stored beef, the combination of *L. plantarum*, and low storage temperature inhibits the survival of this pathogen on beef under vacuum storage.

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List of abbreviations

AHRS	Abattoir Hygiene Rating Scheme
ANOVA	Analysis of variance
APC	Aerobic Plate Count
CE	Competitive Exclusion
CCP	Critical Control Point
CFS	Cell free supernatants
CPS	Coagulase-positive staphylococcus
DWAF	Department of Water Affairs
DoA	Department of Agriculture
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Entero-invasive *E. coli*
DAEC	Diffuse adhering *E. coli*
DNA	Deoxyrobinucleic acid
EAEC	Entero-aggregative *E. coli*
EFSA	European Food Safety Authority
EPEC	Enteropathogenic *E. coli*
FITA	Federation of International Trade Associations
GPDoA:VPH	Gauteng Provincial Department of Agriculture: Veterinary Public Health
HACCP	Hazard Analysis and Critical Control Point
HAS	Hygiene Assessment System
HMS	Hygiene Management System
LPS	Lipopolysaccharides
OM	Outer Membrane
RMAA	Red Meat Abattoir Association
RNA	Ribonucleic acid
SAMIC	South Africa Meat Industries Company
TCC	Total coliform count
VPH	Veterinary Public Health
VTEC	Verotoxin-producing *E. coli*
VFA	Volatile Fatty Acids

CHAPTER ONE:
INTRODUCTION AND PROBLEM STATEMENT

1.1 INTRODUCTION

Meat has been part of human diets for most of mankind's existence. Beef, is a high source of protein, which contains all the essential amino acids and iron. The iron found in meat is in the form of heme iron, which is better absorbed than the non-heme iron found in plants (Schnepf, 2007). Through the ages, a need existed for controlling the activities of producers, processors and manufacturers of food intended for human consumption. This need led to the development of food laws in South Africa (Department of Health, 2006). Furthermore, public awareness of meat associated health hazards, such as heart attacks (Erlinger & Appel, 2003) and fatal *E. coli* bacterial infections (Ethelberg, Smith, Torpdahl, Lisby, Boel, Jensen, Nielsen & Mølbalk , 2009) led to the development of stringent legislative requirements to ensure the supply of safe food products.

In the 1960s, there was a concern about poor hygiene at South African abattoirs. This inadequacy was addressed by creating the Abattoir Commission under the Abattoir Industry Act 54, 1976. This commission then developed hygiene norms and standards for abattoirs and became the only body with the authority to approve applications for building new abattoirs in South Africa (Department of Agriculture, 2007). As a result, the red meat industry became extensively regulated by the Abattoir Commission. The meat industry was characterized by the following: restrictions on the movement of meat between different areas, compulsory auctioning of carcasses according to grade and mass in controlled areas and supply control via permits and quotas (Jooste & van Schalkwyk, 1996). The South African meat industry has gone through many regulatory changes since 1994 (Dittmer, 1998; Red Meat Abattoir Association, 2006).

In South Africa, abattoirs are graded A, B, C, D or E according to throughput (number of animals slaughtered per day). For example, Grade A abattoirs have a throughput > 100 slaughter units, while grade E abattoirs have a throughput of one to eight slaughter units per day (DoA, 2007). To ensure standardized hygiene practices at all abattoirs, the DoA introduced the Hygiene Assessment System (HAS) in 1999. HAS is used to quantify the hygiene standards at abattoirs, against national standards.

The Gauteng Provincial DoA, Directorate: Veterinary Public Health (GPDoA: VPH) introduced a rating scheme, Abattoir Hygiene Rating Scheme (AHRS) in 2003 (Figure 1.1). Although participation in AHRS is voluntary, abattoirs that achieve high ratings receive special certificates and are made known to the public. AHRS ratings range from five stars (90 and above), four stars (75 to 89) and three stars (60 to 74). Abattoirs that score a rating below 60 do not receive any awards (DoA, 2003). The significance of a higher rating is the assurance that the products derived from such an abattoir are of high quality, with a longer shelf life from a hygiene point of view (RMAA, 2004). A rating awarded to an abattoir is valid for twelve months, after which the rating is reassessed.

South African abattoirs that export meat and meat products to the European Union are, in addition to HAS, are required to implement the HACCP system, based on European Union requirements (Van de Venter, 2000). The DoA allocates resident official state veterinarians to these abattoirs and the importing countries periodically carry out physical audits of the HACCP plans of these abattoirs.

1.2 PROBLEM STATEMENT

In 1992, the South African meat industry was deregulated leading to the Abattoir Industry Act 54, 1976 being repealed and the Abattoir Commission being disbanded. The removal of these restrictions brought about a major restructuring of the meat industry. For example, there was a rapid increase in the number of smaller abattoirs in the rural areas mostly on-farm facilities and transportation of livestock to abattoirs became less regulated and therefore easier, responding to the demand (Dittmer,

1998).The deregulation of the industry allowed wholesalers to source live slaughter animals directly from farmers or feedlots, assuming ownership of the animals before the animals are slaughtered. As a result, abattoir owners do not have control over the quality of animals presented for slaughter (RMAA, 2002). Most importantly, meat inspection became a private activity, allowing abattoir owners to employ their own meat inspection personnel (RMAA, 2002). This situation led to a decline in the management of hygiene practices, as adherence to stringent meat inspection procedures was compromised at abattoirs (RMAA, 2002).

To circumvent the declining control of hygiene practices at abattoirs during slaughtering, the RMAA offers training on good hygienic practices, good manufacturing practices and food safety systems at all registered abattoirs in South Africa. The European Commission recognizes the South African Bureau of Standards (SABS), as the competent certifying body for ISO/SANS including SANS 10330 which is a food safety standard. The HACCP certified abattoirs supply meat and meat products to both the export and the domestic markets. The use of different hygiene management systems at abattoirs may create a perception that fresh beef from abattoirs that use HAS + HACCP may be of superior microbiological quality compared to beef from abattoirs that use the HAS. Standardization of practices at abattoirs is further compounded by the lack of a single food safety system in the South African meat industry, as a result of the absence of a centralized food control government agency. Consequently, the regulatory approach is fragmented, located within a number of government departments and within the three tiers of government, namely, national, provincial and local (Jackson, 2006; Van de Venter, 2000). DoA, Directorate: Veterinary Services, is the competent authority for setting norms and standards pertaining to veterinary hygiene i.e. the minimum standards required for an abattoir to be registered, while the provincial and local governments are competent authorities for the enforcement of all legislative requirements.

Contamination of meat carcasses has pointed to many sources including abattoir workers (Wagude, 1999). In instances where, abattoir workers' remuneration is linked to the number of head of cattle slaughtered a day, workers tend to increase the

slaughter line speed (Wagude, 1999). A line speed that is too high implies inadequate time for slaughter operatives to carry out their functions, which may lead to increased chances of spillage during evisceration and inadequate sterilization of slaughter equipment leading to increased risk of contamination of carcasses. The generation of abattoir specific microbiological data can be used for training purposes and illustrations so that abattoir workers understand the link between their practices and meat safety.

Foodborne diseases commonly occur without being reported and South Africa is no exception. The first case of *E. coli* O157:H7 infection was reported in 1990 in South Africa (Browning *et al.*, 1990) followed by sporadic cases of bloody diarrhoea (Effler, Isaäcson, Arntzen, Heenan, Canter, Barret, Lee, Mambo, Levine, Zaidi & Griffith 2001; Galane & Le Roux, 2001). Low levels of *E. coli* O157:H7 have been isolated from meat and meat products in South Africa (Abong'o & Momba, 2009). Food security is more critical than food safety among both rural and urban poor. The lack of vigorous surveillance of food pathogens in South African meat and meat products presents a challenge for food hygiene trainers, as it becomes difficult to demonstrate the magnitude of contamination with these pathogens. There is a need to generate more data from abattoirs, supermarkets, street vendors and butcheries to ascertain the prevalence of *E. coli* O157:H7 in the meat industry and such information must be made available to the public.

Although little is known about consumer perception and awareness of food safety in South Africa, generally freshness of meat is often cited as one of the most influential variables impacting on consumers' decisions to purchase fresh meat (Glitsch, 2000).

It is assumed that there would be more control at abattoirs that use the HAS + HACCP system thereby reducing opportunities for contamination of carcasses during the process of slaughtering, resulting in carcasses of superior microbiological quality compared to carcasses from abattoirs that use only HAS. The aim of this study was therefore, to assess the effectiveness of the combination of the HAS and HACCP system in preventing the microbiological contamination of beef carcasses during

slaughter, as opposed to using HAS alone. *E. coli* O157:H7 has been isolated from faeces of slaughter animals at abattoirs, as well as in processed, ready to eat meat products in South Africa. As a result, the ability of spoilage microorganisms to inhibit the survival of *E. coli* O157:H7 on fresh meat, aerobically and under vacuum storage was also investigated.

Figure 1.1: Example of an abattoir hygiene rating scheme certificate

CHAPTER TWO: LITERATURE REVIEW

2.1 *E. COLI* O157:H7, A FOOD PATHOGEN

2.1.1 Origin of *E. coli* O157:H7

Escherichia coli were first isolated by a German paediatrician, Theodore Esherich, in 1884 from faeces of human neonates (Khan & Steiner, 2002). The bacterium was then called *Bacterium coli commune.* Most *E. coli* strains are harmless commensals, however, some strains are pathogenic and cause diarrhoeal diseases. *E. coli* strains that cause diarrhoeal illness are categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O:H serogroups. These categories include enteropathogenic *E. coli* strains (EPEC), enteroinvasive *E. coli* strains (EIEC), diffuse adhering *E. coli* strains (DAEC), enteroaggregative *E. coli* strains (EAEC) and enterohaemorrhagic *E. coli* strains (EHEC).

E. coli O157:H7 and many *E. coli* produce verotoxins, hence they have been named VT-producing *E. coli* (VTEC). The first confirmed isolation of *E. coli* O157:H7 in the United States of America (USA) was in 1975 from a Californian woman with bloody diarrhoea, while the first reported isolation of the pathogen from cattle was in Argentina in 1977, while the bacterium was first identified as a human pathogen in 1982 (Fernandez, 2008). The spread of *E. coli* O157:H7 in North America coincided with the importation of infected cattle from Argentina, where the rates of human infection were previously about three times higher than those found in North America (McMichael, 2001).

E. coli O157:H7 is usually a harmless bacterial strain present in the intestines of cattle. Probably, the potentially lethal variant of *E. coli* O157:H7 evolved in Argentinian cattle reservoir when the harmless variant of the bacterium acquired a gene from the deadly *Shigella* bacterium through a viral agent, resulting in the lethal

form (Heymann & Rodier, 1997). The resultant strain soon became prevalent in key cattle-growing regions throughout North America. However, for a new bacterial strain to become an infectious disease, it has to be able to survive the media through which it is transmitted to humans (Whittam, McGraw & Reid, 1998). In the Walkerton, Canada waterborne outbreak of 2000, which affected more than 2,300 residents and resulted in seven deaths (Hrudey & Hrudey, 2000), the pathogen had travelled from the intestines of cattle, through the surface water pathways, through the soils into ground water pathways and through the constructed drinking water system to be ultimately consumed by humans, where the pathogen survived the hosts' gastric acid defences (Whittam *et al.,* 1998). Contaminated food and water cause 700,000 deaths in Africa annually (Kertesz, 2009).

2.2 RECORDED *E. COLI* O157:H7 ASSOCIATED FOODBORNE OUTBREAKS

E. coli O157:H7 outbreaks have been reported most frequently in developed countries and in over 30 countries on six continents (Boyle, Swerdlom & Griffith, 1995; Chapman, 1994; Griffith & Tauxe, 1991; Mead & Griffith 1998; Nataro & Kaper, 1996; Water, Sharp & Dev, 1991). In Africa, *E. coli* O157:H7 associated outbreaks have been reported in South Africa (Browning *et al.,* 1990), Swaziland (Isaäcson *et al.,* 1993) and in the Central African Republic (Germanii, Soro, Vohito, Morel & Morvan, 1997), Kenya (Sang *et al.,* 1996), Gabon (Presterl, Zwick, Reichmann, Aichelburg, Winkler & Kremsner, 2003, Nigeria (Olorunshola, Smith & Coker, 2000) and the Ivory Coast (Dadie, Karou, Adom, Kette & Dosso, 2000). The outbreak that occurred in Swaziland in November 1992 was the largest recorded in Africa (Effler *et al.,* 2001). The infection rate was 42% of 778 screened residents (Raji, Minga & Machangu, 2006), many of whom had bloody diarrhoea and abdominal pains which distinguished these patients from those who had cholera. Symptomic cholera infection almost always manifests as profuse watery diarrhoea (Effler *et al.,* 2001).

The major problem with *E. coli* O157:H7 is that it is not detected by the usual methods used to isolate and identify traditional enteric bacterial pathogens.

Moreover, microbiology laboratories in many African countries do not routinely test *E. coli* O157:H7. Hence, many infections may go unrecognized (Raji *et al.,* 2006). Raji *et al.* (2006) suggested three public health measures (educating the public on dangers of eating undercooked meat, increasing awareness among clinicians of *E. coli* O157:H7 infection and mandating case reporting) to control infections associated with *E. coli* O157:H7 in Africa. The impact of gastroenteritis is dramatic for developing countries where an estimated 2.5–3.2 million children <3 years die each year (Bresee, Widdowson, Monroe & Glass, 2002).

2.3 CHARACTERISTICS OF *E. COLI* O157:H7

2.3.1 Acid tolerance

Ground beef has been implicated as the major primary vehicle of the transmission of *E. coli* O157:H7 (Griffith & Tauxe, 1991). *E. coli* O157:H7 has been implicated in outbreaks associated with high acid products such as apple cider (Besser, Lett, Weber, Doyle, Barrett, Wells & Griffith, 1993), mayonnaise (Weagant, Bryant & Bark, 1994) and yoghurt (Morgan *et al.,* 1993). The safety of high acid products and the acid properties of *E. coli* O157:H7 have been of concern. Acid tolerance is both growth phase dependent and inducible. Cells in the stationary phase of growth are more acid tolerant than cells in the exponential phase (Arnold & Kasper, 1995). Arnold and Kasper (1995) demonstrated that acid tolerance of *E. coli* O157:H7 was not dependent on prior exposure to low pH, as cells of a strain that had been grown overnight at pH 7.2 survived acid challenges. Furthermore, Arnold and Kasper (1995) observed enhanced acid tolerance in starved, late lag and stationary phase cells compared to mid lag phase cells.

2.3.2 Antibiotic resistance

Studies have revealed that *E. coli* O157:H7 are resistant to antibiotics (Galland, Hyatt, Crupper & Acheson, 2001; Wilkerson *et al.,* 2004; Zhao *et al.,* 2001). In a recent

study conducted in the North West Province, South Africa, Ateba, Mbewe and Bezuidenhout (2008) determined the antibiotic resistance of 76 *E. coli* O157:H7 strains from three types of species (pigs, cattle and humans) to nine different antibiotics. Their results demonstrated the prevalence of multiple antibiotic resistances (MAR) and resistance to three or more different classes of antibiotics in 93.4% of the *E. coli* O157:H7 isolated. Ateba *et al*. (2008) recorded that 83.8% to 100% of *E. coli* O157:H7 isolates from pig faeces were resistant to tetracycline, erythromycin and sulphamethoxazole. Resistance to these antibiotics was influenced by the location of the sample, i.e. all samples from two areas were resistant, yet none of the *E. coli* O157:H7 isolates from a third location were resistant to tetracycline. Moreover, none of the isolates from cattle faeces obtained from any of the farms were resistant to either neomycin or norfloxacin. In addition, all *E. coli* O157:H7 isolated from humans were resistant to erythromycin, tetracycline, ampicillin and sulphamethoxazole. These authors suggested that rainfall runoffs could be the mode of transmission of antibiotic resistant *E. coli* O157:H7 strains in the North West Province, particularly in communal set-ups where animals were seen drinking rainfall water and pigs sometimes feed on human excreta. Resistance of *E. coli* O157:H7 may have negative clinical implications by hampering clinical treatment of *E. coli* O157:H7 infections.

2.3.3 Carriage of a 60-MDa plasmid

E. coli O157:H7 isolates associated with human illness harbour a plasmid (pO157) of approximately 60 MDa that contains DNA sequences common to plasmids present in other serotypes of VTEC isolated from patients with haemorrhagic colitis. The plasmid is believed to play a role in the pathogenicity of disease (Fernandez, 2008).

2.4 TRANSMISSION OF *E. COLI* O157:H7

Cattle are the major reservoir of *E. coli* O157:H7 (Ateba *et al.,* 2008) and shed this organism more frequently when they are stressed (Armstrong, Hollingsworth & Morris, 1996). The 1992 Swaziland *E. coli* O157:H7 outbreak was preceded by

several years of drought, which led to a situation where cattle were grazing closer to the remaining sources of surface water and vegetation. With the onset of heavy rains (Mead & Griffith, 1998), water contaminated with cattle faeces (and presumably *E. coli* O157:H7) would have become readily available to both livestock and residents downstream (Effler *et al*., 2001). In addition, meat from dead animals was available for residents to consume. It was reported that much of the beef from dead animals would have been grilled leaving the possibility that portions remained undercooked. It is well documented that most *E. coli* O157:H7 outbreaks have been associated with the consumption of raw or undercooked foods of bovine origin (Fernandez, 2008; Raji *et al*., 2006).

2.5 CLASSIFICATION OF FAMILY *PSEUDOMONADACEAE*.

The *Pseudomonadaceae* family covers one of the most diverse and ecologically significant groups of bacteria that are Gram-negative, rod-shaped and contain polar flagella. Members of the genus are found in large numbers in a wide range of environmental niches, such as terrestrial and marine environments, as well as in association with plants and products of animal origin i.e. milk and meat that may become contaminated with pseudomonads during collection, storage and handling (Gennari & Dragotto, 1992). This almost universal distribution suggests a remarkable degree of genomic diversity and genetic adaptation (Kristoffer, Binnewies, Willenbrock, Hansen, Yang, Jelsback, Ussery & Friis, 2008). *P. fluorescens* encompasses a group of non-pathogenic saprophytes that colonize soil, water and plant surface environments and form biofilms on abiotic and biotic surfaces (Toutain, Caiazza & O'Toole, 2004). However, due to the ability of *P. fluorescens* to form biofilms on surfaces (Sillankorva, Neubauer & Azeredo, 2008; Talsma, 2007), this organism has been implicated in disease outbreaks derived from contaminated medical devices (Gershman, Kennedy, Noble-Wang, Kim, Gullion, Kacica, Jensen, Pascoe, Saiman, McHale, Wilkins, Schoonmaker-Bopp, Clayton, Arduino & Srinivasan, 2008; Pappas *et al.*, 2006). *P. fluorescens* is a group of obligate aerobes, except for some strains that can utilize nitrate (NO_3) as an electron acceptor in place of oxygen (Hedgecock & Costello, 1962; Rediers, Vanderleyden & De Mot, 2007).

P. fluorescens also secrete pyoverdin, a fluorescent yellow-green siderophore under iron-limiting conditions (Meyer, Geoffroy & Baida, 2002).

2.6 CLASSIFICATION AND PHYSIOLOGY OF LACTIC ACID BACTERIA

LAB constitutes a group of gram-positive, acid-tolerant, generally non-sporulating, non-respiring rods (*Lactobacillus* and *Carnobacterium*) or cocci (all other genera) that are associated by their common morphological, metabolic and physiological characteristics. LAB produces lactic acid as the major end product during fermentation of carbohydrates (Axelsson, 2004). The genera that comprise LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus*, which form the core group, while *Aerococcus*, *Carnobacterium, Enterococcus*, *Oenococcus*, *Sporolactobacillus, Tetragenococcus*, *Vagococcus* and *Weissella* are considered principal LAB (Stamer, 1976). *Weissella* is the only LAB genus that includes both rods and cocci (Collins *et al.*, 1993).

The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations and acid or alkaline tolerance, of nonaerobic habit, but aerotolerant (Axelsson, 2004). The genus *Lactobacillus* is the largest of the genera included in LAB. Lactobacilli are widespread in nature and many species have found applications in the food industry. For example *Lactobacillus plantarum* is used in the brewery industry.

2.7 MEAT SPOILAGE

Meat is one of the most perishable foods and its composition is ideal for growth of a wide range of spoilage bacteria. The composition of the meat spoilage flora is greatly influenced by the storage conditions such as temperature and the type of packaging material (Nychas, Drosinos & Board, 1998; Tsigarida & Nychas, 2001). Spoilage of aerobically stored meat is dominated by *Pseudomonas* spp. (Mayr, Margesin, Klingbichel, Hartungen, Jenewein, Schinner & Märk, 2003). Mayr *et al.* (2003)

observed 83 to 100% *Pseudomonas* spp. on aerobically stored meat after 11 days of cold storage. In another study, Ercolini, Russo, Torrier, Masi and Villian (2006) studied microbial spoilage under three different conditions of modified atmosphere packaging, air, (ii) 60% oxygen and 40% carbon dioxed (MAP 1) and (iii) 20% oxygen and 40% carbon dioxed (MAP2) and low storage temperature (5 °C). Spoilage of meat, which varied according to the packaging conditions, occurred between 7 and 14 days of storage. *Pseudomonas* spp. were identified as acting during beef storage in air, while *Pseudomonas* spp and *L. sakei* were the main species found during storage using MAP3. When the numbers of pseudomonads reach around 100 million per gram, at the meat surface, they produce a putrid odour and slime forms (Food Science Australia, 2003). The main defects in meat are off-odours, and off-flavours, but discolouration and gas production also occur (Borch *et al.,* 1996).

The shelf life of meat is considerably increased by vacuum packaging. When oxygen impearmable packaging is used, the growth of gram–positive, mostly LAB is favoured because of increased carbon dioxed levels and lowered oxidative–reduction potential (Borch, Muermans & Blixt, 1996). LAB grow slowly at low temperature. LAB could grow to 10–100 million per gram after about 6 weeks of storage, but do not produce signs of spoilage. After 14–16 weeks, bitter or liver-like flavours develop (Food Science Australia, 2003).

2.8 SOURCES AND CONTROL OF CONTAMINATION OF MEAT AND MEAT PRODUCTS

Red meat animals can be infected or carry a wide range of microorganisms, which are potentially pathogenic for man. The most important of these are zoonotic bacteria, principally pathogenic serotypes of *E. coli*, such as O157:H7, *Salmonella* and *Camplylobacter* spp. (Humphrey & Jørgensen, 2006). However, the presence of *Salmonella* or *Campylobacter* on food does not necessarily mean that infection will result. Many *Salmonella* serotypes common in food animals have not been extensively implicated in human infection, probably because they are less virulent compared to serotypes like *S. enteritidis* and *S. typhimurium*, or maybe they do not

survive in the food chain (Humphrey & Jørgensen, 2006). Risks associated with the consumption of contaminated meat and meat products can be reduced by implementing systematic controls from farm-to-fork. At farm level, strategies including the application of probiotics in animals and diet management may reduce the shedding of *E. coli* O157:H7 by slaughter animals.

2.8.1 Animals presented for slaughter

Slaughter animals may arrive at an abattoir positive for different pathogens. Humane treatment of food animals impacts on meat safety and should receive increased attention worldwide (Gradin, 2006). Animal stressing may damage meat quality and lead to more contamination and cross contamination with pathogens due to resultant increased pathogen shedding. Excretion levels of pathogens such as *E. coli* O157:H7 can be higher after transportation, which is associated with stress in animal hosts, leading to the spread of faeces containing high levels of pathogenic organisms on the live animal hide, with subsequent contamination of carcasses during slaughter (Sofos, 2008). Furthermore, during hide stripping, some bacteria originating from the animal hide become suspended in the abattoir atmosphere. This contaminated air may come into contact with food products, i.e. carcasses, containers, equipment and other food contact surfaces during processing, where they may adhere strongly (Sutton, 2004). Nevertheless, the major source of carcass contamination is contact with the skin during hide removal or contamination by spillage of stomach contents during evisceration (Humphrey & Jørgensen, 2006). Meat safety can be assured through the development of an integrated control of pathogens, including pre-slaughter strategies, post slaughter or during processing in the plant, at retail and food services and at home (Sofos, 2008). Therefore, the target of control pre-slaughter should be to minimize sources, levels, access and transfer of contamination to animals and produce.

Challenges at pre-slaughter include the diagnosis of animals as carriers of pathogenic organisms. Diagnosing cattle on the farm or the feedlot as being “infected” by pathogenic bacteria is difficult because pathogenic bacteria often have little or no

effect on the health or production efficacy of animals. Therefore such animals are asymptomatic (Sofos, 2008). Zhao *et al.* (1998) observed that all experimental animals inoculated with *E. coli* O157:H7 in their study remained clinically healthy, with no evidence of diarrhoea. Cattle have been found to be insensitive to the deleterious effects of the toxins produced by *E. coli* O157:H7 and other EHEC (Pruimboom-Brees, Morgan, Ackermann, Nystrom, Samuel, Cornick & Moon, 2000). Detection of *E. coli* O157:H7 is also complicated by the fact that faecal shedding can be sporadic, with an animal testing positive for EHEC one day, but not again for several days or even weeks (Callaway *et al.*, 2004; Sofos, 2008).

2.8.2 Abattoir waste

The abattoir industry is one of the industries that contributes to the problem of possible foodborne disease and potential health hazards associated with food especially meat, by improper handling of condemned material (Adeyemi & Adeyemo, 2007). Abattoir waste is defined as waste or waste water from an abattoir which could consist of pollutants such as animal faeces, blood, fat, animal trimmings, condemned organs and carcasses, paunch content and urine (Department of Water Affairs (DWAF), 2001), together with inedible organs. Inedible offal includes skin, ears, gall bladder, foetuses, hair, hooves, snouts and horns (DoA, 2007).

Abattoir waste material carries a high level of microorganisms that may cause disease in humans and animals, such as *Salmonella* and *Escherichia coli* bacteria (Brown, 2006). According to South African legislation (DoA, 2007; DWAF, 2002), abattoir waste has to undergo pre-treatment before it can be disposed of. The Red Meat Regulations of 2004 (DoA, 2007) stipulate that any condemned material must be disposed of by means of a total incineration, denaturing and burial at secure sites. The burial sites have to be approved by the Provincial Executive Officer and the local government, wherein access should be controlled to avoid illegal removal of condemned material. Furthermore, in cases where condemned material is sterilised, the sterilization facility has to be registered. However, enforcement of these regulations is questionable because raw abattoir waste including animal carcasses has

been found on landfill sites in some provinces of South Africa (Makana Municipality, 2004). Roberts, de Jager and Blight (2009) demonstrated that the majority of the abattoirs in their study did not adhere to the legislation. For example, in cases where condemned material was disposed of by burying, it was evident that it had not been burnt before burying as a legislative requirement. Furthermore, incineration was not properly achieved because in most cases both abattoir managers and workers failed to adhere to the required temperature for proper incineration of abattoir waste. Moreover, in cases where municipal landfill site was used, it was found that access was not controlled.

South Africa is an upper-middle-income country (Federation of International Trade Associations (FITA), 2010). Despite its wealth, the experience of the majority of South African households is either one of outright poverty or of continued vulnerability to becoming poor (Agbola, 2003). This situation forces poor communities to seek any possible food source which includes landfill sites, dumping grounds and disposable sites used by abattoirs (Derbyshire, 2003). This situation creates an opportunity for human beings (scavengers) living off refuse sites to consume condemned abattoir products and carcasses, which could result in significant health problems leading to possible disease and death (Roberts & De Jager, 2004). Hepburn, McRae and Ogden (2002) demonstrated that *E. coli* survives in abattoir waste products stored at a low temperature and increases in numbers at abusive storage temperature. In addition, Avery *et al.* (2009) showed that heat treatment (60 and 50 °C for 10 min) of abattoir waste that has been artificially inoculated with *E. coli* O157:H7 did not achieve a complete kill of the pathogen. Therefore, it is imperative for the South African meat industry to develop strategies and mechanisms that may ensure proper handling of abattoir waste. In addition, governmental policing strategies need to be improved.

2.8.3 On-farm control strategies

On-farm pathogen reduction programmes contribute to the control of food safety problems by reducing both the probability of pathogen presence and environmental

pollution. Such control measures directly minimize water and produce contamination and direct animal-human transmission of pathogens (Sofos, 2008; Zhao, Doyle, Harmon, Brown, Muller & Parks, 1998). Different synergetic strategies can be used to reduce the levels of pathogens in live animals including the use of probacterial and antipathogen strategies and dietary and management strategies.

2.8.3.1 Use of probiotics in animals

Probiotics are defined as commensal (harmless or beneficial) bacteria used to reduce pathogenic bacteria in the gut (Fuller, 1989). Probiotics beneficially affect the host by improving its microbial balance. Such commensal organisms in the gut can be competitive or antagonistic to foodborne pathogenic bacteria. Probacterial strategies can be categorized into two groups: 1) the introduction of a "normal" (non-pathogen containing) intestinal microbial population (probiotics) or 2) providing a limiting substrate (prebiotic) that is not digestible by the host animal but which may allow an already existing microbial population to expand its niche in the gastrointestinal population. The concept of addition of an exogenous bacterial population to the intestinal tract is called Competitive Exclusion (CE). The CE culture may be composed of a single or multiple strains of a single bacterium, or even several different species of bacteria. The CE culture will limit the population of pathogenic bacteria by competing for limited nutrients, by binding sites along the gut epithelium or by producing toxic compounds (Crittenden, 1999; Nurmi, Nuotio & Schncitz, 1992; Steer *et al.,* 2000).

Zhao *et al.* (1998) carried out a study in which experimental animals were inoculated with probiotic bacteria and the control animals were not. These authors used *E. coli* strains, excluding *E. coli* O157:H7 for their probiotic population. Zhao *et al.* (1998) detected *E. coli* O157:H7 in rumen fluid of experimental animals for an average of 14 days post-inoculation with probiotic bacteria, whereas from the rumen fluid of control animals *E. coli* O157:H7 was detected for 26 days. Zhao *et al*. (1998) concluded that treatment of cattle with probiotic bacteria can reduce the level of carriage and faecal shedding of *E. coli* O157:H7 and may thereby reduce environmental contamination

with *E. coli* O157:H7. In another study, Brashears *et al.* (2003) reported the effective use of *L. acidophilus* culture in the diet of finishing feedlot animals in decreasing *E. coli* O157:H7 shedding by more than 50%. Faecal shedding of *E. coli* O157:H7 is directly correlated with levels of carcass contamination (Elder *et al.,* 2000), emphasizing that the live animal is a critical link in the production chain.

2.8.3.2 Diet management effects on levels of E. coli O157:H7

Changes in feeding frequencies are associated with changes in the rumen microenvironment. Feeding cattle concentrated diets high in starch and soluble carbohydrates results in a more acidic rumen fluid, mainly because of increases in volatile fatty acids (VFA) (acetate, butyrate, and propionate) and lactate production by ruminal bacteria. In contrast, feeding cattle roughage diets high in cellulose, hemicellulose and pectin, results in only slightly acidic rumen fluid as a result of lower concentrations of VFAs (Church, 1979). Increased shedding of pathogens by animals has been linked to raising the fibre content of ruminant diets, fasting and other forms of stress (Nicholson *et al.,* 2000).

Cattle in the USA are often fed high-grain diets to maximize growth efficiency (Huntington, 1997). In South Africa, the feedlot industry produces approximately 75% of all beef (South African Feedlot Association, 2010) (Figure 2.1). Typically, grain finishing diets are high in energy and low in fibre. The abrupt switch of diets from high-grain diets to 100% hay-based diets resulted in a reduction of faecal populations (Diez-Gonzalez, Callaway, Kizoulis & Russell, 1998). Similarly, Keen, Uhlich and Elder (1999) compared a group of naturally infected cattle using high grain and hay as variables in their study. They recorded that of the grain-fed cattle 52% remained *E. coli* O157:H7 positive compared with 18% of the cattle abruptly switched to hay. Based on such comparative studies, it has been stated that "the most effective way of manipulating gastrointestinal counts of *E. coli* was to feed hay" (Gregory *et al.,* 2000). In contrast, Hovde, Austin, Cloud, Williams & Hunt (1999) observed longer *E. coli* shedding by hay-fed animals compared to grain-fed ones. In their study Hovde *et al.* (1999) exposed eight 1-to-2-year-old Holstein to different

diets: a typical finishing diet (82 to 90% grain); a medium-quality diet (100% alfalfa hay); and low-quality diet (100% timothy grass hay). After three weeks of adaptation to a particular diet, the animals were inoculated with *E. coli* O157:H7. Regardless of the diet, animals shed similar titers of faecal *E. coli* O157:H7. The average duration that grain-fed cattle were culture positive was 4 days, whereas cattle fed alfalfa or grass hay diets shed faecal *E. coli* for longer times, averaging 39 and 42 days, respectively. In addition, *E. coli* O157:H7 populations collected from the faeces of cattle in both dietary groups were equally acid tolerant. Animal rumen is a highly complex and competitive environment that allows the survival and proliferation of a specific anaerobic population, thereby creating a difficult environment for transient organisms to survive (Hungate, 1966). Thran, Hussein, Redelman and Fernandez (2003) reported that rumen pH as a function of fermentation of dietary components was less effective in decreasing numbers of *E. coli* O157:H7 entering cattle rumen. Thran *et al.* (2003) concluded that the reduction in *E. coli* O157:H7 was rather due to competitive exclusion by the rumen microorganisms.

Figure 2.1: Feedlot animals during feeding and relaxation periods

2.8.4 Control measures at abattoir level

2.8.4.1 Animal coats in carcass contamination

Animal coats are a significant source of microbial contamination of the carcass (Hudson, Mead & Hinton, 1998). Many countries apply a subjective ordinal visual rating system to food animals presented for slaughter, categorizing animals by degree of coat cleanliness (Small, Wells-Burr & Buncic, 2005). Animals assessed as clean are normally processed for human consumption. However, research on cleanliness of the animal coat has demonstrated that visually clean cattle often carry pathogens, for example, *E. coli* O157:H7 and *Salmonella* spp (Avery *et al.* 2002; Small *et al.,* 2002) confirming that hides should always be considered as posing a serious risk to meat safety.

All operations during slaughter and dressing of food animals that involve penetration of the skin such as penetrative stunning of animals, also in South Africa, carry a risk of introducing pathogenic bacteria from the skin onto edible parts of the animal as penetrative stunning pistols are not sterilized between animals (Buncic, McKinstry, Reid & Anil, 2002). Buncic *et al*. (2002) demonstrated in their study investigating the role of the penetrative pistol in spreading microbial contamination to edible parts and the abattoir environment that microbial contamination association with penetrative stunning can spread from the brain to the edible parts of the same animal, including muscles via the blood circulation. In addition, Buncic *et al*. (2002) observed that positive detection of the marker organisms in their study varied between individual animals, between types of tissues/organs and between types of the marker organism. The authors then speculated that the variation could have been due to factors including: (a) differences in stunning-associated damage of the brain blood vessels between individual animals, which could cause different counts of the bacteria to enter the blood circulation, (b) differences in post-stunning rate and/or duration of the heart activity between individual animals, which could cause different transfer of bacteria through the animal bodies, and (c) difference in volume/mass of blood, tissue

and organs between individual animals, which could result in different "dilution" factors for the counts of the marker organisms. Blood and liver were most commonly contaminated (in 90% of animals), followed by the lungs and spleen (in 80% of animals), deep muscle (in 20% animals) and on the carcass surface (in 50% of animals). In this study penetrative stunning was also positively linked to the spread of contamination to the environment. The marker organisms were present in protective clothing samples collected from the slaughtermen conducting the stunning of animals and samples collected from the pelt (leg, breast and shoulder) of the stunned animals. Buncic *et al*. (2002) further demonstrated that penetrative stunning could spread contamination to subsequently stunned animals if equipment was not cleaned and sterilized between animals.

Small *et al*. (2005) evaluated the effects of pre-skinning hide decontamination on carcass contamination. They recorded a positive correlation between the microbial loads on skinned carcasses with those on the hide of the same animal. They concluded that pre-skin hide decontamination would reduce overall microbial loads introduced into the slaughter line environment and onto the dressed carcasses, and hence, improve meat quality and safety.

2.8.4.2 Effect of line speed on carcass hygiene

According to Roberts (1980), line speed may have serious implications in relation to carcass contamination. The faster the line operates, the more opportunities there are for mistakes to be made and hence for more contamination to occur. The relationship between line speed and carcass contamination is influenced by a large number of factors including operator fatigue, knife skills, length of working day, levels of boredom and the presence or absence of proper management structures such as HACCP. The most important aspect is whether or not the operatives have sufficient time to carry out their jobs. In some countries, the speed line is regulated by the number of carcasses that an inspector can examine in an hour (Roberts, 1980).

2.8.4.3 Abattoir workers

The hygienic status of dressed carcasses is largely dependent upon the general slaughterhouse hygiene and the skills of the workers (Mothershaw, Consolacion, Kadim & Ahmed, 2006; Rahkio & Korkeala, 1996. The interviews conducted by Mothershaw *et al.* (2006) revealed that 89% of workers at the abattoir where they conducted their study had no training in safe food handling, and as a result, personal hygiene standards were also found to be low. Furthermore, Desmarchelier, Higgs, Mills, Sullivan and Vanderlinde (1999) showed that the incidence of coagulase-positive staphylococcus (CPS) at one of the abattoirs in their study increased by 33.5% after evisceration compared to counts enumerated before evisceration. This increase corresponded to the heavy contamination of the hands of workers performing the evisceration task. Desmarchelier *et al.* (1999) found that the hands of 75% of workers at trimming of visible contamination step were contaminated with CPS. Therefore, Desmarchelier *et al.* (1999) concluded that workers' hands could have been a source of carcass contamination with CPS, hence a large increase in counts after chilling for 72 hours. The significance of workers' contribution to carcass contamination was also illustrated by Wagude (1999) who observed a great improvement in the microbiological quality of beef after training workers on sanitation, personal hygiene and hand washing techniques.

2.9 HYGIENE MANAGEMENT SYSTEMS AT ABATTOIRS

Cattle slaughter operations, such as bleeding, dressing and evisceration expose sterile muscle to microbiological contamination that is present on the skin, in the digestive tract and in the environment (Gill & Jones, 1999; Sofos *et al.,* 1999). With a view to reducing the risks associated with the presence of food pathogens on carcasses, the need to achieve standardized control systems, and the desire to access international markets, the South African meat industry approached the government for assistance. The DoA co-ordinated a task team representing South African Meat Industries Company (SAMIC), RMAA and GPDoA: VPH. This task team developed the

Hygiene Assessment System (HAS) based on the United Kingdom's evaluation/audit system. HAS became a mandatory requirement for all registered abattoirs in 2000 upon its inclusion in the Meat Safety Act 40 of 2000 (RMAA, 2008).

2.9.1 Hygiene Assessment System

In the South African context, HAS is a tool, that is used in conjunction with the hygiene management system (HMS) to improve hygiene standards at abattoirs. The assessments are carried out using a HAS form (Figure 2.2) by hygiene managers (HM) at abattoirs and verified by provincial veterinary public health (VPH) inspectors. Has is a visual inspection of monitoring the 10 criteria on the HAS form to assess compliance to Meat Safety Act 40, 2000 (DoA, 2009). In South Africa, all animals presented for slaughter at any registered abattoir are examined before slaughter, anter mortem inspection. Antermortem is the first line of defence in protecting the public from potentially harmful meat products by accepting and allowing for slaughter only those animals that are healthy and capable of being converted into wholesome products for consumers (Du Preez, 2009). HAS monitors hygiene and animal welfare status of all registered abattoirs in South Africa to maintain uniform standards of hygiene performance (DoA, 2009). Abattoir operatives at dehiding and evisceration are subjected to training offered by RMAA to ensure that bacterial populations from the hide and intestinal contents are prevented from being transferred onto animal carcasses. Some South African abattoirs that export meat and meat products to the European Union member states, in addition to the use of HAS, have also implemented the Hazard Analysis Critical Control Point (HACCP) system.

ABATTOIR:_____ ABATTOIR NO: _____ GRADE :_____DATE: _____

DAILY THROUGHPUT: C _____P _____S _____ Other_____

INSPECTION COMPONENT

RANK	NAME	DESIGNATED YES	DESIGNATED NO	EMPLOYER
VETERINARIAN:				
MEAT INSPECTORS:				
MEAT EXAMINERS:				
MEAT CLASSIFIER				

OWNER / MANAGER

NAME	CAPACITY	ADDRESS / PHONE / FAX

HYGIENE MANAGER

NAME	CAPACITY	ADDRESS / PHONE / FAX

HAS - SCORE SHEET

CATEGORY	CATEGORY SCORE	WEIGHT	WEIGHTED SCORE
A. ANTE MORTEM		.10	
B. SLAUGHTERING AND DRESSING		.20	
C. MEAT INSPECTION / MARKING		.15	
D. CHILLING / DISPATCH		.20	
E. OFFAL PROCESSING		.05	
F. SANITATION / PEST CONTROL		.10	
G. PERSONNEL		.10	
H. GENERAL CONDITIONS		.05	
I. RECORDS		.05	
	FINAL	SCORE	

VERIFICATION BY PROVINCIAL INSPECTOR

Date:.....

Signature:.....

Comments:.....

.....

.....

.....

.....

	A 10	B 20	C 15	D 20	E 5	F 10	G 10	H 5	I 5	FINAL SCORE	
100											EXCELLENT
95											
90											
85											GOOD
80											
75											
70											FAIR
65											
60											
55											POOR
50											
45											
40											BAD
35											
30											
25											CRITICAL
20											
15											
10											
5											

Figure 2.2: A HAS form used at South African abattoirs (Du Preez, 2009)

2.9.2 Hazard Analysis Critical Control Point

HACCP was jointly developed in the USA by the Pillsbury Corporation and the United States Army Laboratories as a system that would provide a degree of certainty that food was free from pathogens and toxins (Crossland, 1997). HACCP identifies the potential hazards (physical, chemical or microbiological) in the process and then designs the process and control systems to minimize the risks. The implementation of an HACCP system is based on seven principles: conduct a hazard analysis; identify the Critical Control Points (CCP); establish the critical limits; establish monitoring systems; establish corrective action; establish documentation concerning all procedures and records appropriate to these principles and their application; and establish verification procedures (Codex Alimentarius Commission, 1997).

HACCP is a preventative control system wherein hazards are identified, critical control points (CCPs) are determined and the methods for control and compliance are clearly specified (Kinsella, Sheridan & Rowe, 2006). International Standards Organization (ISO) 22000 and most other HACCP guides, specify that there are other prerequisites necessary before HACCP plans should be developed, including appropriate sanitation and hygienic practices and assembly of a multidisciplinary HACCP team, identification of products , process flow diagram, and controls already practiced. The decision tree technique should be used to identify CCPs followed by the prescription of corrective measures that should be implemented to control biological hazards. Misidentification of CCPs in a HACCP plan may render the prescribed standard operating procedures ineffective, resulting in an HACCP system that may give variable and inadequate control over microbiological conditions of raw meat (Bryant, Brereton & Gill, 2003). Finally, the implementation of HACCP systems at abattoirs has to be preceded by the establishment of microbiological data specific to the abattoir for the objective assessment of risks. Wagude (1999) compared the levels of bacterial contamination before and after the implementation of HACCP at a South African abattoir. After the implementation of HACCP, the author reported a reduction in the incidence of *Staphylococcus aureus* and *Salmonella* spp. after the carcass splitting step and further reductions in *S. aureus* count after chilling.

However, total bacterial counts and *Escherichia coli* counts remained similar after the implementation of HACCP. The author attributed most contamination of carcasses to a lack of training of abattoir operatives. Wagude (1999) reported that the unhygienic practices were aggravated by the link between the number of animals processed and the renumeration of operatives that resulted in the line speed being high.

A foodborne outbreak in the USA in late 1992, which was linked to the consumption of undercooked beef patties, triggered the introduction of stringent regulatory requirements by the United States Department of Agriculture (Eustace & Vanderlinde, 1999). The Pathogen Reduction Final Rule, gazetted in 1996, required the implementation by all meat and poultry processing plants of sanitation standard operating procedures, the adaptation of HACCP programmes and sampling of carcasses for generic *E. coli* and *Salmonella* for HACCP verification as part of the Rule. Furthermore, performance standards for those organisms were established for most slaughter classes. Although the final rule does not mandate decontamination treatments (acid washes, chemical dehairing, steam vacuuming, trimming, hot water spray washes and steam pasteurization), a large number of USA meat packers use these decontamination interventions. According to Smulders and Greer (1998), one of the advantages of the application of organic acids is that residual antimicrobial activity is demonstrable over extended periods of storage.

Contrary to the USA, the European Union advocates strict control of processing hygiene, to ensure the safety of meat and meat products. The European Union authorities view the inclusion of decontamination intervention as a way of masking evidence of inadequate hygienic processes (Eustace & Vanderlinde, 1999). As a result, exporting countries to the European Union, including South Africa could not use chemical decontamination interventions during animal slaughter, dressing and processing of primal cuts. However, according to the European Directive EC/471/2001, all red meat slaughterhouses within the European Community are obliged to operate according to HACCP principles (European Union, 2001). As a result of food crises and international trade requirements, the international community has had to review its food safety laws, with the sole intention of providing the highest

level of protection of human health (Hugas & Tsigarida, 2008). Similarly, at European level, Regulation No. 853/2004, which was brought into force in 2006, permits the use of substances other than potable or clean water to remove microbial surface contamination from foods of animal origin. However, the European Commission authorises the use of such substances only after the European Food Safety Authority (EFSA) has provided chemical and microbiological risk assessments.

2.10 COMMON CONTROL MEASURES USED AT HAS ALONE AND HAS AND HACCP ABATTOIRS IN SOUTH AFRICA

The South African meat industry employs trimming of visible contamination with sterile knives and chilling to control microbial growth on carcasses (RMAA, 2002). As an additional measure, one abattoir also uses spot steam vacuuming to remove localized faecal and ingesta contamination on carcasses.

2.10.1 Hot water sanitation of slaughter equipment

One common practice at most meat facilities, including those in South Africa, is to sanitize meat-cutting equipment (knives, neck splitters, bung tiers and saws) by dipping it into containers of hot water (82 °C) adjacent to processing lines to reduce the carcass-to-carcass spread of pathogenic and spoilage bacteria. Gill and McGinnis (2004) demonstrated the potential of tools used for carcass dressing to contaminate carcasses during slaughter and dressing. However, the presence of organic materials on slaughtering equipment reduces the antimicrobial activity of hot water. Hot water tends to coagulate protein, which allows organic material to adhere to equipment surfaces and leads to a greater difficulty in removing meat residues. Taormina and Dorsa (2007) found that brief (1 s) dip treatments of slaughter equipment had limited efficacy, compared to longer immersion time (5 s).

2.10.2 Trimming of visible contamination

During animal slaughter, contaminated carcasses are transferred from a processing to a detaining rail where visible contamination is removed a procedure called trimming. Trimming is an on-line process used to remove excess fat, small faecal spots and smears from beef (Sheridan, 2007). Trimming, which removes enteric pathogens associated with the contaminating matter (Bacon *et al.* 2000), is followed by visual inspection to ensure that contamination has been adequately removed after which the trimmed carcasses are returned to the processing line. Since, in South Africa, decontamination treatments such as acid washes are not used visible contamination is removed by trimming, followed by cold-water washing. Subsequently, carcasses are sent to the coolers. Gill and Landers (2004) documented the effectiveness of the trimming of visibly contaminated carcasses on the reduction of both total bacterial counts and of *E. coli* counts on beef carcasses.

2.10.3 Steam vacuuming

Steam vacuuming uses steam and/or hot water to loosen soil and kill bacteria. Then the application of a vacuum removes the wastewater and contaminants (Bolton, Doherty & Sheridan, 2001; Meat Industry Services, 2006). Steam vacuuming equipment is a hand-held device consisting of a vacuum wand with a hot spray nozzle delivering water at 88–94 °C to the carcass surface under pressure while simultaneously vacuuming the area (Dorsa, Cutter & Siragusa, 1996). Steam continually sanitises the device (Bolton *et al*., 2001). According to Kochevar, Sofos, Bolin, Reagan and Smith (1997), steam vacuuming reduced the mean aerobic plate counts (APC) and total coliform counts (TCC) by 0.72 and 0.26 $\log_{10}$ cfu/cm^2 respectively, from carcasses which did not have visible faecal contamination. A combination of knife trimming and steam vacuuming showed a higher reduction of the mean APC and TCC from visibly contaminated carcasses, by 1.38 $\log_{10}$ cfu/cm^2 and 1.59 $\log_{10}$ cfu/cm^2, respectively.

The effectiveness of steam vacuuming depends on the diligence of application of the operative and the operational status of the equipment. For example, Kochevar *et al.* (1997) observed increased reduction in APC after the operatives had been sufficiently trained in operating the steam-vacuuming device. Moreover, the curvature of some surfaces may make proper contact with the vacuum head difficult which can reduce the effectiveness of the treatment. Furthermore, at least 10 s is required for a pasteurising effect and operatives on-line may not have sufficient time for the job (Bolton *et al*., 2001). Bolton *et al*. (2001) recommended critical limits for steam vacuuming systems (Table 2.1).

Table 2.1: Critical limits for steam vacuuming systems (Bolton *et al*., 2001)

Water temperature	≥82 °C
Water pressure	3.4–10.3 Pa
Air vacuum	-0.093 Pa
Steam pressure	20.7–34.5 Pa
Area decontaminated	No more than 2.5 cm^2

2.10.4 Chilling

During animal slaughter, carcasses are pl aced in the chillers immediately after the final wash until the temperature of the deep round reaches 7 °C or lower to retard bacterial growth. Carcass chilling controls bacterial growth via extrinsic (temperature, relative humidity (RH), air speed and carcass spacing) and intrinsic factors water activity (a_w). Chilling is monitored by checking the deep round temperature of a number of randomly selected carcasses per rail in the chillers. There have been different reports on the effectiveness of chilling in controlling bacterial growth on beef carcasses. According to McEvoy, Doherty, Sheridan, Thomson-Carter, Garvey, McGuire, Blair and McDowell (2003), chilling is effective in controlling the growth of bacterial pathogens on beef carcass surfaces. In their study, three carcasses tested positive for *E. coli* O157:H7. After chilling, McEvoy *et al*. (2003) observed a change from positive for *E. coli* O157:H7 before chilling to negative after chilling on one beef carcass, whereas, with the other two carcasses,

there was an increase in the prevalence of *E. coli* O157:H7. These authors suggested three reasons for the negative result, namely, the irreversible attachment of bacteria to meat surfaces, the effect of chilling and probably the failure of the sampling method to recover the organism from the meat surface.

Chilling may stress bacterial cells because of the synergistic effect of low water activity and temperature. The low detection of *E. coli* O157:H7 from carcasses after chilling could be a subject of the analytical method used. For example, the presence of bile salts and antibiotics in the enrichment media (Hara-Kudo, Ikedo, Kodaka, Nakagawa, Goto, Masuda, Konuma, Kojima & Kumagai, 2000; Stephens & Johnson, 1998) may inhibit the growth of injured cells, resulting in the false presence of *E. coli* O157:H7. Nevertheless, McEvoy *et al*. (2003) observed an increase in *E. coli* O157:H7 prevalence on carcasses after chilling, which they conclude was a result of cross-contamination during chilling. In another study, Kinsella *et al.* (2006) did not find chilling effective in reducing the levels of artificially inoculated *Salmonella typhimurium* on beef after 24 h chilling. These authors recorded only 1 log or greater reduction in *S. typhimurium* count after 72 h. In general, the maximum chilling period of carcasses is 24 h to avoid weight loss (Kinsella *et al.,* 2006).

2.11 COMPETITION BETWEEN SPOILAGE AND FOOD PATHOGENS DURING BEEF STORAGE

2.11.1 Bacterial attachment to meat

Meat is a complex food ecosystem of which the chemical and physical properties can allow the colonization and development of a great number and variety of organisms (García-López, Prieto & Otero, 1998; Holzapfel, 1998). Bacterial attachment is probably the first step in the contamination of solid surfaces. Attachment is generally considered to be a two-stage process. The first stage is reversible attachment that occurs when the bacteria are trapped in a water film on the contact surface. The second stage is irreversible attachment that occurs as the bacteria form a more permanent physical attachment (Davies & Geesey, 1995). Benito *et al.* (1996)

demonstrated that attachment of LAB to beef muscle was influenced by both the immersion time and the cell concentration in the adhesion medium. Interestingly, with *E. coli* O157:H7, attachment to beef surfaces was rapid, occurring within the first few minutes of incubation with little increase in attachment occurring when exposure times were extended (Fratamico, Schultz, Benedict, Buchanan & Cooke, 1996). Bacterial surface structures that assist their adhesion to surfaces include outer membrane proteins, capsular polysaccharides, lipopolysaccharides, curli, non-hemagglutinating pili, fibrillae and flagella. However, Chen, Rossman and Pawar (2007) did not observe major differences in attachment by curliated and noncurliated cells of *E. coli* O157:H7 to beef.

2.11.2 Factors influencing growth of bacteria on meat

The shelf life of meat depends on the number and type of bacteria initially present and their further growth in the ecological conditions applied during storage, particularly the storage temperature, pH and gaseous atmosphere (Russo *et al.*, 2006). Meat stored aerobically at chill temperatures is dominated by *Pseudomonas* spp. (Dainty & Mackey, 1992; Gill & Newton, 1978), while the typical microbiota of vacuum-packed fresh meat products stored at chill temperatures consists of LAB and Enterobacteriaceae at levels of 10^8 and 10^6 $\log_{10}$ cfu/g, respectively (Sutherland, Patterson & Murray, 1975).

When different microbial species live in the same environment they certainly influence each other and in the case of meat, the interactions between microbial groups during storage play an important role in both the development and prevention of spoilage. This antagonistic activity is defined as antibiosis. Russo *et al*. (2006) demonstrated antagonistic activity of LAB against *Brochothrix thermosphacta* in vitro at 5 °C for 7 days. The authors concluded that the LAB antagonistic activity was not due to bacteriocins but rather to the effect of decreased pH and competition for substrate, as substrate competition and antagonism are important in the selection of microflora in any given ecological niche (Fredrickson & Stephanopoulos, 1981).

2.12 BACTERIAL INTERACTIONS ON MEAT

Competition between microflora is an effective way to preserve food and guard against pathogenic microbial growth (Sun & Ockerman, 2004). The inhibition of pathogens can be due to the production and excretion of substances that are inhibitory or lethal to other microbial cells, competition for attachment/adhesion sites, rendering the environment unfavourable/undesirable to other microorganisms, competition for oxygen and other nutrients or combinations of the above (Raghubeer, Campbell & Meyer, 1994). Jay (1996) suggested that low levels of background organisms do not have much influence on pathogens. Jay (1996) hypothesized that increasing levels of background flora may inhibit the survival of pathogenic microorganisms on meat due to microbial interactions among these microorganisms. Jay (1996) suggested protecting carcasses with appropriate mixtures of harmless bacteria, which may compete by occupying attachment sites, thereby reducing colonization by pathogens.

2.12.1 Interactions between *P. fluorescens* and *E. coli* O157:H7 when grown together on foods

Pseudomonas species is a spoilage causing bacterium present in a variety of food environments and foods (Robinson, Batt & Patel, 2000; Shaw & Latty, 1982). In the dairy industry, *P. fluorescens* is one of the most commonly isolated psychrotrophic bacteria that dominate the microflora of pasteurised milk at the time of storage (Ogier, Son, Gruss & Tailliez, 2002; Reddy, Bills, Lindsay, Libbey, Miller & Morgan, 1968). *E. coli* has been used as an indicator of pathogenic bacteria in beef products (Ray, Johnson & Field, 1984) and is also used as a faecal indicator because of its close association with intestinal waste materials (Beerens, 1998).

Pseudomonas spp. use several competitive mechanisms including bacteriocin production i.e. pyocin from *P. aeroginosa*, a phenazine antibiotic and non-nitrogen containing compounds and siderophore-mediated competition for iron (Henry, Lynch & Femor, 1991; Leong, 1986). They preceded other meat borne bacteria in the utilization of glucose, amino acids and lactic acid on fresh meat medium (Gill &

Newton, 1978). Sun and Ockerman (2004) used different levels (zero, 2 and 4 $\log_{10}$ cfu/ml) of *P. fluorescens* to investigate its antagonism and inhibition of *E. coli* on sterile ground beef. These authors used similar levels of *E. coli* and the inoculated samples were incubated at 4 and 25 °C for 14 days and 30 h, respectively. *P. fluorescens* showed an extended lag phase whereby growth was observed only at day 7 for 2 log treated samples and at day 10 for 4 log treated samples.

2.12.2 Interactions between LAB and *E. coli* O157:H7 when grown together on foods

LAB are known to have inhibitory effect against the growth of a wide range of foodborne pathogens (Lewus, Kaiser & Montville, 1991). Kalalou, Zerdani and Faid (2010) investigated the interaction between LAB and *E. coli* O157:H7 on camel meat. In their study these authors observed the growth of *E. coli* O157:H7 when inoculated alone and when inoculated with LAB on vacuum packaged camel meat stored at abusive temperature (10 °C) for 4 days. The LAB counts of inoculated samples dropped followed by the development of spontaneous lactic flora. Towards, the end of vacuum storage, *E. coli* counts were lower than those in the control (Lewus *et al.,* 2010).

E. coli O157:H7 has been associated with produce in 21% of the foodborne outbreaks that occurred between 1982 and 2002 (Amêzquita & Brashears, 2002). In the mid 1990s, fresh produce was recognized as a vector for foodborne illnesses caused by *E. coli* O157:H7 (Deliquis & Dinu, 2007). Gragg, Brook and Brashears (2010) compared the efficacy of LAB and water on the inhibition of *E. coli* O157:H7 on fresh spinash stored at 7 °C for 24 hours. Water alone reduced the level of *E. coli* O157:H7 by 0.88 $\log_{10}$ cfu/g, while the LAB cocktail showed a reduction of 1.03 $\log_{10}$ cfu/g. Gragg *et al*. (2010) concluded that LAB treatment was the most effective intervention in controlling *E. coli* O157:H7 in fresh produce.

2.13 INFLUENCE OF STORAGE CONDITIONS OF MEAT ON ITS MICROBIOTA

Meat is a good support of bacterial growth due to its composition: 75% water and many different metabolites such as aminoacids, peptides, nucleotides and sugars (Labadie, 1999). It is, however, a relatively poor source of sugar for bacteria, but an important source of proteins (Labadie, 1999). The types and proportion of bacteria growing on meat during storage result from the type of contamination introduced by the processing of meat and from the physico-chemical factors applied during storage. These factors (temperature, pH, nutrients, A_w and composition of the atmosphere) constitute hurdles that play a crucial role in the activity and growth of microorganisms. Following attachment, packaging influences the microflora of meat during storage (Chenoll, Macián, Elizaquível & Aznar, 2007).

2.13.1 Microbiota of aerobically chilled stored beef

In aerobically stored meats, milk, fish and vegetables, members of the *Pseudomonas* genus are the microorganisms that rapidly dominate the flora. Although the species that grow on different food products are often different they share important common features (Labadie, 1999). When bacteria coexist within a given ecological niche, substrate competition and antagonism are important in the selection of the resultant microflora (Gram, 1993). Gram (1993) demonstrated the antibacterial activities of 209 *Pseudomonas* strains isolated from spoiled and fresh fish in agar assays against organisms including food pathogens. The antimicrobial activity was more pronounced in strains that produced siderophores. However, the addition of iron to the substrate resulted in the elimination of antibacterial activity of two-thirds of those strains. Therefore, the inhibitory activity of those strains may have been siderophore-mediated competition for iron.

In another study Jurkevitch, Hadar and Chen (1992) suggested that the sequestering of iron by *Pseudomonas* spp. was an advantage to other bacteria that were able to utilize the siderophores. For example, Gram (1993) observed dense growth of *L.*

monocytogenes and *S. aureus* around the wells containing *Pseudomonas* spp. Gram (1993) then suggested that antagonistic *Pseudomonas* spp. could have created a more advantageous nutritional composition, either through the supply of iron (Jurkevitch *et al*., 1992) or by increasing the availability of low-molecular weight nutrients (Cousin & Martha, 1977). In addition, even among the *Pseudomonas* species competition exists. For example, *Pseudomonas fragi* does not synthesize pyoverdine (the green fluorescent pigment) but is able to synthesize its receptors on the outer membrane. This feature enables *P. fragi* to compete for *sidorephore* produced by the growth of other *Pseudomonas* species (Labadie, 1999).

2.13.1.1 Effect of storage temperature on the competitive activity of P. fluorescens on beef

Low storage temperature increases the lag phases of both spoilage and pathogenic bacteria on meat (Sun & Ockerman 2004). Sun and Ockerman (2004) investigated the influence of different levels of *P. fluorescens* on the growth of *E. coli* O157:H7 when co-cultured on sterile meat. These authors used different inoculation levels (zero, 2 and 4 cfu/ml) for both bacteria and incubated the inoculated samples under 4 and 25 °C for 14 days and 30 h, respectively. Under low temperature storage (4 °C), Sun and Ockerman (2004) observed an expected interaction between storage time and the *P. fluorescens* level in the inoculum. *P. fluorescens* count increased as storage period increased. The capacity of *P. fluorescens* to grow faster than their competitors at temperatures below 2 and 15 °C is due to their ability to utilize glucose, amino acids and lactic acid (Gill & Newton, 1978). However, Sun and Ockerman (2004) also observed a slow growth rate by *P. fluorescens* with significantly ($p<0.05$) higher counts enumerated at day 7 for 2 log *P. fluorescens* group and at day 10 for log 4 *P. fluorescens* groups. The authors concluded that the extended lag phase was due to low incubation temperature. For the first 7 days of storage at 4 °C, *E. coli* counts remained the same for samples treated with both 2 and 4 $\log_{10}$ cfu/ml.

The *E. coli* O157:H7 growth curve showed a slight decline at day 7, followed by an increase in growth recorded at day 14. Low temperature could have been responsible

for the lack of growth shown by *E. coli* while decline in growth at day 7 could have been due to limited nutrient availability (Sun & Ockerman 2004). *P. fluorescens* have the ability to change their metabolism to utilize amino acids and lactic acid upon the depletion of glucose (Gill, 1996), therefore increasing availability of nutrients for *E. coli* to utilize. Moreover, oxygen could have been depleted due to high numbers of *P. fluorescens* recorded at day 14, creating a favourable environment for *E. coli*. Hence there was a recorded subsequent increase in *E. coli* counts at day 14.

At higher storage temperature (25 °C), *P. fluorescens* counts increased and reached 10^7 $\log_{10}$ cfu/cm^2 by 20 h, while there was a gradual increase in the growth of *E. coli* O157:H7. The level of *P. fluorescens* in the inocula enhanced the growth of *E. coli* on ground beef stored at 25 °C by way of degrading meat, therefore providing available nutrients for *E. coli* O157:H7 (Jurkevitch *et al*., 1992). Gram (1993) observed that the addition of iron did not eliminate the antibacterial activity of all siderophore producing *Pseudomonas* spp. Therefore, Gram (1993) concluded that the activity of such strains could be attributed to the production of other bacterial inhibitors such as antibiotics or bacteriocins.

2.13.2 Micro biota of vacuum packaged chilled stored beef

Meat and meat products are frequently commercialized by packaging under vacuum or in modified atmosphere to extend their shelf life. Hurdle technologies are frequently used to extend the shelf life of meat. For example, the combination of vacuum packaging and chilling produces an environment where only those organisms able to grow at low temperatures in the absence of oxygen can grow (Jones *et al.*, 2008). Storage under vacuum restricts the oxygen supply, depending upon the permeability of packaging film and thus has a selective effect on the microbial population (Labadie, 1999). As the composition of the gaseous phase changes during vacuum storage, the microbiota undergoes selection towards CO_2-tolerant growing species such as *Carnobacterium*, *Lactobacillus* and *Leuconostoc* species (Chenoll *et al*., 2007). Chenoll *et al.* (2007) did not detect LAB from samples analysed close to the date of packaging, but towards the end of storage LAB counts reached 10^8 $\log_{10}$

cfu/g. Similarly, Jones (2004) had also observed an increase in LAB count at the end of vacuum-packaged storage (16 weeks) compared to the beginning of storage. Furthermore, Jones (2004) observed succession dynamics of LAB on chill-stored vacuum-packaged beef, which was correlated with the production of fermentation products. This author concluded that succession dynamics could be used in developing strategies to improve meat storage properties (Jones, 2004). LAB competes by producing metabolites that may inhibit the growth of their competitors (Ouwehand & Vesterlund, 2004).

2.13.2.1 Low molecular weight acids, lactic and acetic acids produced by LAB

LAB organisms have the ability to inhibit the growth of spoilage bacteria, and occasionally pathogens such as *S. aureus* and *L. monocytogenes* (Schillinger & Lücke, 1989; Stiles & Holzapfel, 1997), yet they also contribute to flavour development and the preservation of fermented sausages (Hammes, 1986; Liepe, 1983). In most cases, the formation of starter-derived inhibitors such as lactic and acetic acids from carbohydrates and the resulting decrease in pH are responsible for the antagonistic effect (Schillinger & Lücke, 1989) coupled with competition for nutrients (Ray & Daeschel, 1992). Fermentation reduces the amount of available carbohydrates and results in a range of small molecular mass organic molecules that exhibit antimicrobial activity, the most common being lactic, acetic and propionic acids (Blom & Mörvedt, 1991). Thus, the type and amount of fermentative products produced differ among LAB species. For example, in an anaerobic environment, homofementative LAB produces only lactic acid from glucose whereas heterofermentative species produce a variety of organic acids, ethanol and carbon dioxide (Stamer, 1976). LAB is used widely as starter cultures in the manufacture of dairy, meat and vegetable products.

When a mixture of acids is present in a food ecosystem, lactic acid contributes mainly to the reduction in pH, while acetic and propionic acids, which become dissociated, are the actual antimicrobial agents. Furthermore, in the presence of oxygen (O_2), LAB also generates hydrogen peroxide (H_2O_2), carbon dioxide and diacetyl. The

bactericidal effect of H_2O_2 is due to the scavenging of O_2 during some H_2O_2 producing reactions, which creates an anaerobic environment that is unfavourable for certain organisms (Egan, 1983; Ouwehand & Vesterlund, 2004).

2.13.2.2 Other low molecular weight antimicrobial substances produced by LAB

LAB produce a variety of antagonistic factors that include metabolic end products, antibiotic-like substances and antibacterial proteins called bacteriocins (Cleveland *et al.*, 2001; Klaenhammer, 1988) and protect themselves from desiccation, bacteriophages and protozoan attack by the production of exopolysaccharide substances (Axelsson, Chung, Dobrogosz & Lindgren, 1989). Bacteriocins are ribosomally synthesized, a heterogeneous group of biologically active proteins or protein complexes. They display bactericidal activity towards closely related species (Oliveira, Oliveira & Glória, 2008; Tagg, Dajan & Wanamaker, 1976). Bacteriocins produced by LAB differ in molecular weight, genetic origin and biochemical properties (Abee, Kröckel & Hill, 1995) and are sensitive to proteolytic enzymes such as pepsin and trypsin.

When proteolytic enzymes are added to bacteriocins, the latter lose their inhibitory effect (Chang, Lee & Chang, 2007; Lash, Mysliwiec & Gourama, 2005). The mode of action of bacteriocins may include the disruption of the membrane integrity of the target organism (Kramer *et al.*, 2004) or the disruption of enzyme functions (Kussendrager & Van Hooijdank, 2000). For example bacteriocins can inhibit the cell wall synthesis of the target cell (McAuliffe, Ross & Hill, 2001). Class IIa bacteriocins show strong antilisterial activity. They have a narrow inhibitory spectrum and thus do not kill starter cultures (O'Sullivan, Ross & Hill, 2002). Class III bacteriocins have only been isolated from members of the genus *Lactobacillus* (Klaenhammer, 1993).

2.13.3 Production of bacteriocins by *L. plantarum*

L. plantarum produces an antibacterial agent that inhibits the growth of some gram-negative bacteria including *E. coli* and gram-positives including *S. aureus* (Fricourt, Barefoot, Testin & Hayasaka, 1994; Lash *et al*., 2005). Lash *et al*. (2005) confirmed that the inhibitory substance produced by *L. plantarum* was a bacterion. These authors assessed the antimicrobial activity of that compound against a range of bacteria representing both gram-positive (*Staphylococcus aureus*, *Listeria innocua*, *Staphylococcus epidermidis*, *Micrococcus luteus)* and gram-negative bacteria (*E. coli, P. aeruginosa*, *Serratia marcescens*, *Shigella flexneri* and *S. typhimurium*) and also determined the effects of both changes in temperature and pH, its molecular weight and the effects of proteolytic enzymes on the inhibitory substance.

Lash *et al.* (2005) observed a greater inhibition of gram-negative, measured as more than 90% compared to the control, an unusual observation because inhibitory effects of gram-positive strains on gram-negative bacteria are less prevalent. In addition, the antimicrobial agent also inhibited Gram-positives although to a lesser extent at less than 90% compared to Gram-negatives. This compound exhibited a wide-range inhibitory effect on non-taxonomically related species including food-borne pathogens. *L. innocua*, used in the food industry as an indicator of potential *L. monocytogenes* contamination was also inhibited (Lash *et al*., 2005).

The characterization of this compound revealed that its inhibitory effect was heat labile, losing activity when the temperature was raised to 30 °C, and also lost at pH values below 4 and above 5, indicating a narrow pH range for inhibitory activity. Lash *et al*. (2005) established that the inhibitory compound had a large molecular weight, which they used to explain both the heat sensitivity and narrow pH activity. These authors suggested that due to the size of the compound, it could have been more rapidly degraded by subtle changes in temperature and pH.

2.13.4 Activity spectrum of bacteriocins

Major classes of bacteriocins produced by LAB include: lantibiotics, small heat stable peptides, large heat labile proteins and complex proteins whose activity requires the association of carbohydrate or lipid moieties (Klaenhammer, 1993). Bacteriocins of LAB are generally considered to be active only against gram-positive bacteria and not gram-negative ones and yeasts (Abee *et al*. 1995; Jack, Tagg & Ray, 1995; O'Sullivan *et al*., 2002). The inability of bacteriocins to act against gram-negative bacteria could be due to the fact that gram-negative bacteria possess an additional layer, the outer membrane (OM). This OM is composed of phospholipids, proteins and lipopolysaccharides (LPS) and functions as a permeability barrier that is able to exclude macromolecules such as bacteriocins or enzymes (Gong, Meng & Wang, 2010). The porins on the OM allow the free diffusion of molecules with a molecular mass below 600 Da. The smallest bacteriocins produced by LAB bacteria are approximately 3 kDa and are thus too large to reach their target, the cytoplasmic membrane (Klaenhammer, 1993; Stiles & Hasting, 1991). However, lactic acid is a disintegrating agent that causes LPS release, therefore permeabilizes the OM allowing bacteriocins and enzymes to effectively penetrate the Gram-negative bacterial OM (Alakomi *et al.*, 2000). These compounds kill the target cell by interference with energy transduction occurring at the cytoplasmic membrane and also inhibit biosynthesis processes of macromolecules such as DNA, RNA, proteins and polysaccharides (Sahl & Brandis, 1982). Héchard and Sahl (2002) suggested that some bacteriocins may interact with the cytoplasmic membrane leading to both pore formation or localised disruption of the membrane.

Several researchers have reported action of bacteriocins from LAB against Gram-negative bacteria and or yeasts, in combination with pH values below 5.5 (Gänzle, Weber & Hammes, 1999; Lash *et al*., 2005; Messi, Bondi, Sabia, Battini & Manicardi, 2001). In addition, Schillinger and Lücke (1989) demonstrated the inhibitory activity of the bacteriocins to lactic acid bacteria only, with the exception of *L. monocytogenes.* These authors attributed the apparent sensitivity of *L. monocytogenes* to bacteriocins as due to the fact that taxonomically, *Listeria* is closely

associated to the genus *Lactobacillus*. These authors reported that the activity of the antibacterial compound produced by *L. sake* was destroyed by protease treatment (trypsin and pepsin) but was resistant to heat. There was no reduction in antibacterial titer after heating for 20 minutes at 100 °C. These authors then concluded that *L. sake* antibacterial compound was a heat-stable peptide. Several LAB bacteriocins offer potential applications in food preservation and the use of bacteriocins can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties (Gálvez, Abriouel, López & Ben, 2007).

2.13.5 Role of bacteriocin-sensitive LAB strains on the bacteriocin production of other LAB strains

LAB produces bacteriocins in an environmentally dependent manner (Anderssen, Diep, Nes, Eijsink & Nissen-Meyer, 1998; Barefoot *et al.*, 1994; Biswas *et al.*, 1991; Chang *et al*., 2007; De Vuyst, Callewaert & Crabbé, 1996; Franz, Stiles & Belkum, 2000). Maldonado, Ruiz-Barba and Jiménez-Díaz (2003) demonstrated that the production of plantaricin NC8 by *L. plantarum* NC8 was induced in the presence of different gram-positive bacteria. In addition, in recent years, Chang *et al*. (2007) showed that kimchicin GJ7 (a bacteriocin produced by LAB isolated from a fermented Korean vegetable product) production was significantly enhanced by the presence of a sensitive *L. plantarum* strain. Temperature and pH also influence bacteriocin production (Oliveira *et al.,* 2008).

2.13.6 Effect of heat on bacteriocins

Oliveira *et al.* (2008) determined the influence of heat treatment and pH on the inhibitory effect of cell free supernatants (CFS) derived from selected LAB on *L. acidophilus*. *L. acidophilus* was found to be the most sensitive strain. Overall, the inhibitory compound present in the CFS were resistant to heat treatment at varying intensities. CFS retained 72% inhibitory activity after exposure to heat at 100 °C for 10 min. Moreover, CFS from some LAB isolates showed even increased heat

resistance by keeping 76 to 86% of original activity when heated at 100 °C for 30 min, while the inhibiting substance from different strains of *Pediococcus acidilactici* showed different resistance to heat treatment.

2.13.7 Effect of pH on bacteriocins

The CFS obtained from isolated LAB strains showed antimicrobial activity in a wide pH range (Table 2.2). Three LAB strains showed inhibitory activity up to pH 9. These results showed the possible application of LAB in food products with high pH like frankfurters. The combination of a wide pH range and high temperature tolerance of bacteriocins is a remarkable property to be used for biopreservation because many food procedures involve a heating step (Oliveira *et al.,* 2008).

Table 2.2: Influence of pH on the inhibitory activity of CFS of selected LAB isolated from vacuum-packaged beef (n=9) against *L. acidophilus,* using the agar well-diffusion test (Oliveira *et al.,* 2008).

pH	Diameter of inhibition zone (mm)/CFS from selected LAB isolates			
	Pediococcus acidilactici	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus casei*	*Pediococcus acidilactici*
4.0	10.1 ±0.7[a]	11.0±0.7[a]	11. ±30.6[a]	12.5±0.7[a]
5.0	9.8±1.2[ab]	10.0±1.2[b]	11. ±20.7[a]	12. ±01.5[a]
6.0	9.5±1.1[ab]	9.0±0.7[c]	11. ±01.0[a]	11.5±0.8[a]
7.0	9.2±0.9[ab]	-	9.9±1.0[b]	10.3±1.2[b]
8.0	8.9±0.7[b]	-	8.3±0.7[c]	8.6±0.4[c]
9.0	6.7±0.3[c]	-	6.6±0.2[a]	6.9±0.4[d]

- = no inhibition zone

Mean values (± standard deviation) with different letters in the same column are significantly different at $p<0.05$

2.14 HYPOTHESES

- Beef from high-throughput abattoirs where food safety systems are in place, will have reduced levels of pathogenic bacteria, compared to beef from similar abattoirs, but without such systems. During the implementation of a food safety system, microbial hazards are identified and reduced to an acceptable level by controlling critical control points, such as evisceration and chilling during the slaughtering process. Food safety systems minimize the incidence of pathogens on meat.

- Selected *E. coli* serotypes will be able to compete more effectively with spoilage bacteria such as *Pseudomonads* spp. and LAB on beef with low (10^2 $\log_{10}$ cfu/cm^2) spoilage bacteria contamination levels than on beef with high (10^6 $\log_{10}$ cfu/cm^2) spoilage contamination levels. High levels of spoilage microorganisms in a food matrix will out-compete pathogens. Pathogen growth rates and maximum densities are a function of the total microbial community composition and density in the food due to competition for nutrients, the production of inhibitory substance and overall density (Powell, Schlosser & Ebel, 2004).

2.15 OBJECTIVES

- To determine the incidence of total bacterial counts, *Pseudomonas* spp., Enterobacteriaceae, LAB *E. coli* serotypes, *Salmonella,* and *S. aureus* on fresh beef after chilling, from high throughput abattoirs with food safety systems and beef from high throughput abattoirs without safety systems.

- To determine the relationship between different levels, high ($10^6\log_{10}$ cfu/cm^2) and low ($10^2\log_{10}$ cfu/cm^2) of spoilage bacteria, i.e. *Pseudomonas* spp. on the survival and growth of *E. coli* O157:H7 isolated from beef and inoculated at a constant level of $10^2\log_{10}$ cfu/cm^2 on beef stored aerobically for 7 days at 4 °C.

- To determine the relationship between different levels, high ($10^4 \log_{10}$ cfu/ cm^2) and low ($10^2 \log_{10}$ cfu/cm^2) of spoilage bacteria, i.e. lactic acid bacteria on the survival and growth of *E. coli* O157:H7 isolated from beef and inoculated at a constant level of 10^2 cfu/cm^2 on beef under vacuum storage for 2 months at 4 °C.

CHAPTER THREE:
EFFECT OF HYGIENE AND SAFETY MANAGEMENT SYSTEMS ON THE MICROBIOLOGICAL QUALITY OF FRESH BEEF

(Submitted for publication in the Journal of Environmental Health)

Abstract

The effect of the Hygiene Assessment System (HAS) alone and the HAS combined with Hazard Analysis and Critical Control Point (HAS + HACCP) on the bacteriological quality of beef in abattoirs were assessed. Sponge swab samples were collected from chilled beef carcasses for indicator organisms: Aerobic Plate Count (APC) and Enterobacteriaceae, and spoilage organisms, *Pseudomonas* spp. and lactic acid bacteria. Swabs were also collected for pathogenic bacteria: *E. coli* O157:H7, *Staphylococcus aureus* and *Salmonella* spp. For each management system, two abattoirs were assessed. There was no significant difference ($p<0.05$) between the microbiological quality of beef carcasses processed in the abattoirs with the HAS system and that of beef carcasses processed in abattoirs with combined HAS + HACCP. However, *E. coli* O157:H7 was isolated from carcasses processed in an abattoir with combined HAS + HACCP system. Although overall *S. aureus* counts at all abattoirs were comparable, the higher incidence (47% of carcasses) was obtained at an abattoir with combined HAS + HACCP. *Salmonella* spp. was not detected during the study, indicating that if the HAS system is effectively implemented and, perhaps, monitored by a third party, as done in South Africa, it can be effective in reducing the incidence of pathogens as well as in producing beef carcasses with acceptable microbiological quality.

Keywords: HAS, HACCP, quality, abattoir, beef

3.1 INTRODUCTION

In South Africa, particular attention has to be placed on food safety in the meat industry, due to risks posed by emerging food pathogens such as *Escherichia coli* O157:H7. *E. coli* O157:H7 is primarily associated with the consumption of contaminated ground beef and is an important food safety concern worldwide (MacRae, Rebate, Johnston & Ogden, 1997). Human infections with this pathogen can lead to a wide range of clinical illnesses, including asymptomatic shedding, non-bloody diarrhoea, haemolytic uraemic syndrome and death (Mead & Griffith, 1998). Infection with *E. coli* O157:H7 poses particular dangers to vulnerable groups such as children, the elderly and those with compromised immune systems (Paton & Paton, 1998a). Sub-Saharan countries, like South Africa that have high proportions of vulnerable individuals resulting from high HIV/AIDS infections (Statistics South Africa, 2009) need to strengthen practices that prevent contamination of foodstuffs with pathogenic bacteria. Food pathogens can arise at any stage of the food chain, from primary production through to consumption (Jaykus, Woolridge, Frank, Miraglia, Mcquatters-Gollop, Tirado, Clarke & Friel, 2008). There is a need to evaluate the effectiveness of current hygiene management systems, used during animal slaughter in preventing or reducing the risk of food pathogens entering and proliferating in the food chain.

The slaughter process for cattle and other meat-producing animals involves the removal of bacteria-free meat from between two contaminated surfaces, the hide and gastrointestinal (GI) tract (Buege & Ingham, 2003). The removal of the hide during slaughter is one of the main steps in slaughtering that may influence the level of contamination on carcasses. Hides carry environmental and faecal microorganisms (Bouttier *et al.,* 1994; Doyle, 2002) that can be spread to meat during slaughter and further processing (Bolder, 2007). Newton, Harrison and Wauters (1978) reported that the bacterial load they recovered on beef and sheep carcasses was a constant fraction, 0.3% of those on the hides. Other factors affecting the microbiological quality of carcasses are the design of abattoirs, equipment, which does not always allow thorough cleaning and disinfection, aerosols or condensation forming on

equipment and ceilings (Bolder, 2007; Shale, Jacoby & Platjies, 2006). Too high slaughter line speeds may also enhance contamination of carcasses by providing insufficient time for slaughtermen to wash their hands, arms and aprons and sterilize equipment properly. Bell (1997) found that the level of microbial contamination on the hands of workers making the cut on the carcass after hide removal was similar to those recorded on the hide, which creates an opportunity for bacteria to be transferred from workers' hands to sterile carcasses. Once introduced to carcass surfaces, microorganisms adhere and are subsequently difficult to remove (Bouttier *et al.,* 1994). From a hygiene and food safety point of view, the goal of the slaughter process is to minimize bacterial contamination of the carcass and effectively remove contamination (Buege & Ingham, 2003).

In South Africa, the majority of abattoirs use the Hygiene Assessment System (HAS) as a hygiene management system. HAS audits focus on 10 categories: ante mortem, slaughter and dressing, meat inspection/marking, chilling/dispatch, offal processing, sanitation/pest control, personnel, general conditions, maintenance and hygiene management. The South African HAS system uses a 5 to 100 scale, which is categorized into excellent, good, fair, poor and critical. Although some South African abattoirs are certified for HACCP, the HACCP system implemented in abattoirs in South Africa lacks a decontamination step, such as chemical rinses. This is because chemical rinsing of carcasses at the end of slaughter is perceived to mask unhygienic handling of carcasses during slaughter. Similarly, the European Union (EU) legislation views interventions requiring chemical decontamination as a means of concealing poor hygienic practices during slaughter (Bolton *et al.*, 2001). Notably, in January 2006, the EU Regulation (EC) No. 853/2004 permitted the use of substances other than potable water to remove microbial surface contamination from foods of animal origin (Hugas & Tsigarida, 2008). Rinsing beef carcasses with hot water reduces the levels of both spoilage and pathogenic bacteria on carcass surfaces (Castillo, Lucia, Goodson, Savell & Acuff, 1998). Castillo *et al.* (1998) inoculated beef cuts with bovine faeces containing 10^6/g each of rifampicin-resistant *E. coli* O157:H7 and *S. typhimurium*, or with uninoculated bovine faeces. Contaminated surfaces then were exposed to a carcass water wash or a water wash followed by hot

water spray (95 °C). Counts of rifampicin-resistant *S. typhimirium* and *E. coli* O157:H7, APC and coliform counts were conducted before and after each treatment. All treatments significantly reduced levels of pathogens from the initial inoculation level of 5.0 $\log_{10}$ cfu/cm^2. Treatments including hot water sprays provided mean reductions of initial counts for *E. coli* O157:H7 and *S. typhimurium* of 3.7 and 3.8 log, APC reductions of 2.9 log, and coliform reductions of 3.3 log.

HAS, like HACCP, is based on prerequisite programs (PRPs) that are components of Good Hygienic Practices (GHP). HAS is a hygiene auditing tool of the PRPs that are used to assess performance using categories that are scored and weighted according to their importance in relation to carcass hygiene (Bolton *et al*., 2001). HAS inspections are a mandatory function of the Department of Agriculture (DoA) under the Directorate: Veterinary Public Health in South Africa. Each abattoir has permanent meat inspectors on the line. In addition, meat inspectors are dispatched periodically from the DoA to carry out HAS inspections at the abattoirs. However, HAS is based on visual inspection, where officials who assess the level of contamination on the carcasses in an abattoir use their individual judgement, which creates opportunities for human error.

HACCP systems identify all the steps throughout the chain where contamination may occur, including measures taken on the farm, the quality of animal feed and handling of animals from farm to the abattoir. In the abattoir the HACCP system seeks to identify all steps in the slaughtering process where biological hazards can either be minimized or eliminated during slaughter. These steps are referred to as critical control points (CCPs). The HACCP system, therefore, allows for quick corrective measures in those situations where a deviation from the set controls in the CCPs are noted, thus assuring the safety and quality of the final product (Pinillos & Jukes, 2008). In the USA, HACCP systems involve interventions such as the application of heat, organic acids or a combination of both to reduce bacterial contamination on carcasses (Bolton *et al.,* 2001). Non-intervention systems employ effective sanitary dressing procedures during slaughtering (Buege & Ingham, 2003).

In South Africa, implementing HACCP is not mandatory in abattoirs. Most of the abattoirs that are HACCP certified are exporting abattoirs and have implemented this system to meet export requirements, particularly establishing auditable and documented hygiene management systems. The last step in the slaughtering process in a South African abattoir is the final wash with chlorinated water. Visible contamination is removed by trimming and not by washing. Carcass washing does not remove contamination but redistributes bacteria from one area to another (Jericho, Bradley & Kozub, 1995). Some abattoirs, including one included in this study, also use vacuum steamers to remove visible faecal contamination on carcasses (Du Preez, 2009).

It is assumed that there would be more control at abattoirs that use the HAS + HACCP system thus reducing opportunities for contamination of carcasses during the process of slaughtering and resulting in carcasses of superior microbiological quality compared to carcasses from abattoirs that use only HAS. The aim of this study was therefore to assess the effectiveness of the combination of the HAS and HACCP system in preventing the microbiological contamination of beef carcasses during slaughter, as opposed to using HAS alone.

3.2 MATERIALS AND METHODS

3.2.1 Sampling protocol

Four high throughput abattoirs, designated A1, A2, B1 and B2 were selected (Table 3.1). The criterion used for selection was the food hygiene management system employed at the abattoir, which was either HAS or a combination of HAS and HACCP. Each abattoir was visited three times in summer. During each visit, five carcasses were selected randomly during slaughter, after evisceration and also after the final wash. Both sides of each of the carcasses were sampled. Another set of 20 samples were collected from the same abattoirs after a minimum of twelve hours of chilling. After stunning, each carcass is attached by a chain to a moving overhead rail that carries the carcass to successive processing stations, including bleeding, head

removal, dressing, splitting and evisceration, knife trimming and washing before chilling. Abattoir B1 uses steam vacuuming in addition to knife trimming to remove spot contamination on beef carcasses during slaughter.

Table 3.1: Details of the abattoirs included in this study

Abattoir	Hygiene management system	Location
A1	HAS	Free State Province
A2	HAS	North West Province
B1	HAS combined with HACCP	Gauteng Province
B2	HAS combined with HACCP	Gauteng Province

3.2.1.1 Sampling after chilling

Sponge swabs were collected from both sides of the selected carcasses. The right side of each carcass was swabbed with a moist 25 cm^2 Envirogen sponge (Analytical Diagnostics, USA). Prior to swabbing, the sponge swabs were moistened by placing them in a sterile stomacher plastic bag (Analytical Diagnostics, USA), followed by dispensing 10 ml sterile buffered peptone water (Analytical Diagnostics, USA) into the bags (one at a time). An additional 5 ml of BPW was added to the sponge after sampling to bring the total volume to 15 ml. The sponges were then gently moistened by massaging the plastic to ensure an even distribution of the peptone water. Sterile hand gloves (Analytical Diagnostics, USA) were used to remove the moistened swabs from the plastic bag. The area for swabbing on the carcass was delineated by using a 100 cm^2 sterile disposable plastic template (10 cm x 10 cm) (Analytical Diagnostics, USA) for each of the flank and the neck areas of the carcasses. At each sampling site, a moistened swab was rubbed vertically, horizontally and diagonally across the sampling site. As a result, each of the ten samples collected from each abattoir during each visit was a composite sample.

Subsequent to swabbing, each sponge swab was placed back into a sterile plastic bag and the remaining peptone water was used as a transport medium. Each plastic bag containing the swab was closed tightly to avoid spillages during chilled transport.

Another set of swab samples was collected from the left sites of each randomly selected carcass. The sponges were prepared using the same procedures as those mentioned above. The moistened swabs were then used to collect surface samples from the whole forequarter of the left side. Those samples were used to determine *Salmonella* spp. All the swab samples were packed into a cooler box containing ice packs to maintain a temperature of 4 ± 1 °C followed by immediate transportation to the destined laboratory, which was either the Agricultural Research Council Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, South Africa (SA) where they were examined for the presence of S*almonella* spp, or the microbiological laboratory at the Department of Food Science of the University of Pretoria, Pretoria SA, where the rest of the analyses were performed.

3.2.1.2 Sampling during the slaughtering process

Ten whole carcasses were selected randomly. Both sides of the whole selected carcass were used to collect samples at different steps during slaughter. Samples were collected after evisceration and also after the final wash. All samples collected from the right side were analysed for *E. coli* O157:H7 analysis and samples collected from the left side of the same carcass were analysed for other *E. coli* serotypes. After approximately 12 hours of chilling, an additional set of ten samples was collected from the same lot for the examination of the presence of *E. coli* O157:H7 after chilling. The same sampling procedure and transportation as previously described were used.

3.2.2 Sample preparation and bacterial analysis

Envirogen sponges (25 cm^2) used for collecting the swab sample were blended in 225 ml sterile BPW (CM0509, Oxoid, Hemisphere, England) using a stomacher (Seward 400, Seward, London, United Kingdom) to achieve an initial 1:9 dilution. Further dilutions were prepared in BPW and 0.1 ml aliquots plated onto media described below using the spread-plate method (Herbert, 1990).

3.2.2.1 Total aerobic counts and Enterobacteriaceae

For the enumeration of Total Aerobic Counts, Standard Plate Count Agar (CM0463, Oxoid, New Hampshire, UK) plates were incubated at 25 ± 1 °C for 72 hours. Violet Red Bile Glucose Agar (CM0485, Oxoid) was used for the enumeration of Enterobacteriaceae at 37 ± 1 °C for 24 hours.

3.2.2.2 Staphylococcus spp., Pseudomonas spp. and LAB

A twenty-five gram sample was homogenized in a stomacher (Seward 400) for 2 minutes in 225 ml sterile peptone-water (CM0509, Oxoid). Consecutive serial dilutions were prepared and 0.1 ml aliquots plated onto Baird-Parker (Biolab, Midrand, South Africa) plates using the spread-plate technique (Herbert, 1990). Plates were incubated at 35 ± 1 °C for 48 hours. All *S. aureus* colonies were confirmed using the Rapid Latex Agglutination Test. *Pseudomonas* Agar Base (CM0559, Oxoid) plates, supplemented with C-F-C Supplement (SR0103E, Oxoid) were incubated at 25 ± 1 °C for 24 hours for the enumeration of *Pseudomonas* spp. and MRS agar (Biolab) plates were used for the enumeration of lactic acid bacteria and incubated at 30 ± 1 °C for 48 hours.

3.2.2.3 Salmonella spp.

The swab sample was mixed with 225 ml buffered Peptone Water (CM0509, Oxoid), homogenised for 2 minutes and incubated at 37 ± 1 °C for 16 to 20 hours. The pre-enrichment broth culture (10 ml) was added to 100 ml of Tetrathionate Brilliant Green Broth (CM0263, Oxoid) and 100 ml of Selenite Cystine Broth (Oxoid), which were then incubated for 24 to 48 hours at 43 ± 1 °C and 37 ± 1 °C, respectively. A loopful of each enrichment broth culture was streaked onto Brilliant Green Agar (CM0263, Oxoid) and Xylose-Lysine-Desoxycholate Agar plates (CM0469, Oxoid). The plates were then incubated at 37 ± 1 °C for 24 to 48 hours (Arvanitidou, Kanellou,

Katsouyannopoulos & Tsakris, 2002). Positive and negative quality control organisms were included with each batch of samples tested.

3.2.2.4 E. coli serotypes

Samples were transferred into sterile buffered peptone water (CM0509, Oxoid BPW), a non-selective liquid medium for pre-enrichment at 37 ± 1 °C for ± 24 hours. A special quantity of the incubated sample suspension was transferred to a liquid standard strength selective medium followed by incubation at 44 ± 1 °C for 48 hours. The samples were examined for gas production after 48 hours. If gas was observed, an inoculum (0.1 ml) was transferred to a MacConkey agar plate (CM0109, Oxoid) streaked and incubated at 37 ± 1 °C for 24 hours.

Presumptive pathogenic-like colonies (smooth-colonies) were scraped off a MacConkey plate (MC0109, Oxoid) and suspended in 0.5 ml sterile distilled water in a microfuge tube. The suspension was boiled for 20 minutes to extract the DNA followed by centrifugation at the speed of 12,000 g, where g represents the relative centrifugal force (RCF) for 2 minutes to remove cell debris. The supernatant was tested by polymerase chain reaction (PCR) assay using primers specific for the four virulence determinants of Shiga toxin producing *E. coli* and the crude extract as a template (Paton & Paton, 1998b). Following DNA amplification, 15 µl aliquots of the PCR mix were analysed by submarine gel electrophoresis with 1.0 to 1.2% agarose gels containing 0.25 µg of ethidium bromide stain per ml. The samples were electrophoresed for 60 minutes at 100 V, visualized by ultra violet UV trans-illumination.

The presence of all four shiga-toxin genes (*stx 1, stx 2, eaeA* and *hylA*) or any of the four products of the molecular weights of 180 bp (*stx 1*), 255bp (*stx 2*), 384 bp (*eaeA*), and 534bp (*hylA*) were considered as *E. coli* and the absence thereof was considered as non-Shiga toxin-producing *E. coli*. The rough colonies were discarded as non-pathogenic *E. coli* colonies. Positive and negative quality control organisms were included with each batch of samples tested.

3.2.2.5 Detection of E. coli O157:H7

Pathogenic *E. coli* is identified based on its unique virulence properties; hence analytical procedure for these pathogens requires the isolation and identification of the organism as *E. coli* before testing for specific virulence traits. Plating of samples directly onto selective agar is not appropriate due to the additional complication of competing flora. The method of Chapman and Siddons (1996) was used. Briefly, 25 g Envirogen swab samples were pre-enriched in 225 ml of buffered peptone water (CM0509, Oxoid) supplemented with Vancomycin (8 mg/l), Cefixime (0.05 mg/l) and Cefsulodin (10 mg/l) (BPW-VCC). The antibiotics were used to inhibit the growth of gram-positive organisms. The enrichment broth suspensions were incubated at 42 ± 1 °C for 18–24 hours to resuscitate injured cells, followed by immunomagnetic separation (IMS), whereby Dynabeads coated with antibodies to *E. coli* O157 (Dynabeads anti-*E. coli* O157: Dynal, dynabeads, Dynal France, Compiegne, France) were used for the immuno-capture separation technique (IMS). The IMS procedure was performed according to the manufacturer's instructions using 1ml of a 6 h enrichment culture added to 20 µl of Dynabeads.

A volume of 50 µl resuspended Dynabead-bacterial complex was subcultured using the streaking technique onto *E. coli* O157 selective Sorbitol-MacConkey agar (CM0813, Oxoid) supplemented with Cefixime Tellurite (SR0172E, Oxoid), Cefixime Tellurite Sorbitol-MacConkey agar (CT-SMAC) followed by incubation at 37 ± 1 °C for 24–48 hours to yield isolated colonies. Characteristic colourless colonies on CT-SMAC were selected. Positive and negative quality control organisms were included with each batch of samples tested.

All suspect colonies were subcultured on CT-SMAC to confirm its non-sorbitol fermenting properties. Non-sorbitol fermenting colonies were examined for the presence of the genes coding for Stx1, Stx2, enterohaemolysin and *eaeA*. A loopful of these colonies was dispersed in 500 µl water and boiled at 99 ± 1 °C for 10 minutes without further treatment to obtain bacterial DNA for amplification with polymerase chain reaction (PCR) (Sambrook, Fritsch & Maniatis, 1989).

Oligonucleotide primers (Sigma-Genosys Ltd) specific for stx1, stx2, EHEC 1and 2 (*eaeA primers*) and EHEC P1 and P2 (enterohaemolysin plasmid primers) were used in the PCR (Pollard, Johnson, Lior, Tyler & Rozee, 1990). The presence of a product bearing a molecular weight of 497 base pairs was considered as positive for *E. coli.* Each 90 ml PCR reaction mixture contained: 10 µl of Mg-free 10x amplification buffer (Promega Southampton, United Kingdom); 6 µl of 25 mMMM_gCL_2 (Promega); 2 µl of 10 mM dNTP (Promega); 100 pmol of VT1 and VT2 primers; 2.5 of Taq DNA polymerase (Promega) and 10 µl of template DNA. An additional PCR was performed with the same PCR reaction mixture for the detection of the *eaeA* (100 pmol EHEC 1 and EHEC 2 primers) genes. The mixtures were overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Hybaid, OmniGene Thermocycler, United Kingdom). The PCR cycle consisted of an initial 5 minutes DNA denaturation cycle at 94 ± 1 °C followed by 35 cycle of denaturation at 94 ± 1 °C for 1 minute, annealing at 55 ± 1 °C for 1 minute and extension at 72 ± 1 °C for 1 minute (Pharmacia LKB Thermocycler, Washington, U.S.A.). The amplicon (20 µl) aliquots from each amplification) were detected by gel electrophoresis using a 2% agarose (Sea LE) gel suspension stained with ethidium bromide (Sigma). A 100 base pair DNA molecular size marker (Promega) was used. The amplified products were visualized by UV-transillumination (UVP Image store 5000 gel documentation system.

Suspected *E. coli* O157:H7 colonies isolated from CT-SMAC were individually tested for agglutination using a commercial *E. coli* O157 slide-agglutination kit with antisera against *E. coli* O157 (Mast Assure, Mono Factor O157, code M12030). In addition, all colonies were biochemically confirmed as *E. coli* by their ability to produce indole from tryptophan using Kovac's reagent (ISO, 2001).

3.3 STATISTICAL ANALYSIS OF THE RESULTS

Analysis of variance (ANOVA) was performed using Statistica 7 (Statsoft Inc., Tulsa, Oklahoma, USA) to determine the effect of different hygiene management systems, HAS and HAS + HACCP, on Aerobic Plate Count (APC), Enterobacteriaceae,

Pseudomonas spp., lactic acid bacteria (LAB), *Staphylococcus aureus* and *Salmonella* spp. on fresh beef. ANOVA was also used to determine whether visit and abattoir and their interactions significantly influenced the bacterial counts ($p \leq 0.05$). Fischer's least significant difference (LSD) test was used to separate the means.

3.4 RESULTS

3.4.1 Effect of hygiene management systems on bacterial counts

Results concerning the level of contamination on beef forequarters are shown in Table 3.2. Overall, the hygiene management system did not influence any of the bacterial counts on carcasses. The mean log APC counts of beef carcasses processed at abattoirs which use HAS and those which use HAS + HACCP were similar. The prevalence of Enterobacteriaceae on beef carcasses processed at HAS abattoirs was also statistically similar to the count on beef carcasses at HAS + HACCP abattoirs. Similarly, *Pseudomonas* spp., LAB and *S. aureus* levels from beef carcasses at HAS and HAS + HACCP abattoirs were similar. Interestingly, *E. coli* O157:H7 was isolated from 20% of tested carcasses from a HAS + HACCP abattoir, while the highest *S. aureus* was also obtained from a HAS + HACCP abattoir. *Salmonella* spp. were not detected from either HAS or HAS + HACCP abattoirs (Table 3.2).

Table 3.2: Bacterial counts (log_{10} cfu/cm^2) obtained from the forequarters of chilled beef carcasses (n=30 per abattoir) at abattoirs with Hygiene Assessment System (A1 and A2) and those with Hygiene Assessment System combined with Hazard Analysis Critical Control Points (B1 and B2) systems

Abattoir	Aerobic Plate Count	Enterobacteriaceae	*Pseudomonas* spp.	LAB
A1	4.17^{a}± 1.19	0.83^{a}± 0.94	2.70^{a}± 1.58	4.56^{b}± 0.49
A2	5.20^{b}±0.65	0.71^{a}± 0.87	2.90^{a}± 1.26	3.51^{a}± 1.58
B1	4.13^{a}± 0.95	0.55^{a}± 0.65	2.85^{a}± 0.92	3.44^{a}± 0.74
B2	4.95^{b}± 0.73	1.64^{b}± 0.97	3.18^{a}± 1.61	4.73^{b}± 0.74
p value	<0.05	<0.05	0.60	<0.05
HAS (A1 and A2)	4.69± 1.08	0.77± 0.90	2.78± 1.45	4.03± 1.28
HAS + HACCP (B1 and B2)	4.54± 0.94	1.09± 0.99	3.02± 1.31	4.08± 0.98
p value	0.43	0.06	0.38	0.81

[a & b] Means within the same column bearing different letter superscripts differ significantly at level p<0.05

3.4.2 Effect of abattoir on bacterial counts

In general, there were significant differences in microbial counts between the four abattoirs (Tables 3.3 and 3.4). There was no significant difference between the average log APC on chilled beef carcasses processed at abattoir A2 and those at abattoir B2. Counts from both abattoirs were significantly higher than the counts determined on beef carcasses processed at abattoirs A1 and B1. There was no significant difference between APC at abattoirs A1 and A2.

There was a significant difference in bacterial counts between the two abattoirs that use the HAS system. APC counts on beef carcasses from abattoir A2 were significantly higher compared to those at abattoir A1. Likewise, APC on beef carcasses at abattoir B2 were significantly higher compared to counts at abattoir B1. *Pseudomonas* spp counts at abattoirs were similar. A significant difference in bacterial counts between the two abattoirs that use HAS + HACCP system was also noted. The APC on beef carcasses from abattoir B2 was significantly higher compared to that from B1. The LAB counts on beef carcasses produced at abattoir B2 was significantly higher compared to those from B1. Similarly, Enterobacteriaceae counts at B2 were significantly higher compared to those from B1. At abattoir B2, both Enterobacteriaceae and LAB counts contributed to the significantly higher APC compared to APC at B1; as with all the other test microorganisms, the counts were similar. LAB counts at abattoir A2 and at B1were similar and LAB counts at A1 and count at B2 were similar. Generally the counts on beef carcasses at abattoirs A1 and B1, which have different hygiene management systems, were similar except for LAB counts at abattoir A1, which were significantly higher than those at B1.

3.4.3 Effect of visit at abattoirs on bacterial counts

3.4.3.1 HAS abattoirs

Table 3.4 illustrates the effect of visit on the bacterial counts at each abattoir. The APC on beef carcasses, after chilling at abattoir A1 was significantly different between visits V1 and V3. The APC recorded during V2 was statistically similar to counts recorded during V1 and V3. At abattoir A2, the APC from V1 and V2 were similar. The lowest count was recorded during V3.

Enterobacteriaceae counts for beef carcasses after chilling at abattoir A1 was significantly higher during V3 compared to statistically similar counts recorded during V1. At abattoir A2, Enterobacteriaceae counts were higher during V1 compared to V2 and V3. Enterobacteriaceae counts at abattoir A1 were similar during V2 and V3.

Pseudomonas spp. counts for beef carcasses after chilling from abattoir A1 were lower during V1 compared to the counts recorded during V2 and V3. At abattoir A2, *Pseudomonas* spp. counts recorded during V3 were significantly higher compared to those recorded during V3.

At abattoir A1, the LAB count was significantly higher during V1 compared to counts recorded during V2 and V3, which were similar. At abattoir A2, significantly higher counts were recorded during V1 and V2 compared to V3.

3.4.3.2 HAS + HACCP abattoirs

APC for beef carcasses from abattoir B1 were similar during V1, V2 and V3. APC at abattoir B2 were significantly higher during V2, compared to V3. The count enumerated during V1 was statistically similar to V2 and V3.

At abattoir B1, Enterobacteriaceae count was significantly higher during V2, compared to V3. Enterobacteriaceae count recorded during V1 was similar to both V2 and V3. At abattoir B2, Enterobacteriaceae count was similar during V1 and V2, which were significantly higher compared to V3.

Pseudomonas spp count for carcasses at abattoir B1 was significantly higher during V1, compared to V3. During V2, *Pseudomonas* spp. count was similar to V1 and V3. At abattoir B2, *Pseudomonas* count was similar during all visits. At abattoir B1 LAB count recorded during V1 and V3 was similar and significantly higher than V2. LAB count for carcasses at abattoir B2 was significantly higher during V1and V2 compared to V3.

Table 3.3: Pathogenic bacteria isolated from forequarters of beef carcasses (n=30 per abattoir) during slaughter and chilling at abattoirs with HAS (A1 and A2) and those with HAS combined with HACCP (B1 and B2) systems

Abattoir	***E. coli* O157:H7**			***Staphylococcus aureus***	***Salmonella* spp.**
				(log cfu/cm^2)	
	After evisceration	**After final wash**	**After chilling**	**After chilling**	**After chilling**
A1	ND	ND	ND	0.46± 0.78 (33%) of carcasses	ND
A2	<0.05	ND	ND	0.50±0.71 (37%) of carcasses	ND
B1	<0.05	ND	ND	0.33± 0.74 (27%) of carcasses	ND
B2	20% of carcasses	20% of carcasses	ND	0.62± 0.74 (47%) of carcasses	ND
p values	NA	NA	NA	0.46	NA

ND=not detected

NA=not applicable

Table 3.4: Bacterial counts (log_{10} cfu/cm^2) obtained from the forequarters of chilled beef carcasses (n=30 per abattoir) at abattoirs with HAS (A1 and A2) and Hygiene Assessment System combined with Hazard Analysis Critical Control Point (B1 and B2) systems during the three visits (V1, V2 and V3)

Abattoir	Visit	Aerobic Plate Count	Enterobacteriaceae	*Pseudomonas* spp	LAB	*S. aureus*
A1	V1	3.60^{a}± 1.9	0.10^{a}± 0.32	0.96^{a}± 1.55	4.98^{b}± 0.41	0.35^{a}± 1.10
	V2	4.14^{ab}±0.16	0.47^{a}±0.64	3.66^{b}±0.60	4.18^{a}±0.33	0.72^{a}±0.65
	V3	4.78^{b}±0.35	1.93^{b}±0.49	3.48^{b}±0.51	4.51^{a}±0.36	0.30^{a}±0.48
p value		0.08	<0.05	<0.05	<0.05	0.43
A2	V1	5.71^{b}±0.24	1.65^{b}± 0.68	Not tested	4.42^{b}± 1.59	0.00^{a}± 0.00
	V2	5.37^{b}±0.50	0.47^{a}±0.64	3.8^{b}±0.50	4.13^{b}±0.65	0.73^{b}±0.65
	V3	4.51^{a}±0.40	0^{a}	1.98^{a}±1.09	1.98^{a}±1.01	0.30^{ab}±0.43
p value		<0.05	<0.05	<0.05	<0.05	<0.43
B1	V1	4.45^{a}± 0.57	0.52^{ab}± 0.60	3.47^{b}±0.49	3.95^{b}± 0.57	0.00^{a}± 0.00
	V2	4.05^{a}±1.51	0.89^{b}±0.73	2.47^{ab}±0.65	2.74^{a}±0.65	0.20^{a}±0.42
	V3	3.89^{a}±0.39	0.23^{a}±0.49	2.35^{a}±1.15	3.63^{b}±0.41	0.79^{b}±0.73
p value		0.42	0.07	<0.05	<0.05	<0.05
B2	V1	5.09^{ab}± 0.84	2.08^{b}± 0.84	3.19^{a}±2.25	4.99^{b}± 0.45	1.00^{b}± 0.81
	V2	5.27^{b}±0.42	2.24^{b}±0.49	3.80^{a}±0.47	5.13^{b}±0.40	0.73^{b}±0.65
	V3	4.48^{a}±0.68	0.60^{a}±0.51	2.56^{a}±1.49	4.06^{a}±0.81	0.10^{a}±0.31
p value		<0.05	<0.05	0.23	<0.05	<0.05

$^{a\ \&b}$ Means within the same column bearing different letter superscripts, for different visits at the same abattoir differ significantly at level p<0.05

3.5 DISCUSSION

The use of HAS or HAS + HACCP at abattoirs did not significantly affect the bacterial counts for Enterobacteriaceae, *Pseudomonas* spp., LAB, *S. aureus* and APC, as well as the presence of *Salmonella* spp. and *E. coli* O157:H7. APC are enumerated for the assessment of the hygienic quality of beef carcasses (Kinsella *et al*., 2006). APC at abattoirs that use HAS system and abattoirs that use HAS + HACCP were similar. In a previous study in South Africa, Wagude (1999) investigated the microbiological quality of beef at a registered abattoir before and after the implementation of HACCP. The baseline results by Wagude (1999) represent the performance of HAS because registered abattoirs in South Africa use HAS. Wagude (1999) recorded lower APC before (3.33 $\log_{10}$ cfu/cm^2) and after (3.52 $\log_{10}$ cfu/cm^2) the implementation of HACCP compared to 4.69 and 4.54 $\log_{10}$ cfu/cm^2 in this study. The latter was comparable to APC 4.4 $\log_{10}$ cfu/cm^2, recorded previously on beef carcasses in the North West Province in South Africa (De Jesus, 1998).

Enterobacteriaceae counts from abattoirs that use HAS and those that use HAS + HACCP were similar. Enterobacteriaceae counts in this study were lower than those reported by De Jesus (1998) and higher than those reported by Zweifel, Baltzer and Stephan (2005). The latter, following the European Union Decision 2001/471/EC, investigated hygienic quality of 800 beef carcasses at 5 Swiss abattoirs. Zweifel *et al*. (2005) recorded Enterobacteriaceae counts ranging from 0.15 to 0.61 $\log_{10}$ cfu/cm^2. The presence of Enterobacteriaceae on carcasses suggests transfer of faecal material onto the sterile carcass during the slaughter process, which may suggest that currently available dressing procedures at both HAS and HAS + HACCP abattoirs cannot be relied upon to prevent faecal contamination during slaughter. The most pressing food safety issues in the food industry are caused by the presence of *E. coli* O157:H7 and *Salmonella* spp in raw meat and poultry products (Sperber, 2005). HAS + HACCP did not seem to provide an assurance for the production of meat free of *E. coli* O157:H7, since *E. coli* O157:H7 was isolated from one of the abattoirs with HAS + HACCP. A prevalence of 20% of tested carcasses was recorded from samples collected after evisceration and after final wash, while none of the carcasses tested

after chilling were positive for *E. coli* O157:H7. A similar result was reported by Carney *et al.* (2006). In their study they positively isolated *E. coli* O157:H7 on 3.0% of beef carcasses before chilling and 0% after chilling.

The positive isolation of *E. coli* O157:H7 from an abattoir that uses the HAS + HACCP system is in agreement with Josling (2004), that even though a risk management system is a good preventive system, it leaves room for substantial uncertainty over the incidence of risk. Furthermore, Sperber (2005) attributed the occurrence of foodborne outbreaks in the USA, despite the widespread use of HACCP, to failures of cleaning and sanitation, or to the lack of management awareness and commitment in providing the necessary training and resources to support HACCP plans.

The absence of *E. coli* O157:H7 in samples of carcasses collected after chilling may be the result of a prolonged lag phase, where growth of injured cells may have been inhibited by the presence of bile salts and antibiotics in selective enrichment media (Stephens & Johnson, 1998). By observing efficient cell recovery after exposure, Lawrence, Frampton and Spitz (2001) demonstrated that *E. coli* O157:H7 could survive environmental stresses, such as heat and freeze injury. As a result, cells that were isolated after evisceration and washing may not have been killed during chilling, but may have rather been unculturable.

Meat carcasses may be contaminated with *Salmonella* spp during slaughtering, which may result in low levels and uneven distribution (Marlony, Löfström, Wagner, Krämer & Hoorfar, 2008). *Salmonella* spp. were not isolated from any of the samples analyzed. Similar results were recorded during a surveillance study that was conducted in South Africa, where 270 carcasses at nine beef abattoirs were sampled (De Jesus, 1998). In a survey in the USA, *Salmonella* spp were found on 1% of samples from 3075 chilled carcasses of steers, heifers, bulls and cattle, and 5% of samples of calves (Hogue, Dreesen, Green, Ragland, James, Bergeron, Cook, Pratt & Martins, 1993). Marlony *et al.* (2008) reported that chilling at 4 ± 1 °C could reduce

the viability of *Salmonella* spp and consequently hamper the growth on selected media.

Overall, HAS and HAS + HACCP did not affect *S. aureus* counts differently. However, *S. aureus* was detected on 47% of carcasses tested at abattoir B2, which was the highest value compared to those recorded at abattoirs A1, A2 and B1. This observation was contrary to the findings of Wagude (1999) that *S. aureus* counts were significantly reduced after the implementation of HACCP. The *S. aureus* counts at all abattoirs ranged between 0.33 to 0.62 $\log_{10}$ cfu/cm^2, which was lower compared to 2.25 $\log_{10}$ cfu/cm^2 found for abattoirs in the North West Province, South Africa (De Jesus, 1998). The latter involved nine beef abattoirs using HAS. Sources of *S. aureus* in an abattoir can vary from workers, abattoir air and the live slaughter animals (Desmarchelier *et al*., 1999; Mead, 1994; Ravenholt, Eelkema, Mulhern & Watkins, 1961; Schlegelová, Nápravníková, Dendis, Horváth, Benedík, Babák, Klímová, Navrátilová & Ková, 2003; Shale *et al*., 2006; Van Loo *et al.*, 2007; Wagude, 1999).

The hide of the slaughtered animals and the water used to wash the carcasses can be sources of both mesophilic and psychrotrophic microorganisms on carcasses (Nottingham, Penny & Harrison, 1974; Samaha & Draz, 1993), which may reduce the shelf life of resulting meat products. The level of spoilage bacteria, *Pseudomonas* spp. and LAB, were very low for all the abattoirs. These low levels may result in an extended shelf life of the beef. Both the HAS and combined HAS + HACCP systems contributed to low levels of spoilage bacteria indicating effective implementation of the PRP's.

3.6 CONCLUSIONS

The microbiological quality of beef processed at abattoirs that use only HAS is not inferior to that of beef processed at HAS + HACCP abattoirs. The combined HAS + HACCP did not prevent contamination of beef carcasses with *E. coli* O157:H7 and *S. aureus*. This study therefore suggests that abattoirs using only the HAS system are capable of achieving beef carcasses of microbiological quality comparable to that

produced through the use of combined HAS + HACCP system. Additional control measures are required during the secondary processing of meat to ensure that pathogens do not have an opportunity to grow and multiply on meat and meat products.

CHAPTER FOUR:

SURVIVAL OF *E. COLI* O157:H7 CO-CULTURED WITH DIFFERENT LEVELS OF *PSEUDOMONAS FLUORESCENS* AND *LACTOBACILLUS PLANTARUM* ON FRESH BEEF

(Accepted for publication in the Brazilian Journal of Microbiology)

Abstract

The purpose of this study was to investigate the effect of different levels of *Pseudomonas fluorescens* (10^2 and 10^6 $\log_{10}$ cfu/ml) and *Lactobacillus plantarum (*10^2 and 10^4 $\log_{10}$ cfu/ml) on the growth of *Escherichia coli* O157:H7 on beef loins. Beef loins inoculated with *E. coli* O157:H7 and *P. fluorescens* were aerobically stored for 7 days at 4 °C, while those inoculated with *E. coli* O157:H7 and *L. plantarum* were vacuum packaged and stored for 8 weeks at 4 °C. Aerobic Plate Counts (APC), *E. coli* O157:H7 and either *P. fluorescens* or *L. plantarum* counts were determined at different storage intervals. For the aerobically packaged beef loins, *E. coli* O157:H7 was detected throughout the 7-day storage period regardless of the *P. fluorescens* level in the inoculum. For the vacuum packaged beef loins, similar inoculum levels of *E. coli* O157:H7 and *L. plantarum* allowed *E. coli* O157:H7 to survive until week 5 of storage, while a higher inoculum level of *L. plantarum* inhibited *E. coli* O157:H7 from week 3. Once fresh beef has been contaminated with *E. coli* O157:H7 the level of *P. fluorescens* in the background flora does not inhibit its survival and growth. However, under vacuum storage, the application of *L. plantarum* as a biopreservative inhibits the survival of *E. coli* O157:H7 on beef. The higher the level of *L. plantarum* in the system, the earlier will be the onset of the inhibition. Farmers and abattoirs have to strengthen preventive strategies to eliminate contamination of beef carcasses with *E. coli* O157:H7.

Key words: aerobic storage, vacuum package, *E. coli* O157:H7, *P. fluorescens*, *L. plantarum*

4.1 INTRODUCTION

There is evidence that some slaughter animals in South Africa shed *E. coli* O157:H7 at the time of slaughter, which creates an opportunity for this pathogen to be present on meat and meat products (Gauteng Provincial Department of Agriculture: Veterinary Public Health (GPDoA: VPH), 2004). The organism has been found in 19% and 7% of slaughter cattle faeces and carcasses, respectively. Similarly, it was found in 8% and 1% of sheep faeces and carcasses and in 31% and 1% of horse faeces and carcasses (GPDoA: VPH, 2004). Spoilage and pathogenic bacteria compete for available substrates in food to survive. Psychrotrophic bacteria especially of the genus *Pseudomonas* are common on meat. These organisms have been identified in numerous studies as the major spoilage organisms in refrigerated fresh meats (Ayres, 1960; Greer & Jeremiah, 1980) partly due to their ability to form quorum-sensing facilitated biofilms (Jay, Vilai & Hughes, 2003).

In South Africa, the meat industry utilises vacuum packing to extend the shelf life of beef products for transportation, which is at times over considerable distances. The final destination of these vacuum packaged products often includes neighbouring countries (Shale, 2004). Lactic acid bacteria (LAB) are facultative anaerobes that are antagonistic to many microorganisms including spoilage and pathogenic bacteria (Ammor, Tauveron, Dufour & Chevallier, 2006). Previously, research work focused on the inhibitory effect of *L. plantarum* on *E. coli* O157:H7 in a range of food products including acidic fruit juices (Uljas & Ingham, 1998), processed meat products (Riley *et al*, 1983) and ground beef (Muthukumarasamy, Han & Holley, 2003; Smith *et al.*, 2005) but there is no evidence of work conducted on whole beef loins. Since Pseudomonads and LAB are natural contaminants of foods of animal origin, which can also be contaminated by *E. coli* O157:H7, it is important to understand how the different levels of these spoilage organisms will affect the survival and growth of *E. coli* O157:H7 on aerobically and vacuum stored beef. Furthermore, it was suggested that decontamination techniques used in developed countries led to very low numbers of background microorganisms on fresh meat, resulting in reduced competition between such microorganisms and food pathogens

(Jay, 1997). Fresh food products that contain 10^5 cells/g of harmless microbiota are less likely to allow low numbers of pathogens to proliferate than those that contain 10^3 cells/g (Jay, 1997). The aim of this study was to investigate the effect of different levels of competitive, spoilage bacteria, *P. fluorescens* and *L. plantarum* on the survival and growth of *E. coli* O157:H7 on aerobically and vacuum packaged stored (4 °C) beef, respectively.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

E. coli O157:H7 strain UT 10, isolated from meat, was obtained from the Agricultural Research Council, Onderstepoort, South Africa. This strain was used for both experiments 1 and 2. For experiment 1 *P. fluorescens* ATCC 13525 strain was used (Kiwk stik; MediMark, France). For experiment 2 *L. plantarum* ATCC 8014 (MediMark) strain was used.

4.2.2 Preparation of beef loins

Vacuum packaged deboned beef loin (*M. longissimus dorsi*) was purchased from a local butchery. The external layer (approximately 1 cm) of the muscle was removed within a biological safety cabinet (Labaire, France), under aseptic conditions. The internal portion was cut into 25 g blocks using a sterile metallic sampler. Two pieces were subsequently analysed to determine the total aerobic counts after sterilization, which were <10 $\log_{10}$ cfu/cm^2.

4.2.3 Preparation of working cultures

E. coli O157:H7 strain was cultivated in Brain Heart Infusion Broth (BHIB) CM 225 (Oxoid, Hamphire, England) and incubated at 37 °C for 24h. The *P. fluorescens* strain was grown on *Pseudomonas* Agar CM 559 with selective SR 103 and incubated

at 25 °C for 72 hrs, while LAB was cultivated on de Man, Rogosa and Sharpe (MRS) agar CM 359 (Oxoid) and incubated at 30 °C for 48 hours. 0.5 McFarland standard (Andrew, 2005) was used to prepare cultures containing 10^2 $\log_{10}$ cfu/ml of *E. coli* O157:H7, as well as 10^2 and 10^6 $\log_{10}$ cfu/ml of *P. fluorescens* and and 10^2 and 10^4 $\log_{10}$ cfu/ml of *L. plantarum*. Serial dilutions of the bacterial cultures were made using Buffered Peptone Water (BPW) CM 509 (Oxoid) and plated onto Plate Count Agar (PCA) CM 463 (Oxoid) for the determination of the exact number of cfu/ml. A cocktail inoculum of 300 ml was prepared by mixing 150 ml of each bacterial inoculum at the concentration of 10^2 $\log_{10}$ cfu/ml. Another cocktail inoculum was prepared by mixing 150 ml volumes of 10^2 $\log_{10}$ cfu/ml of *E. coli* O157:H7 inoculum with 10^6 $\log_{10}$ cfu/ml of *P. fluorescens* and 10^2 $\log_{10}$ cfu/ml of *E. coli* O157:H7 and 10^4 $\log_{10}$ cfu/ml of *L. plantarum*.

4.2.4 Inoculation of beef loins

For each treatment, sterile pieces of 25 g of beef were individually submerged into the inoculum for 10 min to allow for bacterial attachment. Inoculated beef pieces were air-dried for 5 minutes. For the treatment involving *E. coli* O15:H7 and *P. fluorescens*, the inoculated beef pieces were packaged individually in zip-lock plastic pouches (PVC, O_2 transmission>10000 cm^3/m^2 per 24h/atm) (150 mm × 180 mm× 40 mm) and stored aerobically at 4 °C for a total of 7 days. Samples were collected and analysed after 0, 2, 3, 5 and 7 days of storage to determine *E. coli* O157:H7, *P. fluorescens* counts, as well as the APC. The same inoculation procedure was used to inoculate sterile beef pieces with an inoculation cocktail containing *E. coli* O157:H7 and *L. plantarum*. Subsequently, inoculated samples were separately placed in vacuum bags (150 mm x 200 mm), vacuum-sealed and stored at 4 °C for up to 8 weeks. Samples were analysed weekly for up to 8 weeks for *E. coli* O157:H7, *L. plantarum* and APC. All experiments were carried out in triplicate with duplicate samples analysed at each storage interval on duplicate plates.

4.2.5 Microbiological analysis of inoculated beef loins

During each sampling period, duplicate subsamples of 25 g were homogenised with 225 ml of 0.1% BPW in a stomacher (Seward 400, Seward, London, United Kingdom) and subsequent decimal dilutions were prepared and plated. For the enumeration of *E. coli* O157:H7, sorbitol MacConkey (SMAC) Agar CM 813 (Oxoid) with selective supplement SR 172 (Oxoid) was used. SMAC plates were incubated at 37 °C for 24 hours.

4.2.6 Statistical analysis

Data were analysed by two-way Analysis of variance (ANOVA) including the interaction effect using Statistica 7 (Statsoft Inc., Tulsa, Oklahoma, USA, 2003) to determine if levels of *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml), *P. fluorescens* (10^2 and 10^6 $\log_{10}$ cfu/ml) and *L. plantarum* (10^2 and 10^4 $\log_{10}$ cfu/ml) and storage time 7 days (day 0, 2, 3, 5, 7) under aerobic storage and 8 weeks (1 week interval) under vacuum storage significantly (95% confidence interval) affected survival and growth of *E. coli* O157:H7 on meat. All samples were analysed in duplicate and each experiment was repeated three times. Means were separated by Fisher's Least Significant Difference (LSD) analysis.

4.3 RESULTS

4.3.1 Effect of *P. fluorescens* on the survival and growth of *E. coli* O157:H7 on beef loins

The *P. fluorescens* inoculum level did not influence the survival and growth of *E. coli* O157:H7 ($p=0.62$), while growth of *E. coli* O157:H7 was affected by the storage time ($p<0.05$). There was a significant ($p<0.05$) interaction between the inoculation level and time (level x time) on the growth and survival of *E. coli* O157:H7. Inoculum

level and storage time did not influence *P. fluorescens* count (p=0.20) and APC (p-0.94) (Table 4.1).

Table 4.1: Significance by ANOVA of the growth and survival of *E. coli* 0157:H7, *P. fluorescens* and Aerobic Plate Count on aerobically stored (7 days, at 4 °C) beef pieces inoculated with different levels of *P. fluorescens*, level 1 (10^2 cfu/ml) and level 2 (10^6 cfu/ml), while the level of *E. coli* 0157:H7 was constant at 10^2 cfu/ml

Treatments	***E. coli* O157:H7**		***P. fluorescens***		**Aerobic Plate Counts**	
	Degrees of freedom	**p value**	**Degrees of freedom**	**P value**	**Degrees of freedom**	**p value**
Level	1	0.62	1	<0.001	1	<0.001
Time	4	<0.05	4	<0.05	4	<0.05
Level x Time	4	<0.001	4	0.20	4	0.94

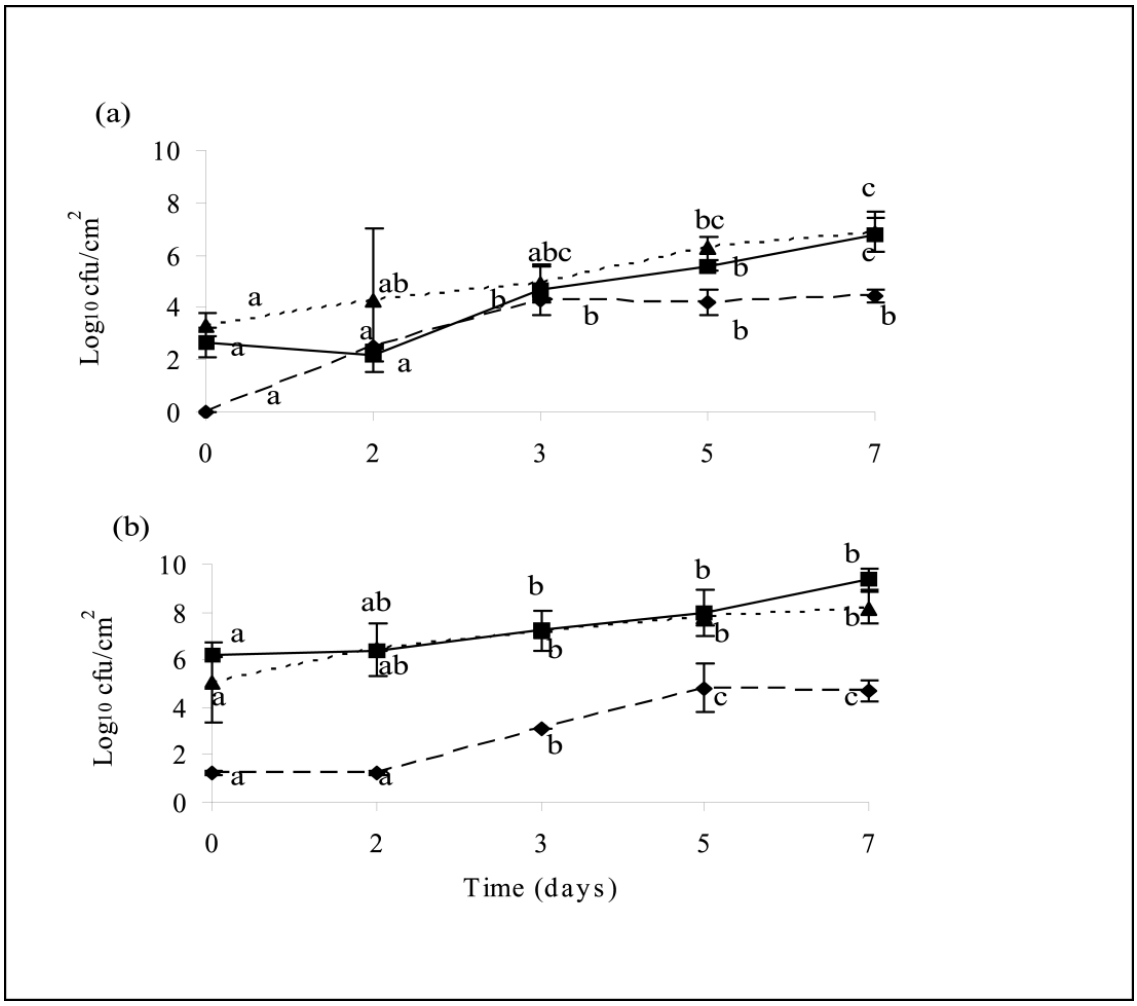
When similar levels (10^2 $\log_{10}$ cfu/ml) of *E. coli* O157:H7 and *P. fluorescens* were combined, *E. coli* O157:H7 was not recovered from beef on day 0 (Figure 4.1a). By day 2 of storage, the *E. coli* O157:H7 count reflected the level that was inoculated. *E. coli* O157:H7 count had significantly ($p<0.05$) increased until day 3. From then on, no further growth occurred. By day 7, *E. coli* O157:H7 was recoverable at the level of 4.44 $\log_{10}$ cfu/cm^2. *E. coli* O157:H7 increased by 2 log when comparing levels at days 2 and 7 (Figure 4.1a).

Under the same experimental conditions, *P. fluorescens* showed a 2-day lag phase, as the counts enumerated during days 0 and 2 remained at the same level (Figure 4.1a). There was a significant ($p<0.05$) increase in growth by day 3 of storage, which remained constant until day 5. The highest *P. fluorescens* growth population was recorded at day 7. Growth of *P. fluorescens* increased by 4 log during the storage period of 7 days.

APC remained relatively constant, with a significant ($p<0.05$) increase between the counts from day 0 to day 5 and day 7. Similar levels of *E. coli* O157:H7 and *P. fluorescens* were recovered on day 3. *P. fluorescens* counts on days 5 and 6 were higher than *E. coli* O157:H7 counts during the same period.

Growth of *E. coli* O157:H7 showed a 2-day lag phase when combined with 10^6 cfu/ml *P. fluorescens* inoculum (Figure 4.1b). By day 3, growth increased significantly ($p<0.05$). The highest *E. coli* O157:H7 growth population was achieved from day 5 and maintained until the end of the experiment. The growth of *E. coli* O157:H7 under both experimental environments (*P. fluorescens* inoculation levels 10^2 and 10^6 $\log_{10}$ cfu/ml) was similar by day 7, indicating that the level of *P. fluorescens* did not affect the growth of *E. coli* O157:H7.

The growth of *P. fluorescens* (Figure 4.1b) showed a similar pattern as that of *E. coli* O157:H7 at the beginning of the storage period with a 2-day lag phase. By day 3, there was a significant ($p<0.05$) increase in growth, which remained constant until day 5. A significant increase ($p<0.05$) in *P. fluorescens* growth was observed at day 7. *P. fluorescens* increased by 3 log over the storage time. APC followed a similar trend to *P. fluorescens* (Fig 4.1b).



In a sub-figure, means for the same bacterium ♦ *E. coli* O157:H7 ■ *P. fluorescens* ▲ Aerobic Plate Count with different letter notations are significantly different at p<0.05

Figure 4.1: Effect of inoculation levels of *P. fluorescens* (10^2 and 10^6) on the growth of inoculated *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml) on sterile beef, aerobically packaged and stored for 7 days at 4 °C. **(a)** Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^2 cfu/ml *P. fluorescens* in the inoculum; **(b)** Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^6 $\log_{10}$ cfu/ml *P. fluorescens* in the inoculum.

4.3.2 Effect of *L. plantarum* on the survival and growth of *E. coli* O157:H7 on beef loins

The level of *L. plantarum* in the inoculum and the storage time significantly ($p<0.05$) affected the survival and growth of *E. coli* O157:H7 and *L. plantarum* on beef loins, while both factors did not affect the APC ($p=0.37$) (Table 4.2). The onset of *E. coli* O157:H7 inhibition was also affected by the level of *L. plantarum* in the inoculum.

Table 4.2: Significance by ANOVA of the growth and survival of *E. coli* O157:H7, *L. plantarum* and Aerobic Plate Count vacuum packaged beef pieces stored for 8 weeks at 4 °C), inoculated with different levels of *L. plantarum*, level 1 (10^2 cfu/ml) and level 2 (10^4 cfu/ml), while the level of *E. coli* O157:H7 was constant at 10^2 cfu/ml

Treatments	*E. coli* O157:H7		*L. plantarum*		APC	
	Degrees of freedom	P value	Degrees of freedom	P value	Degrees of freedom	P value
Level	1	<0.001	1	<0.001	1	0.78
Time	4	<0.05	4	<0.05	4	<0.05
Level x Time	4	<0.001	4	<0.001	4	0.37

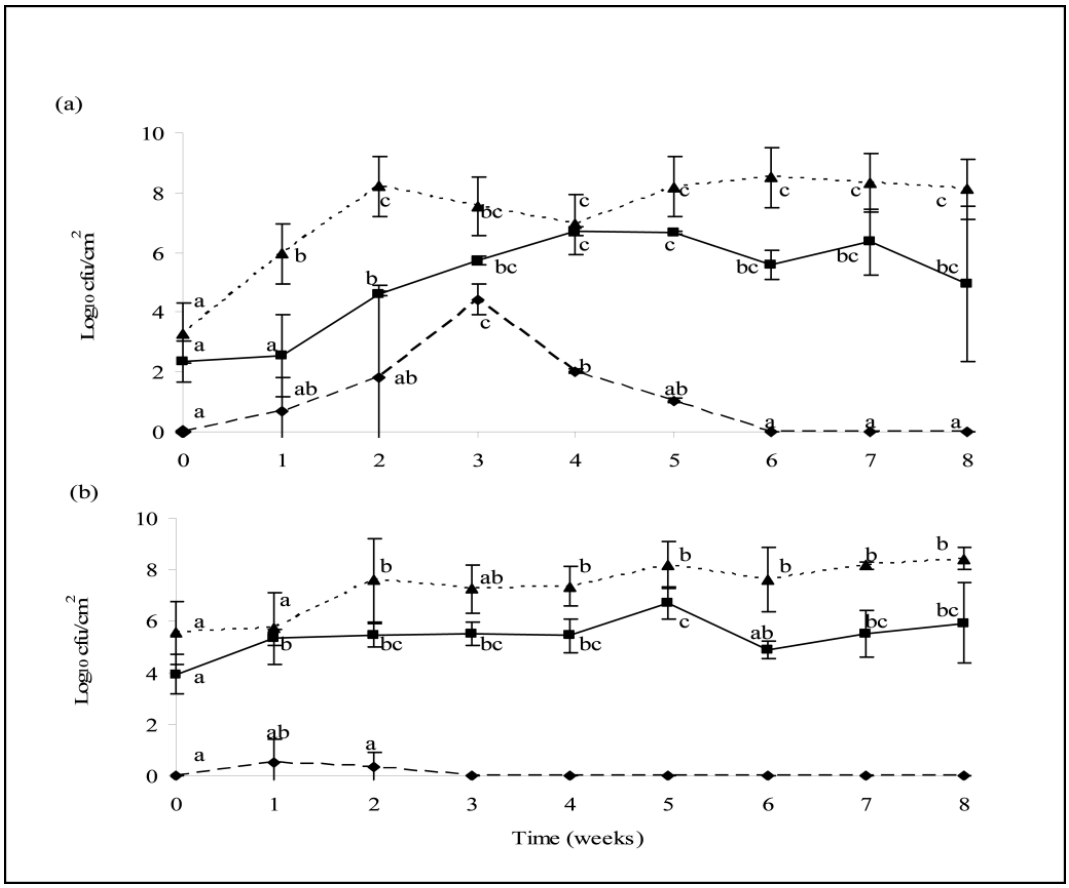
When the same inoculum level was used for *E. coli* O157:H7 and *L. plantarum* (10^2 $\log_{10}$ cfu/ml) in the suspension (Figure 4.2a), *L. plantarum* did not show immediate inhibition of *E. coli* O157:H7. There was a similar numerical increase in the *E. coli* O157:H7 count recorded at weeks 1 and 2. The highest growth population of *E. coli* O157:H7 was noted at week 3 following which no additional growth occurred. Instead, the *E. coli* O157:H7 population declined, on average, by 2 log by week 4 to 2.03 $\log_{10}$ cfu/cm^2. The decline in the *E. coli* O157:H7 population continued up to week 5 and was too low to detect by week 6.

Similar to *E. coli* O157:H7, *L. plantarum* counts remained stable from the beginning of storage until week 1. There was a significant ($p<0.05$) increase in counts at week 2. At week 3, the *L. plantarum* growth curve showed 1.11 log increase compared to week 2. From week 3, *L. plantarum* counts remained constant until week 7, indicating a 5-week-long stationary phase that was followed by a 1.4 log decline in *L. plantarum* counts recorded at week 8. APC showed an increase by week 1 which

continued until week 3. APC then remained constant for the remainder of the storage time.

When a lower level of *E. coli* O157:H7 (10^2 cfu/ml) was combined with a higher level of *L. plantarum* (10^4 cfu/ml) in the inoculum (Figure 4.2b), the adverse effect of *L. plantarum* on the growth and survival of *E. coli* O157:H7 was recorded earlier, compared to the onset of such an effect when the same levels of *E. coli* O157:H7 and *L. plantarum* were used in the inoculum suspension. Low levels of *E. coli* O157:H7 were detected at weeks 1 and 2. From week 3 onwards, *E. coli* O157:H7 was not recovered from beef loin pieces. The *L. plantarum* growth curve showed significant ($p<0.05$) growth of the organism by week 2, which remained at the same level by the end of the experiment. However, levels of *L. plantarum* in week 4 were significantly ($p<0.05$) higher compared to those recorded at week 2. APC were relatively constant throughout the storage period. APC recorded during week 8 was significantly higher than those at weeks 0 and 1. *L. plantarum* count increased by 2 log from the start of the experiment until the end of storage.

By comparing growth of *L. plantarum* with the same initial level as that of *E. coli* O157:H7 in the inoculum to that of *L. plantarum* with a higher initial level, it was noted that the *L. plantarum* count with the same initial count as *E. coli* O157:H7 was approximately 1 log higher compared to that where the initial level was higher than that of *E. coli* O157:H7. APC was relatively constant throughout the experiment.



In a sub-figure, means for the same bacterium, with different letter notations ♦ *E. coli* O157:H7 ■ *L. plantarum* ▲ Aerobic Plate Count are significantly different at $p<0.05$.

Figure 4.2: Effect of inoculation levels of *L. plantarum* (10^2 and 10^4 cfu/ml) on the growth of inoculated *E. coli* O157:H7 (10^2 $\log_{10}$ cfu/ml) on sterile beef, vacuum packaged and stored for 8 weeks at 4 °C. **(a)** Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^2 $\log_{10}$ cfu/ml *L. plantarum* in the inoculum; **(b)** Inoculum cocktail: 10^2 $\log_{10}$ cfu/ml *E. coli* O157:H7 and 10^4 $\log_{10}$ cfu/ml *L. plantarum* in the inoculum

4.4 DISCUSSION

4.4.1 Effect of similar levels of *E. coli* O157:H7 and *P. fluorescens* in the inoculum on the growth of *E. coli* O157:H7 on beef loins

The application of similar levels of *E. coli* O157:H7 and *P. fluorescens* in the inoculum showed interesting growth patterns for both bacteria as well as APC. *E. coli*

O157:H7 could not be quantified immediately after inoculation at day 0, while *P. fluorescens* was detected. The inability to recover *E. coli* O157:H7 from inoculated samples is in agreement with Thran *et al.* (2003) who did not detect *E. coli* O157:H7 in rumen contents when testing directly within 24h of storage. Detection was only achieved after enrichment.

The results of this study showed that 18h cultures of *E. coli* O157:H7 and *P. fluorescens* required 2 days to adapt and start growing on meat stored aerobically at 4 °C. When bacteria are deposited onto meat surfaces, they undergo a period of adjustment in their new environment, where cell damage is repaired and bacteria adapt to utilise nutrients available before they can start to grow (Sentance & Husband, 1993). Temperature influences the lag phase and growth rate of *E. coli* including serotype O157:H7 (Duffy, Whiting & Sheridan, 1999). Duffy *et al.* (1999) showed that the lower the storage temperature, the longer the lag phase for both pathogenic and background bacteria. These authors reported that the *E. coli* O157:H7 lag phase at 15 °C was increased by 6.33 hours compared to the lag phase duration at 37 °C for pure cultures.

By day 3, the growth population of both organisms had increased significantly, implying that there was no antagonism between these organisms before day 3. After day 3, there was an antagonistic interaction between *E. coli* O157:H7 and *P. fluorescens.* The growth of *E. coli* O157:H7 stopped while *P. fluorescens* was still continuing to grow to reach its maximum growth density at day 7. In another study Vimont, Rozand, Montet, Lazizzera, Bavai and Muller (2006) noted that there was competition between *E. coli* O157:H7 and beef background flora during sample enrichment steps. These authors reported that growth of *E. coli* O157:H7 stopped as soon as the maximum bacterial density in the background flora was reached. The retardation of *E. coli* O157:H7 might be due to the effects of the available nutrients on meat, the suppression by background *P. fluorescens* or the combined effect from both nutrients and *P. fluorescens.* Tsigarida, Boziaris and Nychas (2003) reported an accelerated consumption of glucose by pseudomonads when co-cultured with other bacteria. Free glucose is a minor component of meat, 0.1 to 0.5% (Gill, 1976;

Nychas, Dillon & Board, 1988). Therefore, *Pseudomonas* species have a competitive advantage over other organisms due to their ability to transform glucose rapidly to gluconate in chilled muscle foods stored aerobically (Drosinos & Board, 1994). According to Samelis and Sofos (2002), the presence of glucose enhances the inhibition of *E. coli* O157:H7 by *Pseudomonas* spp., particularly at low storage temperatures. Samelis and Sofos (2002) illustrated that in the absence of glucose, the inhibition of *E. coli* O157:H7 was weak at temperatures above 10 °C, while at 25 °C there was minimal inhibition of the pathogen, irrespective of the presence of glucose. *E. coli* O157:H7 is an inhabitant of the gastrointestinal tract; therefore its optimal growth temperature (T_{opt}) would be expected to be at 37 °C. However, Gonthier, Guérin-Faublée, Tilly and Delignette-Muller (2001) reported a T_{opt} value of 40.2 °C in a laboratory medium.

P. fluorescens showed a reduced growth rate between days 3 and 5. Growth of bacteria on the surface of meat depends on the rate of diffusion of fermentable substrates from within the meat to the surface (Gill, 1976). When the rate of transfer of such substrates slows down, the rate of growth declines to the point where the rate at which the substrates become available is only sufficient for cell maintenance and not growth. Therefore, the cessation in growth between days 3 and 5 could signify the period that *P. fluorescens* had to change their metabolism to utilize amino acids and lactic acid upon the depletion of glucose (Gill, 1976). Furthermore, Pseudomonads synthesize high iron-affinity transport molecules called siderophores that function to sequester available iron (Laine, Karwoski, Raaska & Sandholm, 1996). Therefore, *P. fluorescens* could also have exerted a siderophore inhibitory effect (Cheng, Doyle & Luchansky, 1995) on *E. coli* O157:H7, resulting in levels of the latter not increasing after 3 days of storage at 4 °C. The APC curve showed a similar pattern to that of *P. fluorescens,* confirming that the *P. fluorescens* cells contributed mostly to the APC of the sterilized beef loin samples, as would have been expected.

4.4.2 Effect of different levels of *E. coli* O157:H7 and *P. fluorescens* in the inoculum on growth of *E. coli* O157:H7 on beef loins

When beef pieces were inoculated with a higher level (10^6 cfu/ml) of *P. fluorescens*, *E. coli* O157:H7 still required a 2-day lag phase before entering the growth phase. This observation was expected because the level of *E. coli* O157:H7 was constant in both experiments. In addition to the storage temperature, the duration of the lag phase also depends on the inoculum size. The lower the inoculum size the longer the lag phase (Augustin, Delattre, Rosso & Carlier, 2000).

On the other hand, *P. fluorescens* showed a slower growth rate, unlike when similar levels were used, which could probably be due to competition among *P. fluorescens* for growth space on the limited growth area. *E. coli* O157:H7 continued to grow in the presence of *P. fluorescens,* even when the levels of *P. fluorescens* had increased to 10^7 $\log_{10}$ cfu/cm^2, a level contributing to slime and off-flavour formation (Nychas *et al.*, 2008). Therefore, although *E. coli* O157:H7 survived until the end of the experiment, beef loins would have been rejected based on their appearance.

Upon depletion of nutrients on the meat surfaces, pseudomonads have a competitive ability to penetrate meat and acquire access to new growth substrates (Nychas *et al*., 2008). This ability could have allowed *P. fluorescens* growth and 10^9 $\log_{10}$ cfu/cm^2 by day 7. However, the continued increase in *E. coli* O157:H7 growth between days 3 and 5 demonstrates that *E. coli* O157:H7 competes well with *P. fluorescens* on meat. The APC growth curve was similar to that observed when the levels of *E. coli* O157:H7 and *P. fluorescens* were similar. In this study, both inocula levels of *P. fluorescens* did not inhibit the growth of *E. coli* O157:H7 on refrigerated beef loins. These findings are supported by observations made by Saad and Franco (1999), Quinto, Franco, Fente, Vazquez and Cepeda (1997) and Duffy *et al.* (1999) in that pure *E. coli* O157:H7 cultures achieved a similar growth population as when co-cultured with *P. fluorescens* in ground beef and skimmed milk.

4.4.3 Effect of similar levels in the inoculum of *E. coli* O157:H7 and *L. plantarum* on the growth of *E. coli* O157:H7 on beef loins

When beef pieces were inoculated with similar levels of *E. coli* O157:H7 and *L. plantarum, E. coli* O157:H7 growth was detected only after 3 weeks of storage. The ability of *E. coli* O157:H7 to grow under acidic conditions could be a result of acid tolerance. Stopforth, Skandamis, Geornaras and Sofos (2006) found increased acid tolerance of *E. coli* O157:H7 after the pathogen had been incubated in acidic washings of sublethal pH at 4 °C. Likewise, in this study, during the lag phase, the pH of beef could have decreased due to *L. plantarum* activities, exposing *E. coli* O157:H7 cells to acidic conditions. The cells adapted to the acidic conditions and continued to grow, hence, the exponential cell growth by week 3. *E. coli* O157:H7 can survive and be protected in acidic environments such as in apple cider (Miller & Kasper, 1994). In support of their findings in this study, Dykes, Moorhead and Roberts (2001) concluded that *E. coli* O157:H7, unlike generic *E. coli,* was less inhibited by a drop in pH. Furthermore, Dykes *et al.* (2001) also showed that in vacuum packaged beef, low temperature and acidification were able to enhance the acid tolerance of *E. coli* O157:H7.

L. plantarum produces plantaricin, a bacteriocin with inhibitory activity towards both gram-positive and gram-negative bacteria including food pathogens (*Listeria*, *Staphyloccus* and *Salmonella*) (Enan, Alalyan, Abdel-Salam & Debevere, 2002; Fricourt *et al*., 1994; Kodama, 1952; Lash *et al.*, 2005; Miñambres, Rodrigo, Carvajal, Reverter & Tomás, 2007). In this study, as soon as *L. plantarum* cells entered the stationary growth phase, *E. coli* O157:H7 counts showed a steady decline in growth from week 4 until week 6. After week 6, *E. coli* O157:H7 was not recovered from beef samples. The inhibition of *E. coli* O157:H7 growth could be attributed to the effect of plantaricins produced by *L. plantarum* after week 3. This observation corresponds to the reported production of bacteriocins by *L. plantarum* when its growth transcends from exponential to stationary phase, as growth slows down (Bárcena *et al.*, 1998; González, Arca, Mayo & Suárez, 1994).

The growth curve of *L. plantarum* showed that *L. plantarum* cells required 1 week before growth could be detected. Similarly, in another study, Babji and Murthy (2000) detected LAB cells after only 9 days of incubation under vacuum storage at 4 °C on minced goat meat. In this study, *L. plantarum* cells showed a limited growth rate, as there were numerical differences only from week 2 onwards. The inability of *L. plantarum* to grow exponentially could be due to the storage temperature of 4 °C. This is in keeping with the findings of Paynter, Brown and Hayasaka (1997) who found that *L. plantarum* did not grow at 4 °C for 56 days, while growth occurred at higher incubation temperatures from 20 to 37 °C. As expected, the APC growth curve showed cells entering the stationary growth phase from week 2 onwards until the end of the experiment, a similar trend to *L. plantarum.*

4.4.4 Effect of different levels of *E. coli* O157:H7 and *L. plantarum* in the inoculum on the growth of *E. coli* O157:H7 on beef loins

Unlike with similar levels of *E. coli* O157:H7 and *L. plantarum,* the inhibition of *E. coli* O157:H7 at the higher inoculation level (10^4 $\log_{10}$ cfu/ml) occurred earlier. There was an insignificant *E. coli* O157:H7 growth initially and the organism was not recovered from all meat samples from week 2 onwards. The earlier inhibition of *E. coli* O157:H7 corresponded to the earlier entry of *L. plantarum* cells into the stationary phase, signifying a possible earlier onset of the production of plantaricins. This result shows that plantaricins, lower storage temperature (4 °C), high level of *L. plantarum* in the inoculum and vacuum packaging had a combined inhibitory effect on the growth of *E. coli* O157:H7 on beef. In other studies, Yoon and Sofos (2008), Muthukumarasamy *et al.* (2003) and Vold, Golck, Wasteson and Nissen (2000) recorded the adverse effect of high background flora including LAB on meat slices and in ground beef on *E. coli* O157:H7. Higher *L. plantarum* level in the inoculum showed rapid growth by week 1, which could be attributed to their ability to better compete for nutrients and grow faster (Bredholt, Nesbakken & Holck, 1999). However, in this study, *L. plantarum* growth remained constant from week 2 onwards, while APC showed only 1-week growth and remained constant until the end of the experiment. This was expected, as the APC was comprised of *L. plantarum.*

4.4.5 Comparison of aerobic and vacuum packaging on beef loins

When beef was contaminated with *E. coli* O157:H7 and *P. fluorescens,* and stored aerobically at refrigeration temperature, the presence of *P. fluorescens* did not inhibit the survival of viable cells of *E. coli* O157:H7 on the meat. Due to the low infectious dose of *E. coli* O157:H7, even survival without growth on beef may pose a public health risk should contaminated meat not be sufficiently heat-treated. Unlike *P. fluorescens, L. plantarum* successfully inhibited the growth of *E. coli* O157:H7 on vacuum packaged beef loins. The onset of inhibition was influenced by the level of *L. plantarum* in the initial inoculum. Therefore, the effect of spoilage bacteria on the survival and growth of *E. coli* O157:H7 on meat depends on the type and levels of microorganisms present, as well as storage conditions. This study showed that *L. plantarum*, regardless of its levels on the meat, successfully inhibited the growth and survival of *E. coli* O157:H7.

4.5 CONCLUSIONS

There is minimal competitive interaction between *E. coli* O157:H7 and *P. fluorescens* on beef. Once meat has been contaminated with *E. coli* O157:H7, the level of *P. fluorescens* in the background flora does not inhibit the growth of *E. coli* O157:H7 on beef stored under aerobic conditions at 4 °C. *E. coli* O157:H7 was able to survive at levels that can cause foodborne illness regardless of the level of *P. fluorescens* present on meat. Farmers (primary production) who supply slaughter animals need to strengthen preventive strategies (i.e. feed manipulation and the addition of probiotics to animal feed) to eliminate the shedding of *E. coli* O157:H7 by slaughter animals. At abattoir (ante-mortem) level, it is vital that slaughter cattle handling techniques prevent contamination of meat with *E. coli* O157:H7. These include adequate and continual training of slaughtermen, particularly with regard to skinning and evisceration slaughter steps. Under vacuum packaging, *L. plantarum* culture treatment is beneficial in inhibiting the survival and growth of *E. coli* O157:H7 on meat. The higher the cell suspension of *L. plantarum* in the system, the earlier will be

the onset of the inhibition of *E. coli* O157:H7. Further studies to test the application of *L. plantarum* as a bio preservation technology are needed.

CHAPTER FIVE:
GENERAL DISCUSSION

In South Africa, the red meat abattoir industry is divided into those abattoirs that are linked to the feedlot and the wholesale sectors (class A and B) and those which are owned by farmers (classes C, D and E). Classes A and B abattoirs comply with all statutory measures including continual training of slaughter operatives and proper temperature control, while it is questionable if this is the case with the majority of the latter abattoir classes. Insufficient training of slaughtermen can lead to contamination of carcasses during the slaughtering process with pathogenic bacteria, which may proliferate under poor temperature control conditions. Inadequate cooking of contaminated meat could potentially cause diseases when this meat is consumed.

5.1 REVIEW OF METHODOLOGY

5.1.1 Swabbing of carcasses

In this study, the swabbing method (Gill & Jones, 2000) was used to collect sponge swab samples from chilled beef carcasses. *E. coli* O157:H7 and *Salmonella* spp. were among microorganisms under investigation in this study. Food pathogens have a low prevalence and a heterogeneous distribution on beef carcasses (Bolton, 2003). It would have been possible to use the excision method (Gill & Jones, 2000) to collect samples for microbiological analysis. However, the swabbing method was preferred because it provided an opportunity to collect swabs from a larger surface area (whole forequarter) compared to the area that could have been covered by using the excision method. The excision method would only cover a smaller area, usually about 20 cm^2 per carcass. Contradictory observations have been made by previous researchers when comparing swabbing and excision methods. Martínez, Celda, Anastasio, García and López-Mendoza (2010) compared the recovered bacterial loads from fresh beef carcasses by the excision or swabbing method. Martínez *et al.* (2010) recorded higher APC (4.50 log_{10} cfu/cm^2) from excision samples compared to 3.53 log_{10} cfu/cm^2

recovered from swab samples. In addition, Martínez *et al.* (2010) observed that the bacterial count recovered from swab samples was influenced by the type of swab used. Based on their results, the use of cellulose swabs, as used in this study, could not have been a limitation factor on the results. Higher bacterial counts were recovered from swabbing with abrasive sponges compared to viscose ones (Martínez *et al.,* 2010). However, care should be taken that the pressure of sponging should be as if the sampler is removing dried blood from the carcasses, while not being so hard as to crumble or destroy the sponge (Anonymous, 2007). Trenton *et al.* (2010) observed that the fat cover on the sampled site may prevent adequate removal of bacteria by occluding the pores of the sponge, thereby reducing removal of bacteria.

5.2 ISOLATION OF ENTEROBACTERIACEAE FROM CHILLED BEEF CARCASSES

Enterobacteriaceae counts from abattoirs that use HAS and those that use HAS + HACCP were found to be similar. The presence of Enterobacteriaceae on carcasses suggests transfer of faecal material onto the sterile carcass during the slaughter process, which may suggest that the currently available dressing procedures at both HAS and HAS + HACCP abattoirs cannot be relied upon in preventing faecal contamination during slaughter. In this study, the Enterobacteriaceae was enumerated as a whole by using VRBG agar without taking a further step to identify the specific Enterobacteriaceae members prevalent on beef carcass at abattoirs using different HMSs. This additional information could have provided insights into the effects of HAS and HAS + HACCP on different types of Enterobacteriaceae on beef carcasses. Moreover, comparison could have been made between abattoirs using the same HMS. Stiles and Ng (1981) used biochemical testing to identify and characterise 2220 Enterobacteriaceae from meats and identified principal Enterobacteriaceae types to be differentiated in meat.

5.3 ISOLATION OF *E. COLI* O157:H7 FROM BEEF CARCASSES

Beef and beef containing products have been implicated in both VTEC (Sartz, De Jong, Hjertqvist, Plym-Forshell, Alsterlund, Löfdahl, Osterman, Ståhl, Hansson & Karpman, 2008) and non-O157 VTEC outbreaks (Ethelberg, Olsen, Scheutz, Jensen, Schiellerup, Engberg, Petersen, Olesen, Gerner-Smidt & Mølbalk, 2004). In this study, the target VTEC was only *E. coli* O157:H7, whereby a prevalence of *E. coli* O157:H7 of 20% was recorded from samples collected after evisceration, 20% after final wash and 0% after chilling from an abattoir with a combined HAS + HACCP system. The absence of *E. coli* O157:H7 after chilling could be due to the effects of chilling, a step in animal slaughter that is known to reduce the numbers of generic *E. coli* on carcasses. It is reasonable to assume that when present, *E. coli* O157:H7 is on the surface of carcasses, not in internal tissues of intact muscle that normally is protected from surface contamination during slaughter. A combination of rapid chilling of adequately spaced carcasses and surface dehydration during chilling should retard *E. coli* O157:H7 growth on carcass surfaces (Butler *et al.*, 2006).

The occurrence of non-O157:H7 should have been investigated, which could have given a better picture of the prevalence of VTEC on beef. Arthur *et al.* (2002) isolated 361 non-O157 STEC isolates from beef carcasses where 49% of the O serogroups had previously been associated with diseases. It is well documented that many methods used in *E. coli* O15:H7 investigation, as was the case in this study, include the use of selective agents such as novobiocin or acriflavin to inhibit the growth of gram-negative organisms and a combination of vancomycin, cefsulodin and cefixime to suppress the growth of *Aeromonas* spp. and *Proteus* spp. However, Stephens and Johnson (1998) illustrated the toxicity of these agents towards stressed *E. coli* O157:H7 cells due to the presence of bile salts and antibiotics in selective enrichment media (Stephens & Johnson, 1998). Therefore, chilling may not have killed the cells that were isolated after evisceration and washing, but they may have been unculturable.

5.4 ISOLATION OF *SALMONELLA* SPP. FROM CHILLED BEEF CARCASSES

Meat carcasses may be contaminated with *Salmonella* spp during slaughtering (Marlony *et al.*, 2008). In this study, the prevalence of *Salmonella* spp from beef carcasses was only investigated after chilling, whereby, *Salmonella* spp. were not isolated from any of the samples. The chilling process can injure bacterial cells as a result of low carcass surface a_w and low temperature (McEvoy, Sheridan & McDowell, 2010). Therefore, an investigation of the prevalence of this organism during the different steps of animal slaughter could probably have given a better indication of the efficacy of the HMS used in preventing contamination of carcasses with *Salmonella* spp. In this study, the method used was both labour and growth media intensive and relied on *Salmonella* specific biochemical tests, such as the production of hydrogen sulphide, non-fermentation of lactose and sucrose or xylose to distinguish *Salmonella* from other members of the Enterobacteriaceae. This method could have been modified by including a thin layer agar (TAL) of a non-selective medium such as tryptic soy agar (TSA), over layered on pre-poured and solidified selective medium plates of xylose lysine decarboxylase (XLD) medium. This method would have simultaneously facilitated the recovery of injured *Salmonella* cells, while also providing the selectivity of isolation of *Salmonella* spp. (Kang & Fung 2000). The advantage of the TAL method is that it allows injured *S. typhimurium* to be resuscitated and grow on the TSA layer, while selective agents from the XLD medium diffused to the top thin TSA layer. Once the selective agents diffused to the top agar layer, the resuscitated *Salmonella* spp. cells would start to produce a typical reaction (black colonies) and other microorganisms would be inhibited by selective agents diffused from XLD.

Alternatively, chromogenic media could have been used. Chromogenic media are microbiological growth media that contain organism specific enzyme substrates, linked to a chromogen. The target populations are characterized by enzyme systems that metabolize the substrate to release the chromogen, which then results in a colour change in the medium. Perez *et al.* (2003) established that chromogenic media

demonstrate higher specificity compared to conventional *Salmonella* spp. growth media. Similarly, Schönenbrücher, Mallinson and Bülte (2008) recovered 100% *Salmonella* from artificially inoculated raw meat when using chromogenic media (Miller-Mallinson, AES *Salmonella* Agar Plate Agar and Oxoid *Salmonella* Chromogen Media Agar) compared to traditional media. Moreover, Schönenbrücher *et al*. (2008) found that a combination of the enrichment in Selenite Cystine broth for 24 hours and chromogenic plating media significantly increased the recovery of *Salmonella*. The higher specificity of the chromogenic media also reduces the need for confirmatory tests, thereby cutting technical time and reagent requirements.

5.5 SAMPLE PREPARATION FOR *E. COLI* O157:H7 SURVIVAL STUDIES FROM ARTIFICIALLY CONTAMINATED BEEF LOINS

In this study, a constant inoculation level 10^2 cfu/ml of *E. coli* O157:H7 was used together with either 10^2 or 10^6 of *P. fluorescens* or 10^2 and 10^4 cfu/ml of *L. plantarum*, respectively, to contaminate sterile beef loins. Although the infectious dose of *E. coli* O157:H7 is unknown (Food and Drug Administration, 1998), Paton and Paton (1998a) estimated that the infectious dose of *E. coli* O157:H7 ranges from 1 to 100 cfu/cm^2. Since the level of *E. coli* O157:H7 was held constant at 10^2 cfu/cm^2, it would have been interesting to see how a lower inoculation level could have been affected by the lower levels of *P. fluorescens* and *L. plantarum*, considering that natural contamination of these bacteria is lower than the levels used in this study. The reason for using higher levels in this study was to ensure that the effects of *P. fluorescens* and *L. plantarum* were more apparent.

5.6 EFFECT OF HAS ALONE AND HAS COMBINED WITH HACCP ON THE MICROBIOLOGICAL QUALITY OF FRESH BEEF

HACCP systems identify all the steps throughout the chain where contamination deviations may occur, including measures taken on the farm, the quality of animal feed and handling of animals from farm to the abattoir. In the abattoir the HACCP system seeks to identify all steps in the slaughtering process where biological hazards

can either be minimized or eliminated during slaughter. These steps are referred to as critical control points (CCPs). The HACCP system, therefore, allows for quick corrective measures in those situations where deviations from the set controls in the CCPs are noted, thus assuring the safety and quality of the final product (Pinillos & Jukes, 2008). This study demonstrated that the microbiological quality of beef derived from HAS combined with HACCP abattoirs is not microbiologically superior compared to that of fresh beef derived from HAS abattoirs. APC counts from all abattoirs were similar. The highest Enterobacteriaceae count was recovered from one of the abattoirs that use the HAS + HACCP system. In addition, *E. coli* O157:H7 was isolated from that same abattoir. The positive isolation of *E. coli* O157:H7 from an abattoir that uses the HAS + HACCP system is in agreement with Josling (2004) who found that though risk assessment is a good preventive system, it leaves room for substantial uncertainty over the incidence of risk. Furthermore, Sperber (2005) attributed the occurrence of foodborne outbreaks in the USA, despite the widespread use of HACCP, to failures of cleaning and sanitation, or the lack of management awareness and commitment to provide the necessary training and resources to support HACCP plans.

5.7 SURVIVAL OF *E. COLI* O157:H7 CO-CULTURED WITH DIFFERENT LEVELS OF *PSEUDOMONAS FLUORESCENS* AND *LACTOBACILLUS PLANTARUM* ON FRESH BEEF

In this study, *E. coli* O157:H7 was challenged with different levels of *P. fluorescens* and *L. plantarum*, yet in reality *E. coli* O157:H7 may occur simultaneously with other VTEC on beef. The results of this study showed that 18 h cultures of *E. coli* O157:H7 and *P. fluorescens* required 2 days to adapt and start growing on meat stored aerobically at 4 °C. When bacteria are deposited onto meat surfaces, they undergo a period of adjustment in their new environment, where cell damage is repaired and bacteria adapt to utilize nutrients available before they can start to grow (Sentance & Husband, 1993). Temperature influences the lag phase and growth rate of *E. coli* including serotype O157:H7 (Duffy *et al.* 1999). There are instances where the cold chain may be broken, exposing raw meat to temperatures that promote the growth of

both pathogenic and spoilage bacteria. The challenge study with the same levels of *E. coli* O157:H7 and *P. fluorescens* at abusive temperatures (10 °C) would provide an indication of the length of the lag phase of this organism on beef loins. An antagonistic interaction between *E. coli* O157:H7 and *P. fluorescens* was observed, as evidenced by *E. coli* O157:H7 that stopped growing while *P. fluorescens* continued to grow to its maximal growth density at day 7. Nevertheless, the results of this study showed that *P. fluorescens* is unable to affect the growth of *E. coli* O157:H7 on aerobically stored beef, regardless of the levels in the inoculums. Therefore, improper handling of meat and meat products, as well as the lack of maintenance of the cold chain, which is more prevalent under non-commercial conditions in South Africa, may support the growth and spread of *E. coli* O157:H7. It would have been interesting to investigate the survival of *E. coli* O157 in the presence of a low level of a non-O157:H7 strain together with *P. fluorescens*.

Vacuum packaging is a widely used practice in meat marketing. Prime meat cuts are vacuum packaged for distribution and extension of storage life. However, in South Africa, vacuum packaging is used in only the commercial sector, yet the South African meat industry comprises both the commercial and the informal sector, with slaughter of own-use also being allowed. In this study, *L. plantarum,* under vacuum storage resulted in total kill of *E. coli* O157:H7 on beefsteaks. The onset of inhibition was influenced by the level of *L. plantarum* in the initial inoculums, with total kill being recorded earlier when the level of *L. plantarum* in the inoculums was 10^6 cfu/ml. The effect of spoilage bacteria on survival and growth of *E. coli* O157:H7 on meat depends on the type and levels of microorganisms present and storage conditions. *L. plantarum* regardless of the level completely eliminated *E. coli* O157:H7. It would have been interesting to see how an acid-adapted *E. coli* O157:H7 strain could have been affected by *L. plantarum*.

5.8 AREAS FOR FUTURE RESEARCH

Further research work needs to be conducted using molecular techniques to assess the effect of HAS alone and HAS + HACCP on the microbiological quality of beef.

Molecular techniques e.g. are rapid, specific and more sensitive compared to traditional methods, which are lengthy and media intensive. Rapid methodologies may allow for more visits to abattoirs under investigation.

The Enterobacteriaceae family includes indicator organisms for faecal contamination. A study using molecular techniques should be conducted to identify the specific organism within the Enterobacteriaceae family to elucidate the influence of HMS on the range of Enterobacteriaceae species occurring on beef carcasses.

In South Africa, there is a need to conduct an investigation on the occurrence of VTEC on live animals both at feedlots and free range beef animals compared to the levels of VTEC on beef carcasses and retail samples. This must include both *E. coli* O157:H7 and non-O157 *E. coli* that have also been implicated in sporadic foodborne outbreaks. This investigation could be expanded to other animal species such as sheep, goats and pork.

L. plantarum inhibited the growth of *E. coli* O157:H7 on vacuum packaged beef loins. Further research needs to be conducted to determine the level of *L. plantarum* that inhibits *E. coli* O157:H7 without adversely affecting the sensory qualities of vacuum packaged beef.

CHAPTER SIX:
CONCLUSIONS AND RECOMMENDATIONS

The HAS system has been shown to be an effective tool for assessing hygienic practices at abattoirs, as statistically similar bacterial counts, i.e. to those at HAS + HACCP abattoirs, were recorded. However, to provide an increased assurance that pathogens are detected rapidly and corrective action taken, a HAS + HACCP system would be a preferred hygiene management system, which would also function as a base for a proper traceability system. In South Africa, presently, only a single abattoir is listed as an exporter of beef to the European Union. Strengthening of hygiene management systems could improve beef export opportunities.

The level of *P. fluorescens* and low storage temperature do not inhibit the survival of *E. coli* O157:H7 on beef. *E. coli* O157:H7 survives at levels that can cause foodborne illness regardless of the level of *P. fluorescens* present on meat. Slaughter animal farmers, therefore, need to strengthen preventive strategies to eliminate the entry of *E. coli* O157:H7 into the food chain.

Under vacuum packaging, the combination of the influence of the level of *L. plantarum* and low storage temperature inhibited the survival of *E. coli* O157:H7 on beef loins. The higher the cell suspension of *L. plantarum* is, the earlier the onset of the inhibition of *E. coli* O157:H7 survival on beef.

In South Africa, information on food poisoning outbreaks implicating *E. coli* O157:H7 is scanty. Yet there are reports (Ateba *et al*., 2008; GPDoA, 2004) illustrating that *E. coli* O157:H7 was isolated from patients as early as 1988 in Pretoria (Geyer, Crewe-Brown & Greeff, 1993). Moreover, a study carried out in 1993 at Dr George Mukhari Hospital (previously known as Ga-Rankuwa Hospital) in Pretoria revealed that the most prevalent organism in paediatric patients diagnosed with gastroenteritis was ETEC (Sooka, Du Plessis & Keddy, 2004). In addition, *E. coli* O157:H7 has been positively isolated from live animals (both commercial and

communal), their faeces and carcasses and even from ready-to-eat meat products (Ateba *et al*., 2008). The revelation that *E. coli* O157:H7 is prevalent in South Africa does not correspond with the lack of reported cases, a situation that could probably be attributed to inadequate surveillance for this organism in South Africa, where testing is still voluntary. Food regulatory authorities, such as the DoA in South Africa, need to enforce "zero tolerance" towards *E. coli* O157:H7 in raw meat to protect the entry of this pathogen into the domestic market and also to be on par with prospective international trading partners. Contamination of raw meat with *E. coli* O157:H7 is a vehicle of transmission into the food chain. Improved vigorous mandatory testing for VTEC from farm-abattoir to retail needs to be established so that areas that need more control can be identified, such as slaughtering *E. coli* O157:H7 shedding animals separately from non-shedding animals.

Abattoir owners need to invest in training of slaughter personnel to ensure that all workers including management take ownership of hygiene practices during animal slaughter and during further processing. Contamination of raw meat can occur as a result of inadequate cleaning of surfaces, equipment and hands of food handlers. Moreover, it has been reported that at some abattoirs, remuneration of slaughter operatives is illegally based on the number of cattle slaughtered per day. This practice encourages slaughter operatives to increase the line speed to achieve a desired number of cattle slaughtered but unfortunately compromises hygiene. A too high line speed may lead to spillages and cross contamination during evisceration.

One of the reliable means of ensuring safety of meat is thorough cooking. The South African meat industry needs to embark on awareness campaigns for consumers to ensure that raw meat is handled properly at all levels of the food chain. In cases of animals not slaughtered at registered abattoirs (ritual slaughter, slaughtering of animals for own consumption and traditional slaughtering for special occasions such as weddings) cattle are often transported in unsuitable vehicles, a condition that may result in stressed animals leading to increased shedding of pathogenic microorganisms. This situation may increase contamination of animal carcasses with pathogenic bacteria and cross contamination of ready-to-eat foodstuffs. In this regard,

awareness could be created if the relevant authorities worked closely with communities and encouraged the presence of environmental health practitioners to render meat inspection services at community level.

CHAPTER SEVEN:
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