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

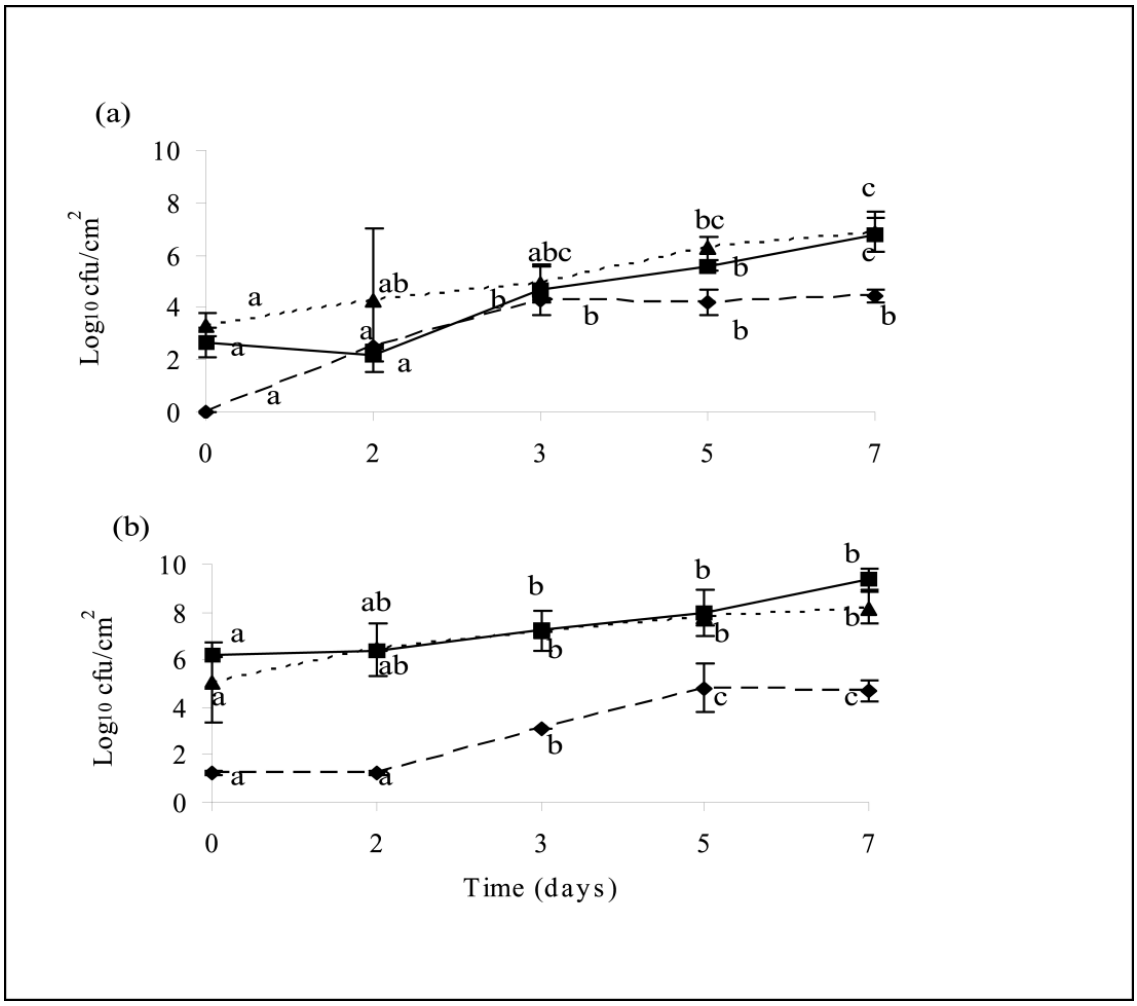
When similar levels (10^2 $\log_{10}$ cfu/ml) of *E. coli* O157:H7 and *P. fluorescens* were combined, *E. coli* O157:H7 was not recovered from beef on day 0 (Figure 4.1a). By day 2 of storage, the *E. coli* O157:H7 count reflected the level that was inoculated. *E. coli* O157:H7 count had significantly ($p<0.05$) 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

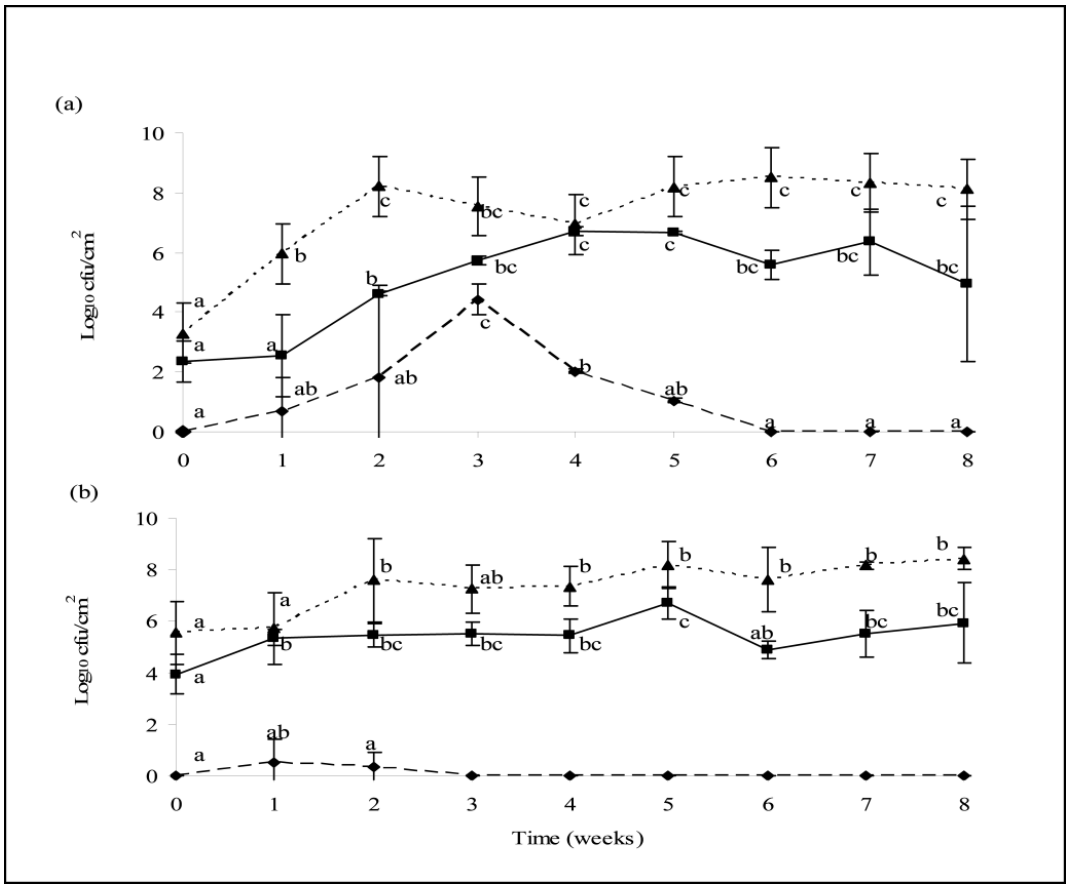
When the same inoculum level was used for *E. coli* O157:H7 and *L. plantarum* (10^2 $\log_{10}$ cfu/ml) in the suspension (Figure 4.2a), *L. plantarum* did not show immediate inhibition of *E. coli* O157:H7. There was a similar numerical increase in the *E. coli* O157:H7 count recorded at weeks 1 and 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.