Microbial quality and safety of ostrich meat

By

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University of the Western Cape

Supervisor: Professor P.A Gouws

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DECLARATION

I, the undersigned, declare that **'Microbial quality and safety of ostrich meat'** is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

Miss Anya Cloete

2203959

Signature………………………….

Date……………………………….

This thesis represents a compilation of articles where each chapter is an individual entity and some repetition between chapters has been unavoidable. The style of this thesis is in accordance with that of the *Journal of Applied Microbiology.*

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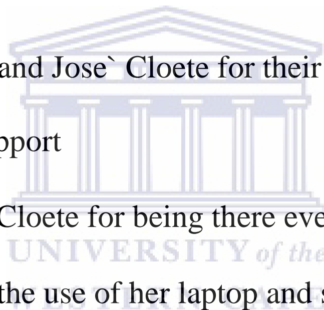

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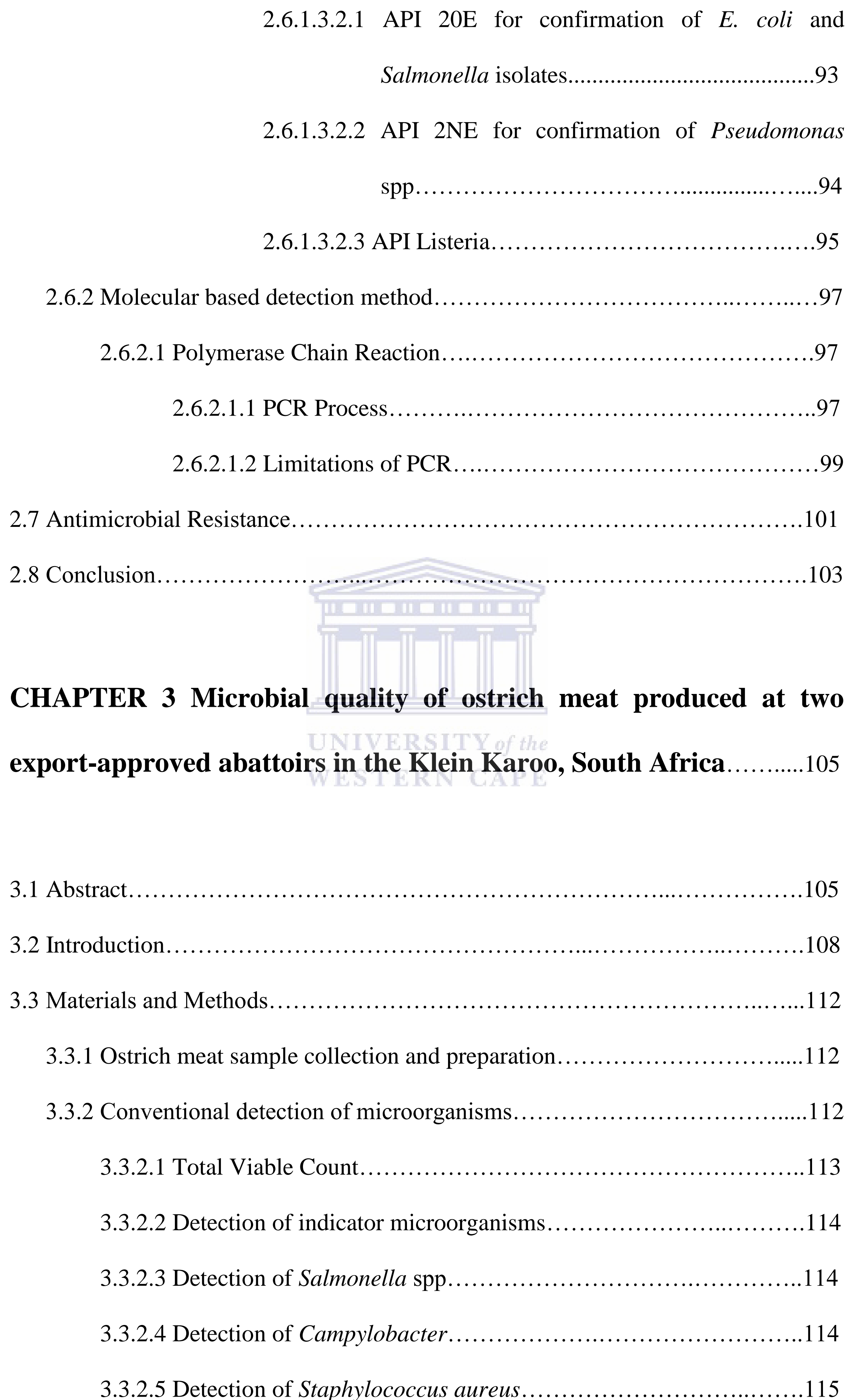
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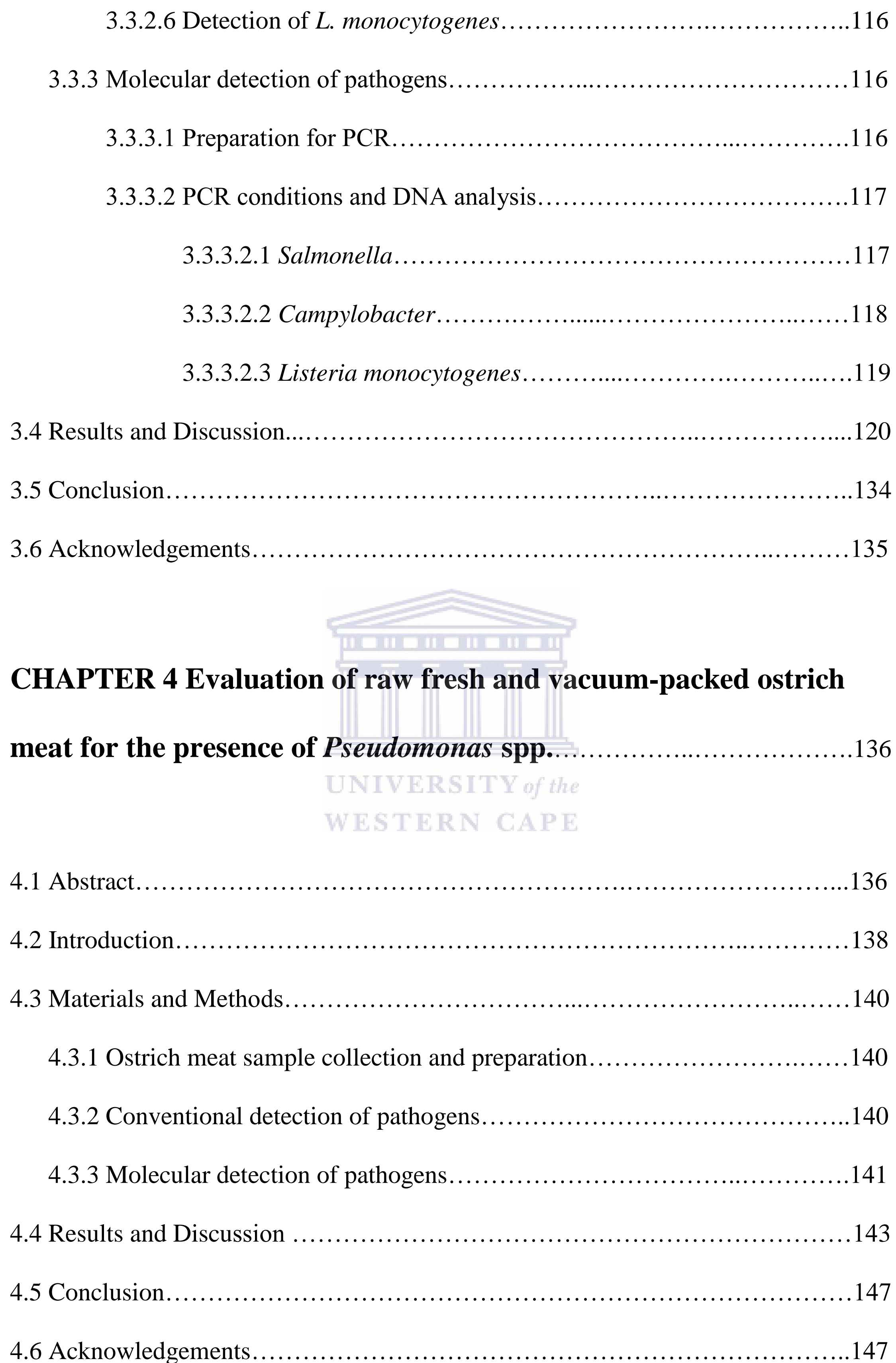

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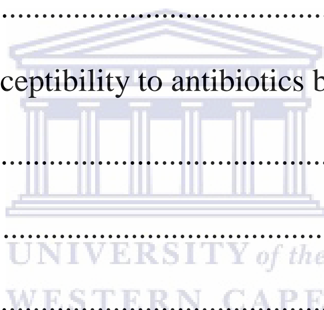
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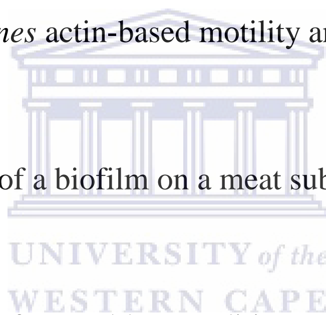
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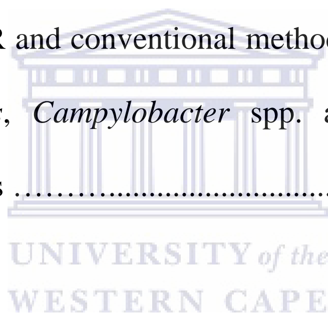
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LIST OF ABBREVIATIONS

- AAF Aggregative adherence fimbriae
- Act A Actin polymerizing protein A
- API Analytic profile index
- α Alpha
- a_w Water activity
- β Beta
- bp Base pair
- BPW Buffered peptone water
- BHI Brain heart infusion
- CDSC Communicable Disease Surveillance Centre
- CFSAN Center for Food Safety and Applied Nutrition
- cfu/g^{-1} Colony forming units per gram
- cfu/cm^2 Colony forming units per centimeter
- DNA Deoxyribonucleic acid
- DFD Dark, Firm, Dry
- eg. Example
- et al. And others
- FB Fraser broth
- FDA Food and Drug Administration
- FSAI Food Safety Authority of Ireland
- GC Gaunine-Cytosine
- g Gram

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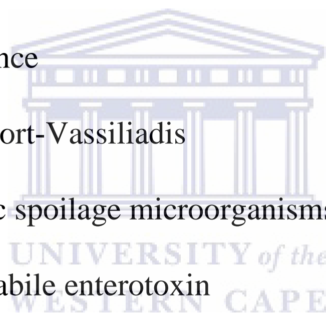
- h Hour
- LLO Listeriolysin O
- LT Heat-labile enterotoxin
- max Maximum
- ml Milliliter
- min Minimum
- min Minute
- mM Millimolar
- MUG 4-methyl-umbelliferyl-β-D-glucuronic acid
- PCR Polymerase Chain Reaction
- R Resistance
- RV Rappaport-Vassiliadis
- SSO Specific spoilage microorganisms
- ST Heat-stabile enterotoxin



- Spp. Species
- S Susceptible
- Taq *Thermus aquaticus*
- TBA Tryptose blood agar
- temp. Temperature
- TSA Tryptone soya broth
- TVC Total viable count
- UV Ultra Violet light
- VRBA Violet red agar
- XLD Xylose lysine deoxycholate
- µ Micro

- μl microliter
- μm micrometer
- ºC Degrees celsius
- < Less than
- > More than

CHAPTER 1

1.1 INTRODUCTION

An emerging public health hazard requiring immediate attention is food safety. Concerns for food safety were once focused only on residues of toxic agents, which can accumulate in meat. The discovery of microbial pathogens that do not affect animals but can cause human illness has changed the focus of concern. The presence of pathogenic microorganisms such as *Salmonella* species, *Listeria monocytogenes*, *Campylobacter* species and *E. coli* on foods pose a food poisoning threat and following a number of recent high publicity food-related health scares, consumer concerns regarding the safety of food are now a priority (Knowles *et al*. 2007; Ellis and Goodacre 2001). Consumers demand the production of high quality, pathogen-free food and failure by industries to produce according to these high hygienic standards has a direct influence on production efficiency by loss of production and consumer confidence of product (Van Donk and Gaalman 2004). This demand and the economic implications of gastro-enteritis are increasing pressure on the food industry to reduce numbers of contaminating and pathogenic organisms in food (Brown *et al.* 2000).

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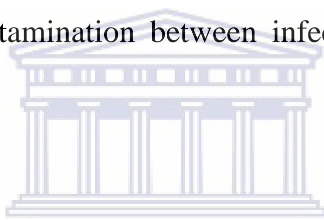
As the ostrich industry turns from sales of breeding stock to the production of ostrich products for revenue, it is important for the industry to know as much as possible about the products that can be derived from these birds. Given the considerable national and international demand for ostrich meat, it is therefore the responsibility of the ostrich meat processing industries to assess and assure microbiological quality and

safety of their meat products. Recognized as the leading cause of human gastroenteritis in developed countries are food-borne diseases. The major bacterial agents causing food-borne diseases include *Campylobacter* species, *Escherichia coli*, *Listeria monocytogenes, Salmonella* species and *Staphylococcus aureus* (Singh and Prakash 2008; Doyle *et al.* 2006). Each of these bacterial agents have uniquely adapted to the conditions established by current food production and distribution systems, and may easily be introduced into slaughterhouses by farm animals that harbour them, food handlers or pests, thus contamination of meat may occur during processing (Gill and Hamer 2004; Adams and Motarjemi 1999). The slaughter process contributes to the prevalence of foodborne pathogens through contamination of the carcass and cross-contamination between infected and uninfected carcasses (Horrocks *et al.* 2009).



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Spoilage caused by bacteria cause significant economic loss for food industries (Rodríguez-Calleja *et al.* 2005). The microorganisms that have been identified as playing a major role in the onset of food spoilage belong to the genus *Pseudomonas.* These psychrotrophic *Pseudomonas* species pose a significant spoilage problem in refrigerated meat and meat products due to their ability to produce and secrete hydrolytic enzymes such as lipases and proteases (Nychas *et al.* 2008; Ellis and Goodacre 2001).

Ostrich meat samples in previously published studies were analysed only by conventional methods (De Freitas Neto *et al.* 2009; Ley *et al.* 2001). These conventional methods were based on the detection of microorganisms by selective media such as Pseudomonads Isolation Agar for isolation of *Pseudomonas* spp.,

Violet Red Bile Glucose agar for isolation of *Enterobacteria*, Violet Red bile Agar Medium for isolation of coliforms and Baird Parker agar for isolation of *Staphylococcus aureus* (Seydim *et al.* 2006; Alonso-Calleja *et al.* 2004). Traditional techniques for the detection of food-borne pathogens and spoilage microorganisms rely primarily on enrichment procedures and the isolation of presumptive colonies of evaluated microorganisms on solid agar media. At present conventional microbiological techniques are increasingly being supplemented by a new generation of rapid or alternative methods, such as the polymerase chain reaction (PCR), enzyme linked immunosorbent assay (ELISA) and mass spectrometry, able to produce results much more quickly and reliably (Yang and Bashir 2008; Mothershed and Whitney 2005). The most powerful tool for ensuring rapid and specific detection of pathogenic bacteria in a wide variety of samples today is the PCR technique (Albuquerque *et al*. 2009). Traditional and molecular analysis for the detection of these above mentioned food-borne pathogens and spoilage microorganisms are essential for implementation of food quality control measures to minimize the risk of infection to consumers' as well as the economic impact of their diseases (Malorny *et al.* 2003).

The supplementation of animal feed with antibiotics such as tetracyclines, macrolides and penicillins to reduce the risk of epidemics is increasing in animal husbandries (Ungemach *et al.* 2006). Antimicrobials administered to animals through feed and water is generally administered as feed additives (Wegener *et al*. 2003). Resistant strains emerge from these widespread abuses of antibiotic supplements in meat industries. The prevalence of antimicrobial resistance among foodborne pathogens may be a potential problem in the treatment of humans with antibiotics. This practice in animal husbandries may lead to the development of resistance to one or even

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multiple antibiotics in foodborne pathogens, which could ultimately be transmitted to human beings via the food chain (Angulo *et al.* 2004; Gouws and Brözel 2000).

Due to the lack of scientific information on the microbiological status of ostrich meat worldwide, this would be the first in-depth scientific study done on the microbiological quality and safety of fresh ostrich meat by means of both conventional and molecular techniques. The study will assure the microbiological quality and safety of ostrich meat. It will also allow for the implementation of food quality control measures if food-borne pathogens are detected. Other benefits include the provision of rapid, sensitive and economic techniques for detection of the principal food-borne pathogens.

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The aim of this study was to determine the quality of slaughtered ostrich meat and to evaluate the ostrich slaughter process, to determine whether ostrich meat are contaminated by the in-house slaughtering practices and if prevalence of microorganisms increase with the succession of the slaughter process. Furthermore, the presence of specific foodborne pathogens and spoilage organisms was explored by means of molecular and conventional methods to determine whether ostrich meat is a source of these microorganisms. Data obtained from this study provides some baseline information that could be used in future studies on system contamination and the extent of downstream processing steps in the production of ostrich meat. Antimicrobial resistance has become a growing area of concern in both human and veterinary medicine, it is therefore necessary that another aim of this study was to determine the antibiotic resistant pattern of *Staphylococcus aureus* in ostrich meat in

order to establish whether *Staphylococcus aureus* strains isolated from ostrich meat samples show resistance to antibiotics.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

South Africa is the most important country in the international industry of ostrich production. Of the worlds demand for ostrich products 90% was supplied by South Africa in 1993 (Trent *et al.* 2003). Ostrich farming started 146 years ago in South Africa in 1864. The focus many years ago was on slaughtering the ostrich only for its feathers and skin (SAOBC 2005(a)). The ostrich meat was given away to the labour force and local people because the bird was not seen as a meat-producing animal. When the ostrich market for feathers collapsed in 1914, ostrich farming disappeared from many countries except South Africa. Revival of the ostrich trade brought upon the importance of skin products and with it an increasing market for ostrich meat (Adams and Revell 2004; Gill 2007).

At present ostrich production has become one of South Africa's top twenty agro-based industries. The values of ostrich meat compared to the rest of the bird (feathers, skin) have increased over the last few years (Hoffman 2005). Ostrich feathers, leather and meat have contributed 5%, 50% and 45% respectively to the income of ostrich production. It was estimated that in 2002, South Africa slaughtered 310 000 ostriches compared to the rest of the world's 80 000 ostriches (SAOBC 2005 (b)).

The most popular ostrich (figure 2.1) farmed in South Africa is the *Struthio camelus var. domesticus,* because it is commonly found in South Africa (Hoffman 2005). Adult males range from 2 to 3 meters tall and may weigh up to 150kg or above. Ostriches are flightless birds and due to their large frame have very long, sturdy legs. The reliance on their legs rather than their wings for movement means that all muscle development occurs in the legs, thighs and back (Adams and Revell 2004). Ostriches in husbandry practice are bred for desirable qualities and are smaller than the wild birds (Gill 2007).

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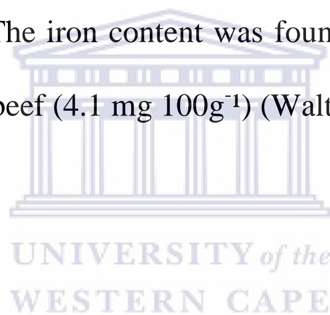
Figure 2.1 Male and female ostrich (*Struthio camelus var. domesticus*)

Ostriches are considered poultry even though they produce a mild fine-grained red meat (Figure 2.2), which tastes similar to beef. Ostrich meat is fast becoming a competitor in the red meat market because of its increasing popularity in western society, due to its nutritional value (Alonso-Calleja *et al.* 2004). The meats tenderness, low fat content and cholesterol levels make it an alternative healthy red meat. It's high protein (22.2%) and low collagen (0.16%) makes ostrich meat more digestible than beef (20.1% and 0.18% respectively). The low fat content of ostrich meat (1.6%) compared to turkey (3.8%) and bovine (4.5%) makes it of particular interest. The cholesterol content of ostrich meat (33.8 mg $100g^{-1}$) was found to be lower than turkey (36.6 mg $100g^{-1}$) and bovine (50.1 mg $100g^{-1}$) (Palearri *et al.* 1998; Hoffman and Mellet 2003). The iron content was found to be higher in ostrich meat (5.9 mg $100g^{-1}$) compared to beef (4.1 mg $100g^{-1}$) (Walter *et al.* 2000).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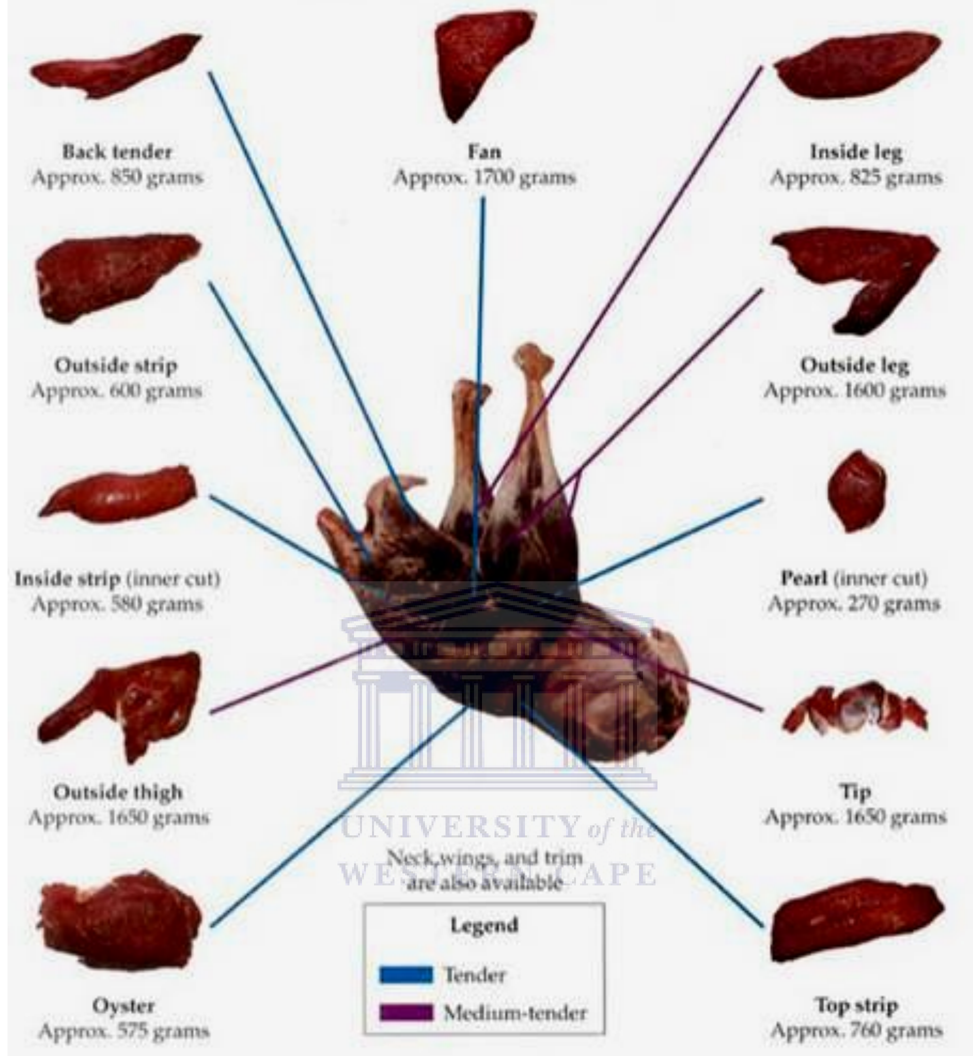

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Figure 2.2 Ostrich portions

2.2 OSTRICH HUSBANDRY IN SOUTH AFRICA

The current status of the ostrich market forces ostrich husbandries in South Africa to concentrate on issues such as improving productivity, reducing incidence of disease, improving overall farm efficiency, increasing yields per bird and reducing costs in order to succeed in the current highly competitive market (Black 2001). The success of healthy flocks on a farm is dependent on factors such as the experience and skill level of the consulting veterinarian, willingness and ability of farm management to respond to veterinary advice, the quality of the ostrich facility's infrastructure and record keeping and the availability and quality of nutrition (Aganga *et al.* 2003; Black 2001).

2.2.1 Ostrich Facilities

Three basic types of farming systems exist for ostrich production; these are governed by the price and availability of land, scale of production and labour and feed costs. The three types include the extensive farming system which requires a large area of land, since birds are kept and raised with little interference and as close as possible to their natural habitat; the semi-intensive farming system which involves birds being kept in relatively small paddocks or territories of 8 to 20 ha in which they are able to roam freely to a certain extent; and the more popular intensive farming system which consists of a small land area divided into small paddocks of 1-2 ha each (Shanawany and Dingle 1999). An ostrich facility that practices the intensive farming system usually contains breeding pens and colonies, incubation and hatching facilities, infant chick rearing pens, older chick rearing pens and juvenile growing paddocks or pens

(Black 2001). A higher incidence of bacterial and fungal diseases in chicks, birds and eggs is generally brought on by wet climates and hot, high humidity climates; it is therefore imperative that shelters, nest site protection, heating and extra feeding are provided in these farming systems (Black 2001; Hermes 1996). Ostriches raised for slaughtering in the Klein Karoo are generally kept intensively in feedlots (80%) and on pasture (20%), while 60% of slaughter birds in the Southern Cape are raised on pasture and 40% intensively in feedlots (Bels 2006).

2.2.2 Ostrich Feed

Nutrition plays a vital role in ostrich production as the relative size of the lower digestive tract as well as the ability to obtain more nutrients from the diet is affected by the type of feed accessible to ostriches (Bels 2006). Common feed ingredients (Table 2.1) include maize meal as a source of concentrated energy; lucern (alfalfa) as a bulking, fibre and protein source; fish meal and peanut meal as a crude protein and essential fatty acids source; and carcass meal as an important source of protein and amino acids (Aganga *et al*. 2003).

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Table 2.1 Composition of ostrich diets and the quantity consumed (g/kg) at various stages of growth (adapted from Aganga 2003)

Ingredient	1 to 6 weeks	3 to 13 weeks	13 to 40 weeks	Maintenance
Maize meal	600	520	550	550
Fish meal	100	65	16.3	0
Peanut oil cake	200	130	32.5	0
Carcass meal	0	30	15	10
Alfalfa	60	260	370	410
Lysine HCl	2.5	1.63	0.41	0
Methionine	2	1.22	0.31	0
Vit/min premix	2.5	1.22	0.41	1
Monocalcium phosphate	13.68	1.7	13.3	17
Limestone	10.6	0	5	8
Fine salt	1.5	2	2.2	2.3
ME MJ/kg	12.23	10.77	9.67	9.17
Protein %	21.5	20.1	13.9	11.8
Lysine %	1.12	0.99	0.62	0.5
Calcium%	1.15	0.96	1.14	1.25
Avail. Phos%	0.64	0.46	0.48	0.49

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Lucerne is the most common cultivated pasture used for grazing ostriches and is mechanically harvested and fed to birds as chopped green feed or as a hay component of a balanced diet or as silage in feedlots (Bels 2006; Black 2001; Hermes 1996). The pelleted form of ostrich feed is the easiest to use in ostrich production as it results in less wastage, minimal dustiness, less separation of feed ingredients and increases in animal performance (Bels 2006; Aganga *et al.* 2003).

2.3 MICROBIAL QUALITY OF OSTRICH MEAT

Healthy animals and poultry carry a very large and diverse microflora that may include human pathogens in their intestines, while their muscle tissues are almost entirely free from microorganisms (Adams and Motarjemi 1999). The growth of several spoilage and pathogenic bacteria are supported by an ideal substrate presented by meat and meat products. These microorganisms are not inhibited by intrinsic factors of fresh meat such as pH (5.5 – 5.9) and water activity (1.00-0.98) (Mataragas *et al.* 2008; Ellis and Goodacre 2001). Many of the microbial pathogens of current concern survive in the environment, in water, on pastures and in food, unless precautions are taken to ensure pathogen control. Contamination of raw meat with human foodborne pathogens is a consequence of a wide range of pre-slaughter, slaughter and post-slaughter factors. Meat may support a mixed population of microorganisms derived from the initial animal's natural microflora, those introduced during slaughter and subsequent handling, processing and storage (Adams and Motarjemi 1999). Hygienic production of carcass meat is essential to ensure that contamination with potentially pathogenic bacteria is minimised (Bolder 2007; Gill 2007; Mead 2004).

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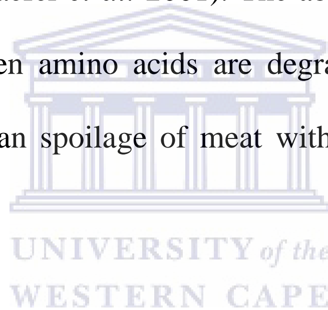
2.3.1 Pre-slaughter sources of meat contamination

2.3.1.1 Dark, Firm, Dry (DFD) meat

Muscular fatigue or excessive stress before slaughter depletes glycogen levels in the muscle and blood. Reduction in glycogen levels result in lower production of lactic acid and a high final pH (Rosenvold *et al.* 2001). These conditions make the meat more favourable for bacterial spoilage and the growth of foodborne bacteria. DFD tissue does not only have a final higher pH, but is also deficient in glucose and glycolytic intermediates (Schaefer *et al.* 2001). The absence of glucose in the tissues results in rapid spoilage when amino acids are degraded by Pseudomonads. DFD tissues spoil more rapidly than spoilage of meat with a normal pH (Jackson *et al.* 1997).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2.3.1.2 Transport and Lairage

Occurrences of *Salmonella* and *Campylobacter* among animals have been shown to increase when amalgamating and stressing animals during transportation and in the lairage (Black 2001). Stressing animals in this way can cause a disturbance of intestinal functions and may lower the resistance of the live animal and increase spreading of intestinal bacteria (Keener 2004). The close proximity of the animals increases the likelihood that bacteria will be transferred from animal to animal (Shea 2004). Infection in infected pens intensifies when the numbers of shedding animals increase through re-activation of latent infections and/ or exposure to environmental pathogens on uncleaned transporting vehicles and lairage pens. Non-infected pens

intensify with infection when animals are exposed to pathogen-contaminated trucks and lairage pens or to amalgamations with infected pens of animals on the transporting vehicle or in the lairage pens. Animals acquiring the infection orally or through aerosols during transportation and lairage shed faeces within a few hours of infection (Anonymous 2000; Reid *et al.* 2002a; Reid *et al.* 2002b).

2.3.1.3 Environmental Contaminations

2.3.1.3.1 Water

An important potential vehicle in spreading potential human pathogens among exposed animals is water when contaminated by animal or human faeces. Water troughs may serve as *E. coli* 0157:H7 (Haemorrhagic *E. coli*) reservoirs for animals and running or stagnant environmental water may serve as *Campylobacter* species sources (Black 2001). The presence of faecal matter in troughs influences the survival of microbes and potentially affects the extent to which water serves as a pathogen vehicle (Doyle and Erickson 2006; Doores 2005).

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2.3.1.3.2 Soil

Fertile soil may contain millions of bacteria per gram. Soil bacteria easily contaminate animals on the outside through either direct contact or aerosols (dust). When animals are slaughtered, these bacteria can contaminate the tissues inside (Macrae *et al.* 1993). Animals held in lairage without adequate drainage frequently results in faecal soiling of the skin (Trent 2003). In order to decrease or prevent variable amounts of manure,

bedding and soil from adhering to feathers and skin, ostrich pens should be sited on well drained soil that never becomes waterlogged (Adam and Revell 2004). Ostrich skin, feathers and feet harbour microorganisms both inhabitant to the skin as well as those from faeces, bedding and soil, which may potentially contaminate the carcass during the slaughter process.

2.3.1.3.3 Wildlife and Pests

Significant sources of enteric pathogens include wildlife, pests, insects and pets roaming unreservedly on farms (Acar and Moulin 2006). Pests such as rodents have been recognised as potential sources, as they easily transfer infections between households and may contaminate stored feeds via their fur, urine, feaces or saliva (Adams and Motarjemi 1999). Wild bird populations infected with salmonellae has been correlated with proximity to farms (Winfield and Groisman 2003). Insects such as flies, cockroaches and ants have been implicated as another potential vector of enteric pathogens. Adult muscoid flies carry *Salmonella*, particularly on dairy farms (infection rate, 67%) and poultry farms (infection rate, 13%) (Mian *et al*. 2002). A study determined that 90% of chickens became infected by *Campylobacter* after consuming a single beetle adult- or larval- infected beetle and 100% of birds consuming 10 infected beetle adults or larvae became *Campylobacter* positive (Doyle and Erickson 2006).

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2.3.1.3.4 Feeds

The occurrence of pathogens in poultry and cattle has been linked directly to contaminated feeds (Callaway *et al.* 2008). Infection and colonisation of food producing animals with enteric pathogens are contributed by the contamination of animal feed before arrival at and while on the farm (Maciorowski *et al.* 2006). Intensification of agriculture has brought upon increased reliance on a wide array of manufactured feed products as food for animals destined for human consumption. Commercially manufactured feeds contain ingredients of animal and plant origin. These ingredients may possibly be contaminated with enteric pathogens and may well serve as the initial vehicle in feed production (Crump *et al*. 2002). Contamination of animal feed with enteric pathogens by the animal itself, insects, rodents or birds serves as a considerable potential pathway for entry of pathogens into the slaughter process (Smith 2008). Feed ingredients and dust in feed mills have been implicated to be sources of *Salmonella* contamination after analyses of commercially manufactured feeds was carried out (Doyle and Erickson 2006).

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2.3.2 Slaughter sources of meat contamination

The current slaughtering process (Figure 2.3) of all animals acts as an amplifier by allowing for contamination of carcasses and for cross-contamination between infected and uninfected carcasses (Anonymous 2000). In some large plants where high speeds of processing takes place the main concern is the assurance of undamaged valuable skin; this results in the prevention of contamination of the meat receiving less attention (Gill 2007). The microbial conditions of meat depend on the circumstances

in which the animal is killed and the conditions under which the carcass is dressed and slaughtered.

2.3.2.1 Defeathering

Feathers that are grossly contaminated increase the amount of faecal material entering the plant. Microbial colonisation of equipment and further contamination of carcasses in the defeathering process is a result of aerosols created, which disperses microorganisms. Aerosols or condensation formed on the equipment or ceilings in this process may contaminate carcases transported by overhead conveyors. Particularly dangerous is the development of microbial growth niches, which hide in unexpected corners and can contaminate subsequent batches. The plucking of clean feathers reduces bacterial load during the first processing steps of unloading, killing and plucking (Bilgili 2006; Bolder 2007; Anonymous 2000; Macrae 1993).

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2.3.2.2 Evisceration

In the evisceration process cross-contamination is usually with bacteria that reside in the faeces of animals. Cross-contamination occurs when the intestines rupture and the faeces come into contact with the carcasses (Hafez 1999). In Danish slaughterhouses the prevalence of contaminated pig and beef carcasses were favourably affected by the covering of the bungs with plastic bags the moment the anuses were cut loose (Anonymous 2000). Evisceration, apart from dressing is regarded as an important step that needs to be hygienically performed to minimize meat contamination (Adams and Motarjemi 1999).

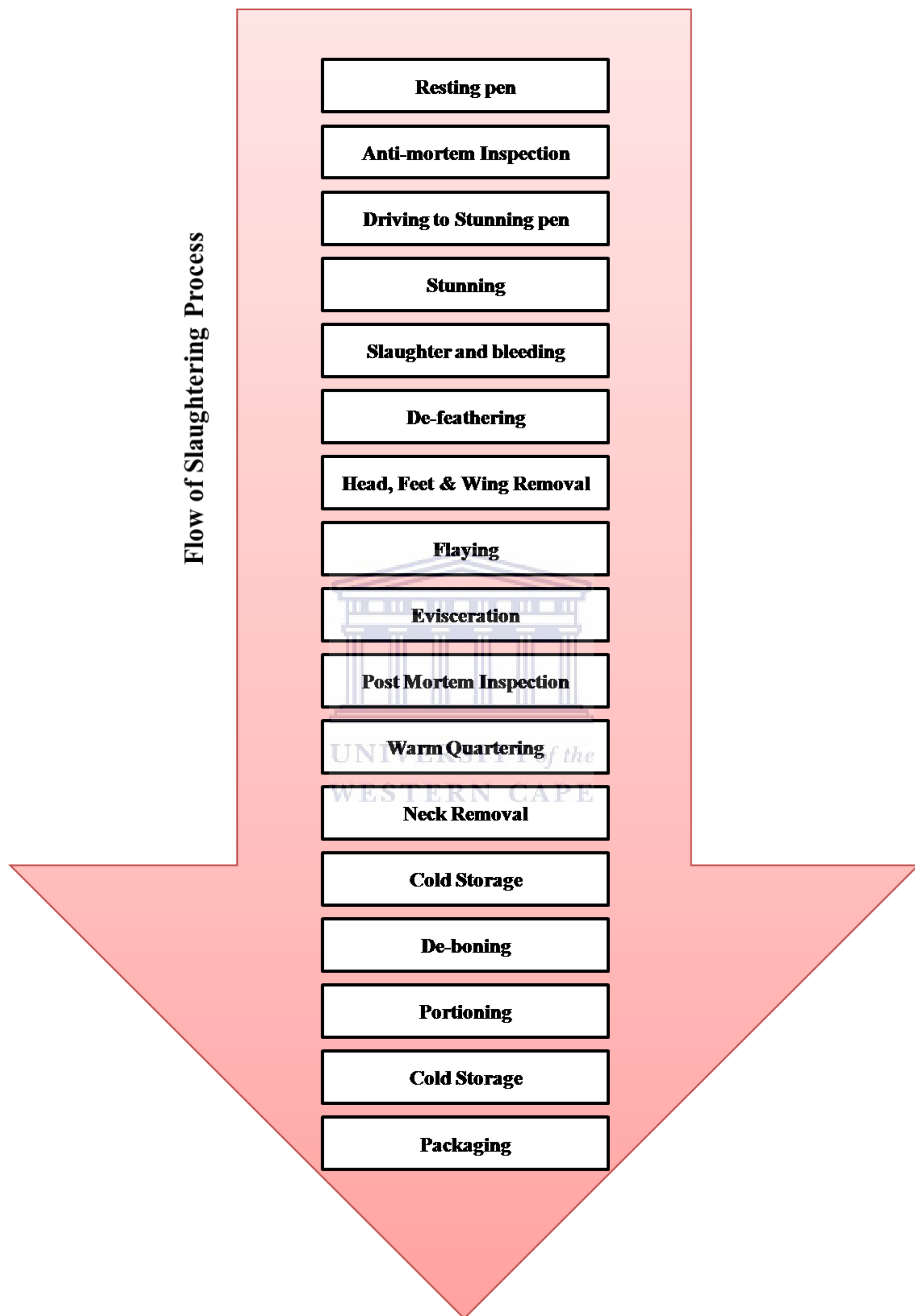

Figure 2.3 The slaughter process of ostriches.

2.3.2.3 Processing equipment, the environment and workers

The handling of meat can introduce and spread pathogenic microorganisms and subsequently cross-contamination of carcasses can originate from the equipment, utensils and the environment that has been contaminated by microbes from hides, feathers and faeces (Paramithiotis *et al.* 2009). Animals entering the slaughterhouse are often dirty on their external surfaces and the knife used for bleeding and de-hiding will have to cut through faecal residues, which ultimately results in potential meat contamination. Millions of bacteria may be present on the surface of un-cleaned and unsanitary equipment. When the meat comes in contact with its surface the bacteria will be transferred. Food handlers who handle animal carcasses may carry pathogens without experiencing any serious ill-effects themselves and can subsequently easily contaminate the carcasses (Bilgili 2006; Trent *et al.* 2006; Macrae *et al*. 1993). Intestinal microorganisms can easily be transferred to food if food handlers fail to wash their hands thoroughly after using the toilet, this may be prevented by washing of hands with soap and water, since these microbes adhere less strongly to the skin (Adams and Motarjemi 1999).

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2.3.3 Post-slaughter sources of meat contamination

Unclean chillers that store meat may cause contamination when contact is made with the chillers walls. When contaminated meat come into contact with the surfaces of clean meat cross-contamination with microbes occur. Under aerobically chilled conditions microbes that predominate at spoilage in low temperatures are pseudomonads accompanied by lower numbers of other Gram-negative bacteria.

Storage conditions and intrinsic biochemical qualities of the meat will determine the microflora that develops. During wrapping and packaging cross-contamination of meat occurs by dirty hands, aprons, overalls, personal adornments and bandages on hands, unsterilised knives, cutting surfaces and tables (Bolder 2007; Gill 2007; Hafez, 1999; Mead 2004).

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2.4 FOODBORNE PATHOGENS CONTAMINATING MEAT

The unfavourable health effects associated with the consumption of biological, chemical and physical hazards in food has been recognised as foodborne illness (Acheson 2009; WHO 2009). All diseases that may be infectious or toxin-mediated that are passively introduced into humans by means of a food vehicle is generally considered foodborne if two or more persons experience similar illness (gastrointestinal) after ingestion of a common food and when epidemiological analysis implicates food as the source of the illness (Gill and Hamer 2004). Ingestion of harmful microorganisms may lead to gastrointestinal illness caused by their invasion and/or toxin production (Figure 2.4) (Giannella 2006; Adams and Motarjemi 1999). The harmful pathogens contaminating food and causing gastroenteritis or inflammation of the stomach and intestinal lining include *E. coli, Staphylococcus aureus, Salmonella* species, *Listeria monocytogenes* and *Campylobacter* species (Prescott *et al.* 2002)

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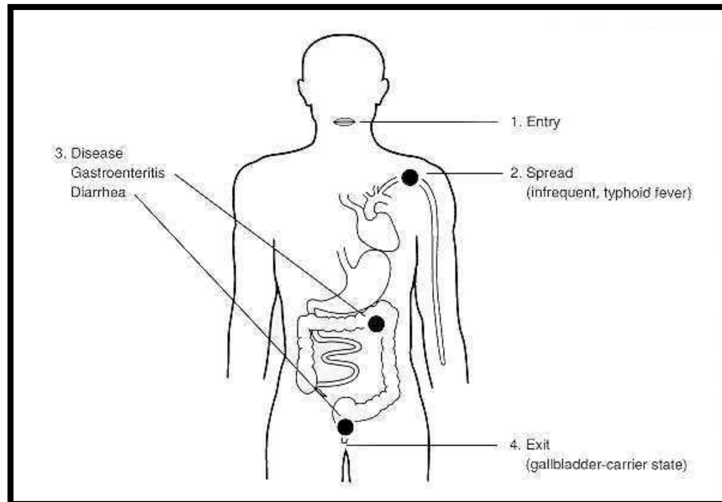

Figure 2.4 Pathogenesis of foodborne pathogens. (adapted from Giannella 2006)

2.4.1 Indicator microorganisms

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The presence of an indicator microorganism indicates the condition of a substance or environment (Prescott *et al.* 2002). The general use of groups or species which are easily enumerated and whose presence in foods indicates exposure to conditions that might introduce hazardous organisms and/or allow their growth are used in order to assess the microbial safety from foodborne pathogens (Schaffer and Smith 2004). Indicator organisms are considered to be faecal contamination and the potential presence of enteric pathogens in water and foods (Tortorello 2003). The sole source of these organisms in faeces of warm-blooded animals was established due to their use as indicators. Due to this establishment, the hygiene in food processing was evaluated using members of the *Enterobacteriaceae* as indicator organisms. *Staphylococcus aureus* as an indicator organism generally indicates contamination from the skin, mouth or nose of human handlers and/or inadequate cleaning and disinfections of processing equipment. The presence of *E. coli* in food is of interest as it indicates that recent faecal contamination has occurred with the possibility of

accompanying pathogens (Çakir *et al.* 2002). The application of these indicator organisms has provided information on the faecal contamination of food, the effect of heat treatments, recontamination, and various other microbiological productions. Aspects of food selected representative microorganisms that are monitored. Testing for indicator organisms produces a simpler means of controlling the hygienic status of foods and helps to ensure production of safe food. *E. coli* and coliform microorganisms are initially used to indicate possible faecal contamination (Smoot and Pierson 1997).

2.4.1.1 Coliforms

Coliforms are members of the family *Enterobacteriaceae* and are defined as facultatively anaerobic, Gram-negative, nonsporing, rod-shaped bacteria that ferment lactose with gas formation within 48 hours at 35˚C (Prescott *et al.* 2002). It has also been defined as ONPG + Enterobacteriaceae (Table 2.2), allowing the assessment of the level of faecal contamination of food and water (Leclerc *et al.* 2001). Coliforms constitute 10% of intestinal flora in humans and animals (Pandey *et al*. 1999). Coliform bacteria are used as a component of microbiological standards to monitor the quality of food and water, their presence and populations especially in the case of *E. coli* therefore indicates the microbiological quality of the raw material as well as the efficiency of the processing techniques (Leclerc *et al.* 2001). Assessing the presence and prevalence of faecal coliforms is essential since non-*E. coli* faecal coliforms may exist and they still may determine that faecal contamination has occurred when *E. coli* does not survive or is not present in the food sample (Doğan-Halkman *et al.* 2003). Contamination with coliforms leads to food spoilage or

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transmission of bacteria to humans, which may result in gastrointestinal problems or food poisoning (Smoot and Pierson 1997).

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Table 2.2 Coliform bacteria in the family *Enterobacteriaceae*

	ONPG[a]	Faecal origin	Occurrence in Water
Arsenophonus	+	−	−
Budvicia	+	−	+
Buttiauxella	+	−	+
Cedecea	+	−	−
Citrobacter	+	+	+
Edwarsiella	−	−	−
Enterobacter	+	+	+
Erwinia	+	−	+
Escherichia	+	+	+
Ewingella	+	−	−
Hafnia	+	−	+
Klebsiella	+	+	+
Khuyvera	+	−	+
Leclercia	+	−	+
Leminorella	−	−	−
Moellerella	+	+	+
Morganella	−	+	+
Obesumbacterium	−	−	−
Pantoea	+	−	+
Photorhabdus	−	−	−
Pragia	−	−	+
Proteus	−	−	+
Providencia	−	+	+
Rahnella	+	−	+
Salmonella[b]	−	+	+
Serratia	+	−	+
Shigella[c]	−	+	+
Tatumella	−	−	−
Trabulsiella	+	−	−
Xenorhabdus	−	−	−
Yersinia[d]	+	+	+
Yokenella	+	−	−

[a] ONPG, o-Nitrophenyl-β-galactopyranoside
[b] *Salmonella* subgroup 3a, 3b (*Arizona*) and 5 (*S. bongorae*) are ONPG positive.
[c] Some *S. sonnei* biovars are ONPG positive.
[d] Most species (except *Y. aldovae*) are often ONPG positive.

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2.4.2 Gram-negative pathogens

2.4.2.1 *E. coli*

2.4.2.1.1 General characteristics and classifications

E. coli are Gram-negative, facultative anaerobic rods that belong to the family *Enterobacteriaceae. E. coli* isolates can be identified and confirmed on the bases of their colony morphology and biochemical characterizations (Table 2.3) (Singh and Prakash 2008). *E. coli* isolates are serologically distinct from each other. This serological differentiation is based on three major surface antigens O (somatic), H (flagella) and K (capsule). The K antigen descriptor has been dropped and only the H and O are commonly employed as descriptors of serotypes (Batt 1999; Doyle *et al.* 1997).

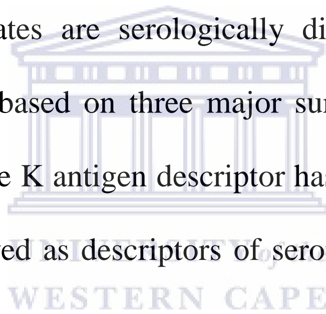

Table 2.3 Biochemical characterization of *Escherichia coli* (adapted from Singh and Prakash 2008)

Biochemical test		Reaction
Lactose Fermentation		+
Catalase		+
Simmon's Citrate		-
Indole Production		+
Nitrate Reduction		+
Methyl Red		+
Voges-Proskauer		-
Urease		-
Acid from sugar	(a) Glucose	+
	(b) Mannitol	+
	(c) Lactose	+
	(d) Salicin	+
	(e) Sucrose	+

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2.4.2.1.2 Ecology/ Origin

Strains of *E. coli* commonly form part of the normal microflora found in the intestinal tracts of mammals and birds, but certain strains such as serotype 0157:H7 have been associated with gastrointestinal diseases in both humans and animals (Capriola *et al.* 2005). The organism does not survive well outside of the intestinal tract, as it fights for survival, facing limited nutrient availability, osmotic stress, large differences in temperature and pH, and predation. The presence of *E. coli* in the environment is therefore considered as evidence of recent contamination with mammalian or avian faeces (Macrae *et al*. 1993). A priority of *E. coli* strains is to survive and multiply in a hostile environment; the pathogen therefore modulates the expression of genes that are necessary for establishing itself in a new niche (Diard *et al.* 2006).

2.4.2.1.3 Importance in food

E. coli diarrhoeal outbreaks have implicated foods such as meat and meat products, fish, poultry, milk and dairy products, vegetables and water (Silva and Gibbs 2010; Taulo *et al*. 2008). Enterotoxigenic *E. coli* (ETEC) infections is the most common type of colibacillosis of young animals, primarily calves and pigs; and is recognised as one of the most frequent causes of diarrhoea among children in developing countries and travellers (Nagy and Fekete 2005; Qadri *et al.* 2005). Enterohaemorrhagic *E. coli* (EHEC) is the only group that has a definite zoonotic origin, with cattle being recognised as the major reservoir for human infection (Caprioli 2005). EHEC such as serotype 0157:H7 is the most common cause of postdiarrheal haemolytic–uremic syndrome (Karch 2001). The principle reservoir of enteropathogenic *E. coli* (EPEC) are people and the serogroups implicated in human disease include 011ab, 0119, 0125ac, 0126, 0127, 0128ab, and 0142 (Doyle *et al.* 2001). EPEC is frequently the cause of infantile diarrhea in the developing world (Garmendia *et al.* 2005). A small number of cells (Table 2.4) are required to begin an infection (Schmid-Hempel and Frank 2007). Symptoms develop 5 to 48h after food consumption. The onset of the illness is a function of the strain as well as the numbers of *E. coli* consumed by the victim (Doores 2005). Complications resulting from *E. coli* (EPEC and EHEC) infections include erythema nodosum, haemolytic-uraemic syndrome and seronegative arthropathy (Adams and Motarjemi 1999).

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Table 2.4 Infectious doses of *Escherichia coli* (Adams and Motarjemi 1999)

Escherichia coli	**Infectious dose**
Enteropathogenic (EPEC)	10^6-10^{10} cells
Enterotoxigenic (ETEC)	10^6-10^8 cells
Enteroinvasive (EIEC)	10^8 cells
Enterohaemorrhagic (EHEC)	10^1-10^3 cells

2.4.2.1.4 Pathogenesis

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Diarrhoeal illness-causing *E. coli* strains are categorized into groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O: H serogroups. The six groups include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EaggEC) and diffusely adherent *E. coli* (DAEC) (Yoon and Hovde 2008).

2.4.2.1.4.1 Enterotoxigenic *E. coli*

ETEC strains are frequently responsible for traveller's diarrhea, contracted while travelling in another country with lower hygienic standards (Batt 1999). Once ingestion of ETEC occurs, the microorganism attaches to the epithelial cells lining the ileum by colonization factor antigens (Sizemore 2004). A morphologically non-destructive attachment of ETEC to the microvilli occurs when the adhesion interacts

with the ligands present on the small intestinal microvilli. Fimbriae are proteinaceous attachments that develop from the bacterial cells outer membrane and are the most common adhesive surface antigens of ETEC. Adhering to the ileum prevents the microorganism from removal by the peristaltic movement of intestinal contents (Nagy and Fekete 2005). Once adherence has taken place in the host, the ETEC producers enterotoxins, which bind to the intestinal cells. Heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST) are released either alone or together. The B subunit of LT binds to the GM1 ganglioside receptor while the A subunit is responsible for the activation of the adenylate cyclase system. This in turn causes the increase of cyclic AMP, which stimulates the secretion of chloride in the crypt cells and the inhibition of neutral sodium chloride in the villus tips. Water follows the secreted chloride generating a profuse loss of fluid from the intestine (Qadri *et al.* 2005; Sanchez and Holmgren 2005). The heat-stable enterotoxins elicit a diarrhoeal response by binding to guanylate cyclase C which activates the guanylate cyclase system causing an increase in the intracellular levels of cGMP (Nagy and Fekete 2005).

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2.4.2.1.4.2 Enteroinvasive *E. coli*

EIEC forms a single pathovar of *E. coli* with *Shigella* and infection with these bacteria causes shigellosis. Following ingestion (Figure 2.4), EIEC strains invade the colonic and rectal epithelium of humans. EIEC's ability to invade epithelial cells (Figure 2.5) is associated with the presence of a large virulence plasmid, generally known as pINV (Lan and Reeves 2002). pINV contains a 30kb region that encodes for the Mxi-Spa TTSS apparatus, the translocators and the effectors, their chaperones and two transcriptional activators (Peng *et al.* 2009).

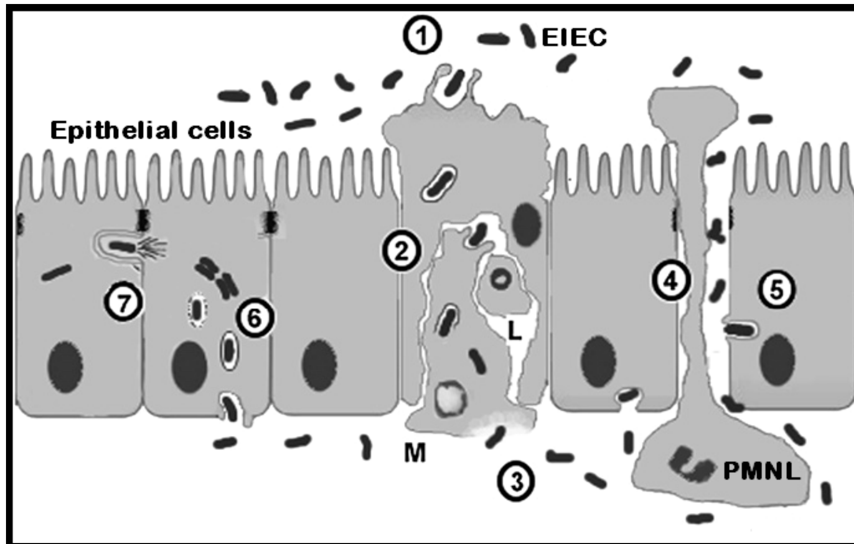

Figure 2.5 Infection of epithelial cells by EIEC**. (1)** Uptake of bacteria into the host cell. **(2)** The bacteria encounter resident macrophages as they arrive at the lymphoid follicles. **(3)** Proliferation and induction of apoptosis give rise to an inflammatory response. **(4)** Transmigration of polymorphonuclear leukocytes (PMNL) through the tight junctions between epithelial cells. **(5)** Bacteria invade the epithelial monolayer, entering the basolateral surfaces of the colonic epithelium as the migration of PMNLs occur. **(6)** EIEC lyse their surrounding membrane bounded vacuole once they have entered the host cell. **(7)** After entrance into the host cell EIEC attain the ability to proliferate and colonize the infected cell and the adjacent ones (adapted from Prosseda *et al.* 2007).

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The microorganism then spreads and proliferates intracellularly after invasion has taken place. This invasion causes acute mucosal inflammation due to the destruction of the colonic epithelial layer, therefore causing the clinical symptoms of shigellosis or bacillary dysentery.

2.4.2.1.4.3 Enterohaemorrhagic *E. coli*

Colonization involves the expression of the adhesin intimin which allows the adhesion of EHEC to the host cell. Once adhesion has taken place a signalling pathway is induced which causes various signalling events in the enterocytes and cytoskeletal rearrangements (Garmendia *et al.* 2005). The effect of these

rearrangements is the effacement of microvilli and its replacement with an organized cytoskeletal structure containing filamentous actin called an actin pedestal or lesion (Figure 2.6). This histopathologic lesion is defined as “attaching and effacing” (A/E) lesion due to the process of its formation. A large pathogenicity island (PAI) defined as the Locus of Enterocyte Effacement (LEE) governs this mechanism of A/E adherence (Caprioli *et al.* 2005).

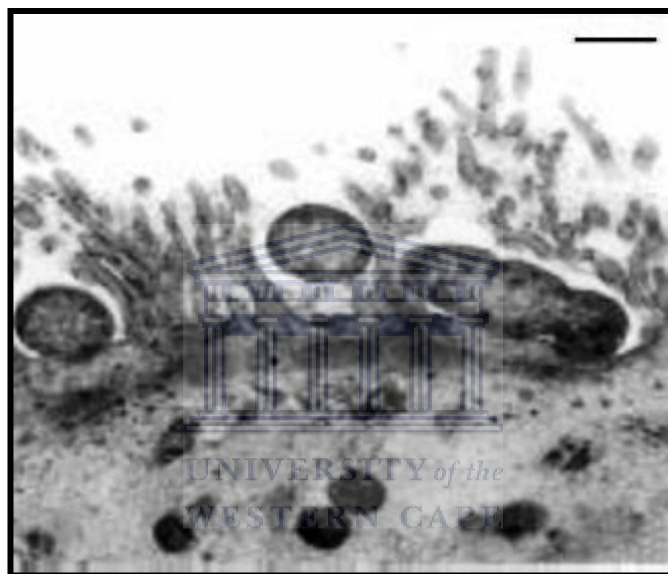
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Figure 2.6 Transmission electron microscopy (bar = 0.5µm) displaying the attaching and effacing lesions caused by EHEC infection (adapted from Phillips *et al*. 2000).

LEE encodes a type III secretion system (TTSS), the expression of proteins EspA, B and D and the adhesin intimin and the translocated intimin receptor (Tir). EspB/EspD is exported through a filamentous cylindrical structure formed by EspA to form a pore in the host membrane. The EspABD therefore functions as the translocation machinery for the transfer of effector proteins (Yoon *et al.* 2008; Garmendia *et al.* 2005). The TTSS injects several effector proteins directly into the eukaryotic cell. Tir is injected into the host cells plasma membrane through TTSS inserts in a hairpin loop typology and is presented on the external face of the plasma membrane, consequently

functioning as the receptor for intimin (Frankel and Phillips 2008; Taylor 2008). Intimin γ is produced by serotypes 0157, 0111 and 0145, while intimin ε is produced by serotypes 0103 and 0121 (Caprioli *et al*. 2005). EHEC strains form pedestals independently of Nck (Figure 2.7) and require the translocation of Tir-cytoskeleton coupling protein (TccP). TccP activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP) by binding to its GTPase binding domain, stimulating Arp2/3-mediated actin polymerisation and pedestal formation (Yoon *et al.* 2008; Caron *et al.* 2006).

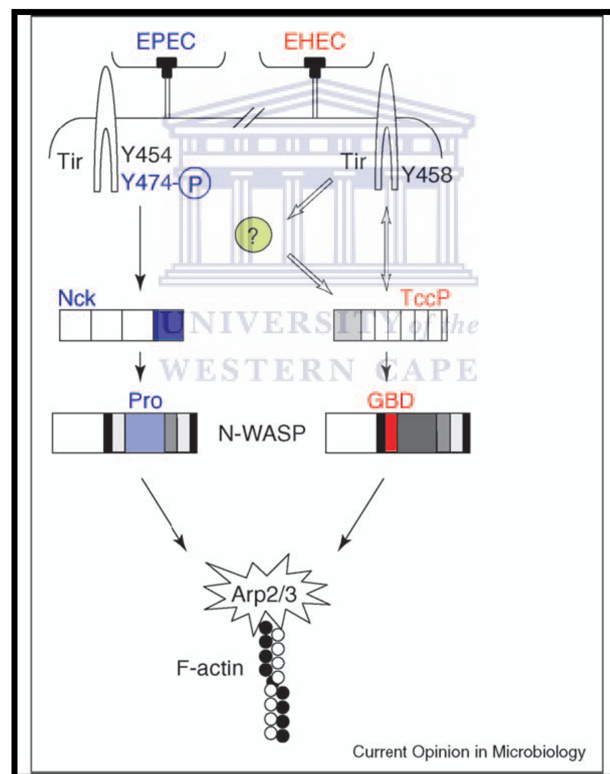

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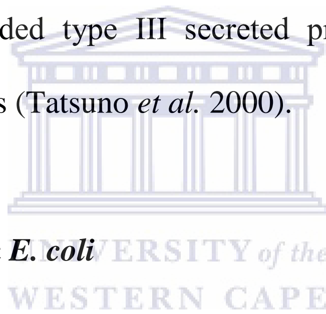
Figure 2.7 Mechanism of Actin polymerisation and pedestal formation by EHEC and EPEC (adapted from Caron *et al*. 2006).

EHECs attachment to the actin pedestal allows closer delivery of shiga toxins (Stx) to host enterocytes. The B subunit of Stxs bind to globotriaosylceramide or globotetra-osylceramide receptors on the host cells membrane, which facilitates in the

endocytosis and intracellular trafficking of the toxin (Karch and Bielaszewska 2005; Karch 2001). The A subunit of Stx is cleaved by furin in the Golgi apparatus. The product of cleavage has RNA N-glycosidase activity, which irreversibly depurinate a specific adenine residue from the 28s rRNA of the 60S ribosome, resulting in the prevention of binding by elongation factor I-dependent aminoacyl-tRNA. This process inhibits the synthesis of proteins and therefore results in cell death, while sub-lethal intoxication causes stress responses in the cell and pro-inflammatory signalling events (Taylor 2008; Yoon *et al.* 2008). ToxB, a virulence gene has been described in a large virulence plasmid possessed by EHEC 0157 isolates termed p0157, its product may be directly involved in the colonisation of the host gut by influencing the expression of the LEE-encoded type III secreted proteins and by inhibiting the activation of host lymphocytes (Tatsuno *et al.* 2000).



2.4.2.1.4.4 Enteropathogenic *E. coli*

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EPEC strains contain the same LEE PAI that governs the A/E lesions and therefore colonises the gut mucosa in the same manner as described in EHEC pathogenesis (Figure 2.8) (Ochoa *et al.* 2008; Garmendia *et al.* 2005). Pedestal formation in EPEC has a different actin polymerisation pathway in that it is Nck-dependant (Figure 2.7). The C-terminus of Tir contains 2 essential tyrosine residues which are phosphorylated by the host cells tyrosine kinases, creating a binding site for the Src homology (SH) 2 domain of Nck. The SH3 domain of Nck then binds to the polyproline region of N-WASP. The activation of N-WASP consecutively stimulates Arp2/3-mediated actin polymerisation and pedestal formation. This cell destruction leads to the subsequent diarrhea (Nougayrède *et al.* 2003; Celli *et al.* 2000).

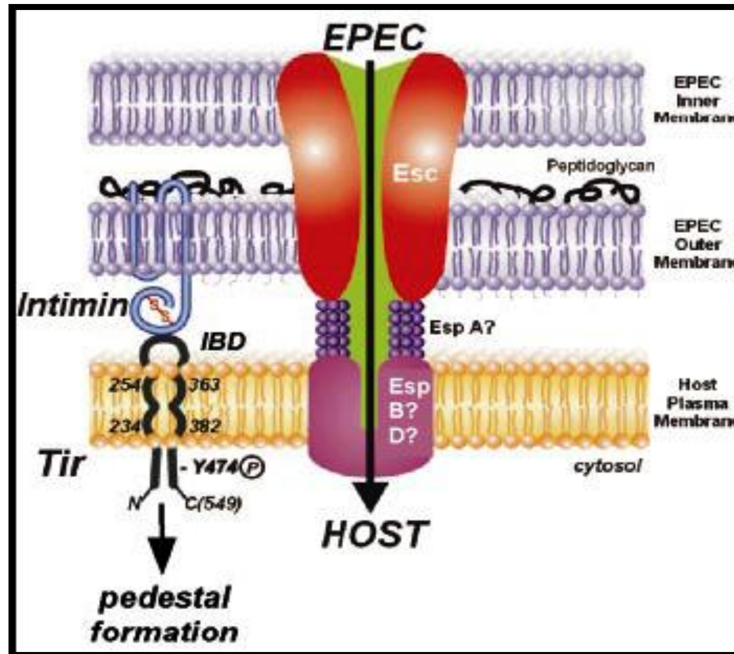
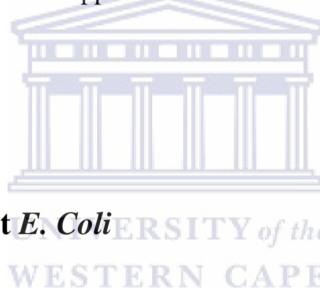


Figure 2.8 EPEC type III translocation apparatus and Tir-intimin binding (adapted by Celli *et al.* 2000).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2.4.2.1.4.5 Diffusely adherent *E. Coli*

DAEC strains uniformly adhere to the entire surface of HEp-2 cells and HeLa cells (Meraz *et al.* 2007) (Figure 2.9). DAEC express virulence factors such as fimbriae Afa-I, AfaE-III, Dr, Dr-II and F-1845 of the Afa/Dr family (Diard *et al.* 2006). Several pathways such as the chaperone/usher pathway and the type IV pilus assembly pathway are responsible for the assembly of these fimbriae (Le Bouguénec 2005).

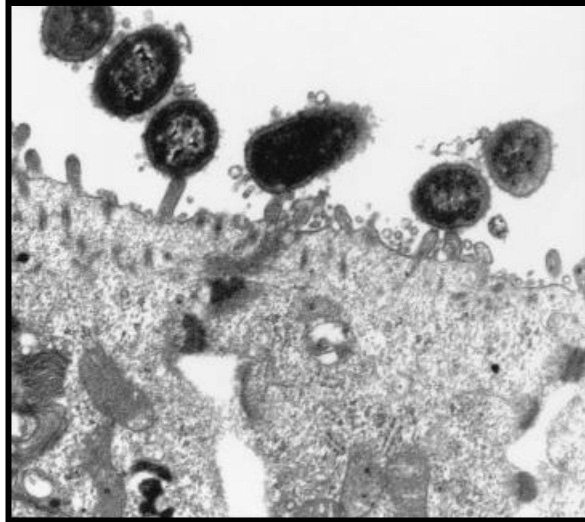

Figure 2.9 Electron microscopy displaying DAEC strains interaction with a host cell (adapted from Bétis *et al.* 2003).

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Attachment of DAEC to fully differentiated epithelial cells (Figure 2.10) take place when adhesions such as the fimbrial adhesin (Afa) produced from DAEC binds to receptors present in the membrane of the host's cell wall. Afa recognises the glycosylphosphatidylinositol-anchored protein (GPI) carcinoembryonic (CEA or CD66e) antigen-related molecule and the Cromer blood group antigen Dr on the GPI-anchored protein decay-accelerating factor (DAF or CD55as a receptor (Le Bouguénec 2005; Bétis *et al.* 2003). This attachment creates a signaling pathway involving the proteins tyrosine kinase(s), phospholipase Cγ, phosphatidylinositol 3-kinase and protein kinase C as well as an increase in Ca^{2+} (Le Bouguénec and Servin 2006).

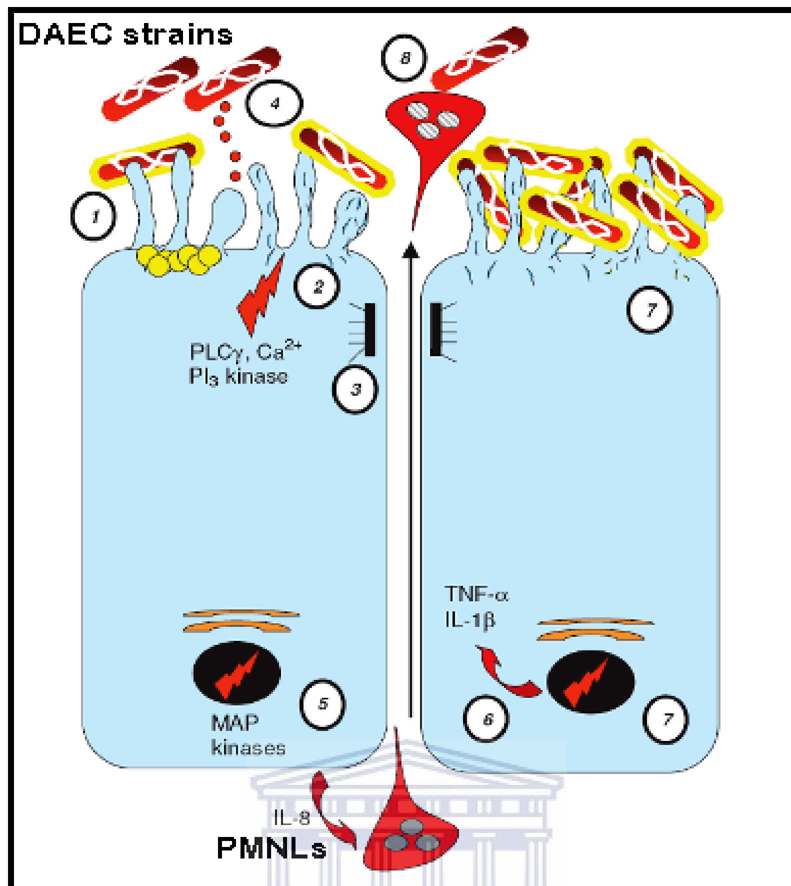

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Figure 2.10 Interaction of Afa/Dr DAEC strains with fully differentiated cells. **(1)** Bacteria interacting with membrane-bound receptors. **(2)** Signal pathway causes the disappearance of the brush border and the induction of lesions. **(3)** Alteration in the distribution of tight junction-associated proteins increases paracellular permeability. **(4)** DAEC isolates produce Sat, inducing fluid accumulation in the intestines. **(5)** Activation of MAP kinase-dependant signalling pathway induces PMNL transepithelial migration. **(6)** PMNL transmigration activate proinflammatory cytokines (TNF-α and IL-1β). **(7)** The upregulation and MHC class 1 chain-like gene is subsequently induced (Le Bouguénec and Servin 2006).

The activated signal pathway induces structural and functional lesions created by the rearrangement of brush border-associated F-actin and villin cytoskeletal proteins and the decrease in the expression and enzyme activities of functional brush border-associated proteins (Hudault *et al.* 2004; Bétis *et al.* 2003). Increased paracellular permeability subsequently arises when the distribution of tight junction-associated proteins are altered. Fluid accumulation in the intestine occurs when DAEC releases a secreted autotransporter toxin (Sat) after its attachment (Le Bouguénec and Servin

2006). A proinflammatory response is generated when activation of the MAP kinase-dependent signaling pathway results in the production of proinflammatory cytokine IL-8, which induces the transepithelial migration of polymorphonuclear leukocytes (PMNLs) (Diard *et al.* 2006; Bétis *et al.* 2003). Proinflammatory cytokines TNF-α and IL-1β are produced as the direct result of the transepithelial migration of PMNLs. These released cytokines induce the upregulation of DAF and MHC class I chain-like gene A (Le Bouguénec and Servin 2006).

2.4.2.1.4.6 Enteroaggregative *E. coli*

EaggEC strains produce characteristic patterns of aggregative adherence (Figure 2.11) on Hep-2 cells (Greenberg *et al*. 2002). The first stage of EaggEC pathogenesis involves adherence by aggregative adherence fimbriae (AAF) or other organism adherence factors to the intestinal mucosa. A 60-65 MDa pAA plasmid encodes the three AAF structural subunits involved in adherence, namely aggregative adherence fimbriae I (AAF/I), aggregative adherence fimbriae II (AAF/II) and aggregative adherence fimbriae III (AAF/III) (Haung *et al.* 2006). AAF/I contains the aggregative phenotype and human erythrocyte hemagglutination, while AAF/II is responsible for intestinal mucosal adherence (Haung and Herbert 2004).

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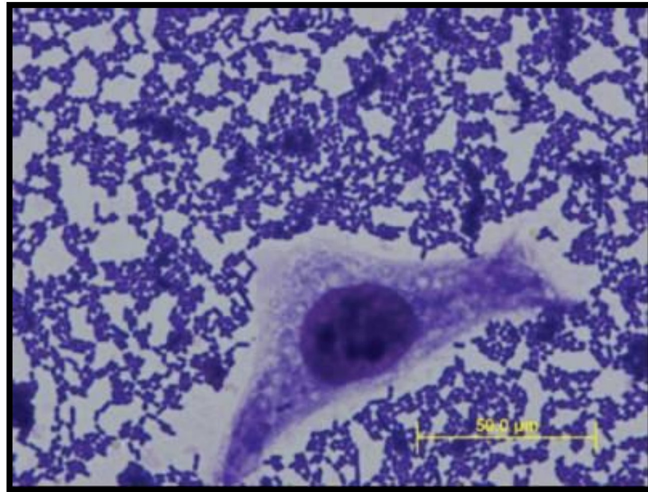


Figure 2.11 Aggregative adherence pattern of EAEC on HEP-2 cells (adapted from Haung *et al.* 2006).

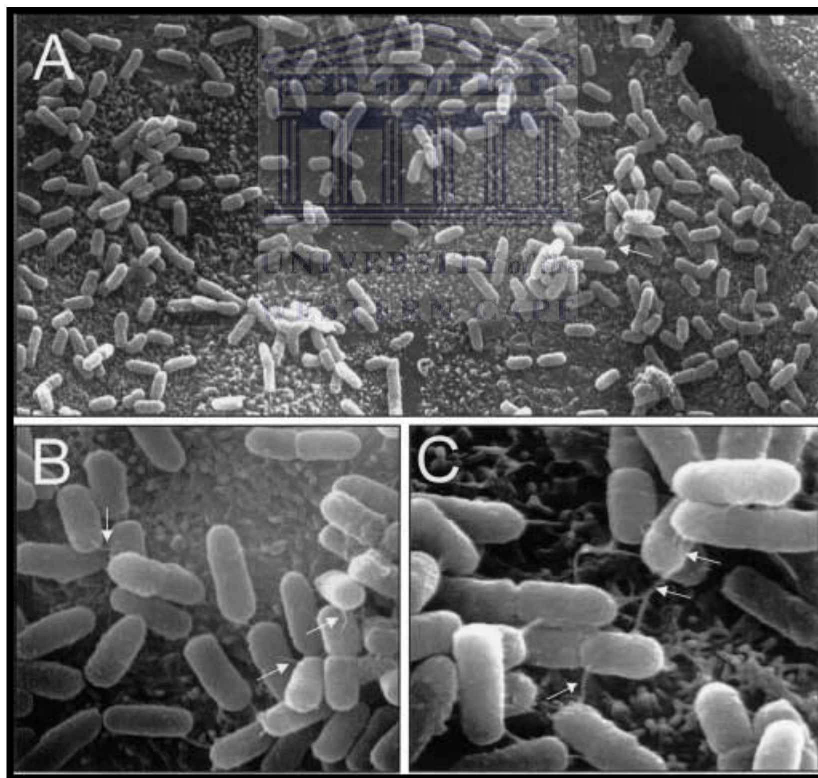
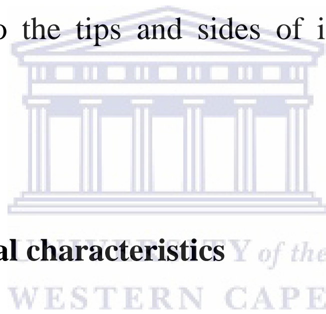


Figure 2.12 Electron microscopy displaying EAEC adhering to host cells. (A-C) Bacteria interact with the cellular microvilli and with one another through rope-like structures (arrows) (Monteiro-Neto *et al.* 2003).

AAF/I and AAF/II have a bundle forming morphology that chains the bacteria to each other and to the host cells wall (Figure 2.12). AAF/III however, emerges as peritrichous and flexible elongated filaments that act as an adhesion (Monteiro-Neto *et al.* 2003). Membrane associated proteins are the other important factors associated with the adhesion process (Okeke *et al*. 2000). In the following stages of pathogenesis the adherence of EaggEC to the intestinal mucosa stimulates the secretion of mucus. This thick layer of mucus encrusts and embeds EaggEC on the surface of enterocytes (Haung and Herbert 2004). The final stages of EaggEC pathogenesis result in mucosal toxicity and intestinal secretion when invasion causes an inflammatory response with cytokine release (Jiang *et al*. 2002). Several toxins are released in the final stages of EAEC that are destructive to the tips and sides of intestinal villi and enterocytes (Haung *et al.* 2006).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2.4.2.1.5 Growth and survival characteristics

E. coli thrives in diverse environments and can therefore survive for long periods of time in water, especially at cold temperatures and can enter a viable but not-culturable state (Wang and Doyle 1998). Under refrigeration conditions *E. coli* strains do not grow, but can survive for weeks at 4ºC or -20ºC. *E. coli* grows at temperatures that range between 7 and 46ºC with optimum growth at 37ºC (Riemann and Cliver 2006). They have a D-value of 60ºC for 0.1min. *E. coli* has the ability to limit internal acidification by producing lactate instead of acetate plus formate and by conversion of formate to H_2 and CO_2 (Poole 2009). It was found that growth of *E. coli* can occur at a pH of 4.5 with optimum growth at pH 7.4 (Dlamini and Buys 2009). *E. coli* has the ability to adapt to extremely acidic pH, as low pH triggers the transcription of several

amino acid carboxylases, which subsequently mediates maintenance at a suitable pH level for cell survival (Prosseda *et al.* 2007). *E. coli* 0157:H7 has three systems involved in acid tolerance: an acid-induced oxidative system, an acid-induced arginine-dependant system and a glutamate-dependent system (Law 2000). The minimum water activity (a_w) for growth of this microorganism is 0.95 (Black and Jaczynski 2008). *E. coli* are more resistant to sodium chloride and sodium nitrate than salmonellae, but growth can occur in 0 to 4% sodium chloride and 0 to 400µg of sodium nitrite per millilitre (Macrae *et al.* 1993; Pandey *et al.* 1999). Changes in environmental signals such as temperature, ion concentration, osmolarity, carbon source, Fe^{++}, pH and O_2 can be sensed by ETEC bacteria, resulting in the expression of virulence factors (Nagy and Fekete 2005).

2.4.2.2 Salmonella

2.4.2.2.1 General Characteristics and Classifications

Salmonella species are Gram-negative, motile, non-sporing rods, which belong to the family *Enterobacteriaceae* (Dunkley *et al.* 2009). Strains of this genus are facultatively anaerobic chemoorganotrophs with a DNA composition of 50-52mol % GC (Wan Norhana *et al.* 2010; D`Aoust 1997). *Salmonella* spp. was first named according to clinical isolates and later from its geographical origin. Biochemical and serological reactions (Table 2.5) can distinguish *Salmonella* spp. from other microorganisms. Kauffmann subdivided the genus into four subgenera based on biochemical reactions (D`Aoust 1997). More than 2300 *Salmonella* serotypes of *Salmonella* exists worldwide (Arvanitidou *et al.* 2005).

Table 2.5 Biochemical and serological reactions of *Salmonella* (adapted from FDA 2007)

Test or substrate	Result		*Salmonella* species reaction[(a)]
	Positive	**Negative**	
Glucose(TSI)	Yellow butt	Red butt	+
Lysine decarboxylase (LIA)	purple butt	yellow butt	+
H_2S (TSI and LIA)	Blackening	no blackening	+
Urease	purple-red color	no color change	-
Lysine decarboxylase broth	purple color	yellow color	+
Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+[(b)]
KCN broth	Growth	no growth	-
Malonate broth	blue color	no color change	-[(c)]
Indole test	violet color at surface	yellow color at surface	-
Polyvalent flagellar test	Agglutination	no agglutination	+
Polyvalent somatic test	Agglutination	no agglutination	+
Phenol red lactose broth	yellow color and/or gas	no gas; no color change	-[(c)]
Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	-
Voges-Proskauer test	pink-to-red color	no color change	-
Methyl red test	diffuse red color	diffuse yellow color	+
Simmons citrate	growth; blue color	no growth; no color change	v

[a] +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.
[b] Majority of *S. arizonae* cultures are negative.
[c] Majority of *S. arizonae* cultures are positive.

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2.4.2.2.2 Ecology/ Origin

Salmonella spp. is excreted by humans, pets, farm animals and wildlife, and is therefore constantly found in environmental samples (Arvanitidou *et al.* 2005). *Salmonella* colonise in environmental sources such as water, soil, insects and seafood (Macrae *et al.*1997). Foods such as raw meat, poultry, eggs and dairy products have often been implicated as the source of *Salmonella* (Dunkley *et al.* 2009).

2.4.2.2.3 Importance on food

Salmonella illnesses in humans are most commonly associated with food products, especially foods of animal origin such as poultry, eggs, meat, and dairy products (Yan *et al.* 2003). Salmonellosis is the disease caused by ingestion of contaminated food. Illness occurs when 100-1000 cells is ingested by healthy individuals (Warriner and Namvar 2009). Symptoms first appear 6 to 48 hours after ingestion of contaminated food and may last a period of 1 to 4 days. Symptoms include diarrhea, vomiting, fever and cramps. Infants and immunosuppressed patients are susceptible and have more severe symptoms than healthy adults (Doores 2005). Complications associated with Salmonellosis include aortitis, cholecystitis, colitis, endocarditis, orchitis, meningitis, myocarditis, osteomyelitis, pancreatitis, Reiter's syndrome, rheumatoid syndromes, septicaemia, splenicabscess and thyroiditis (Adams and Motarjemi 1999). Humans excrete salmonellae long after being clinically cured and asymptomatic carriers who are potentially dangerous may remain carriers for months or years. Faecal contamination of water and food with *Salmonella* persists where hygienic conditions are lacking (Balows *et al.* 1992). Contaminations of meat for human consumption

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with *Salmonella* often occur in slaughterhouses resulting from intestinal contents during evisceration of animals containing salmonellosis (Macrea *et al.* 1993).

2.4.2.2.4 Pathogenicity

Salmonella infections occurs when the microorganism colonizes and invades enterocytes (intestinal columnar epithelial cells) and M-cells overlying Peyer's patches (epitheliolymphoid tissues) (Jepson and Clark 2001). The degree of pathogenesis of *Salmonella* strains depends on the ability to invade cells, a complete lipopolysaccharide coat, intracellular replication and elaboration of toxins (Giannella 2006).

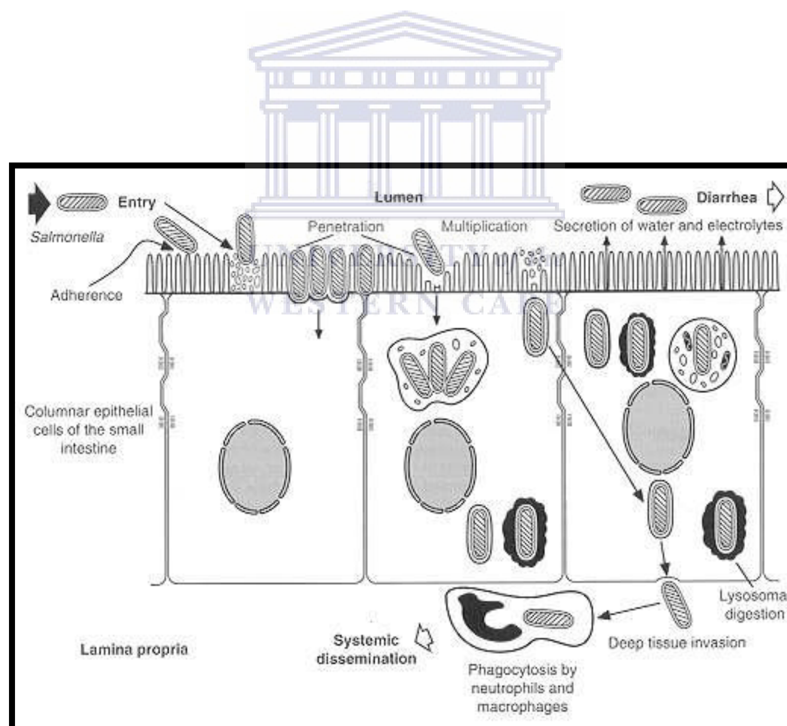

Figure 2.13 Invasion of intestinal epithelial cells by salmonellae (adapted from Giannella 2006).

After ingestion (Figure 2.4) the microorganism migrates from the oral cavity, surpasses the stomach's gastric acid, towards the intestinal tissues. Once in the intestinal tissues, the microorganism must transverse the intestinal mucus layer before

colonising and invading the intestinal epithelial cells (Figure 2.13). The ability of salmonellae to adhere to enterocytes lie in the interaction of several fimbriae, surface adhesions, nonfimbriate hemagglutinins, or enterocytes-induced polypeptides with host glycoprotein receptors (location in microvilli) with the surface of the ileum and colon (Giannella 2006, Galán 2001).

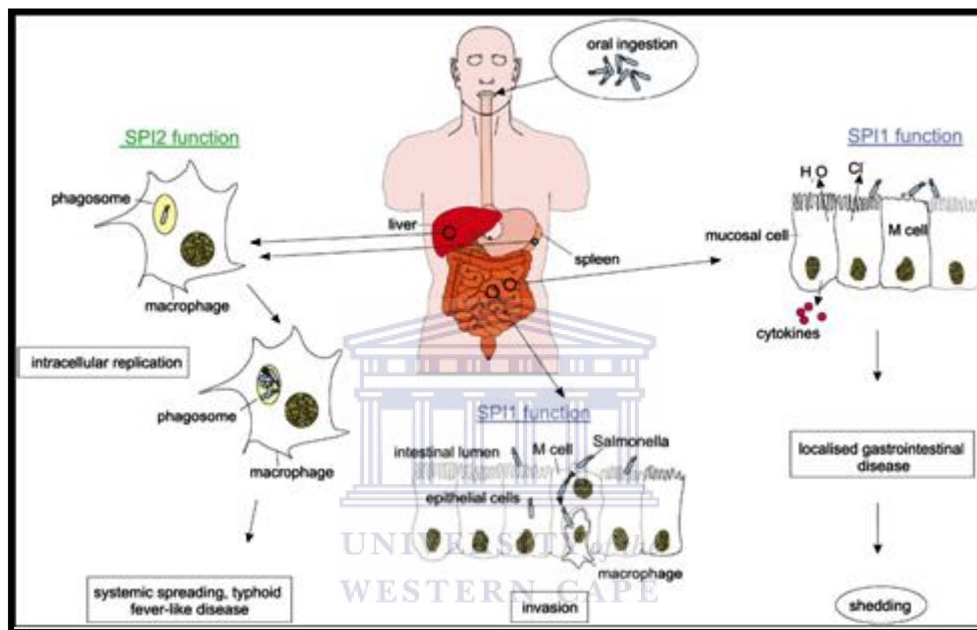

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Figure 2.14 Host-pathogen interactions during pathogenesis of *Salmonella i*nfections (adapted from Hansen-Wester and Hensel 2001).

The *Salmonella* Pathogenicity Islands (SPI) plays a crucial role in the pathogenesis of salmonellae infections (Figure 2.14). Five SPIs have been identified; these include SPI1, SPI2, SPI3 and SPI4. SPI1 and SPI2 contain a large number of genes which encodes a type III secretion system (TTSSs). SPI1 is responsible for the deliverance of bacterial effector proteins into the cytoplasm of the host cell which subsequently induce the cytoskeletal rearrangements that facilitate the uptake of the bacteria into *Salmonella*-containing vacuole (SVC) (Guiney and Lesnick 2005; Hensel 2004; Marcus *et al.* 2000). These infection features of *Salmonella* are termed the "trigger

mechanism" as it involves the injection of effectors into the cytosol of infected cell to induce macropinocytosis (Cossart and Toledo-Alejandro 2008). SPI1's TTSS produces translocator proteins SipA, SipB, SipC and SipD that are involved in the invasion of host cells. SipA Direct binding of SipA's carboxy-terminal domain to the host cell's actin decreases the concentration of actin available for polymerisation and stabilises actin filaments by inhibiting their depolymerisation (Guiney and Lesnick 2005; Lilic and Stebbins 2004). SipB and SipC are inserted into plasma membrane and form a multimeric translocon complex. Both these proteins function as effectors and as translocators for other SPI effector proteins. SipD is thought to quide this mechanism (Schlumberger and Hardt 2006; Hansen-Wester and Hensel 2001).

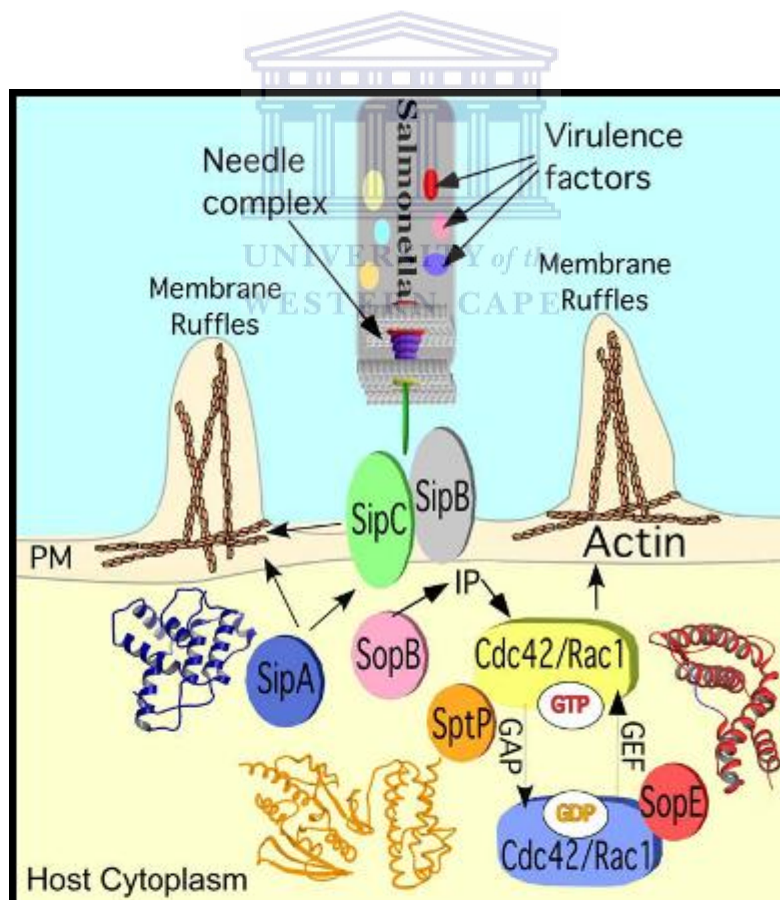

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Figure 2.15 SPI1 virulence factors involved in *Salmonella* invasion of target cells (adapted from Lilic and Stebbins 2004).

The effector proteins SopB, SopE and SopE2 function by activating the Rho family GTPases Cdc42 and Rac1 (Figure 2.15). SopE has the ability to activate both Cdc42 and Rac1 while SopE2 only activates Cdc42. Cdc42 subsequently interacts with N-WASP while Rac1 interacts with WAVE2. This interaction activates the Arp2/3 complex which promotes extensive cytoskeletal rearrangements in the form of membrane ruffles (Figure 2.16) and subsequently bacterial internalisation. Due to SopB's inositol phosphate phosphatase activity, the loss of electrolytes and fluid secretion of intestinal epithelial host cells is triggered which contributes to the diarrhoeal symptoms (Hansen-Wester and Hensel 2001).

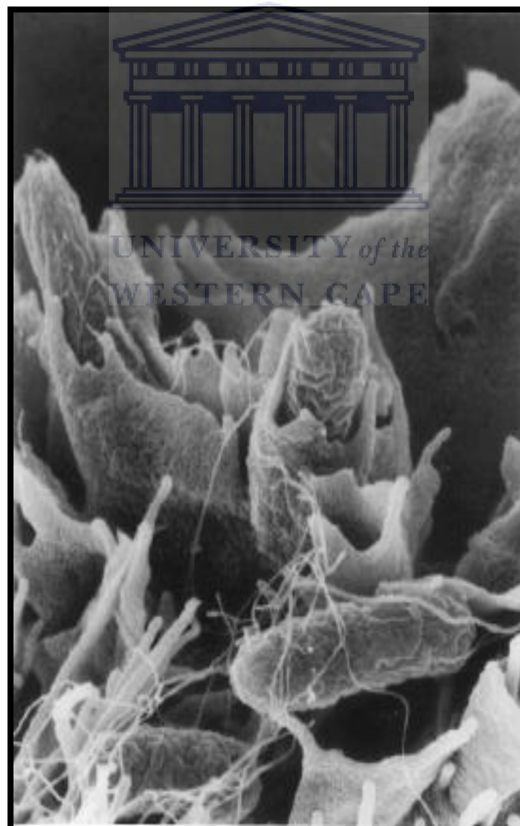
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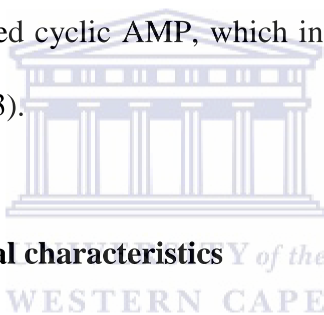
Figure 2.16 Scanning electron micrograph showing infection by *Salmonella* spp. The host cell's membrane ruffles extending for the internalisation of adherent bacteria. (adapted from Ohl and Miller 2001).

Once salmonellae have invaded target host cells, bacterial replication occurs within their endocytotic vacuoles. SPI2 is responsible for the deliverance of virulence effectors proteins produced across the vacuolar membrane of the SVC and into the cytoplasm of the host cell. Replication and survival of *Salmonella* inside the SCV is dependent on the proteins produced by SPI2's TTSS (Figure 2.14) (Guiney and Lesnick 2005; Lilic and Stebbins 2004; Marcus *et al.* 2000). Released salmonellae spread to mesenteric lymph nodes and throughout the body. Invasion results in inflammation of infected tissues. The recruitment and transmigration of neutrophils into the intestinal lumen is initiated when salmonellae invade the intestinal epithelium. A secretory response also occurs when mucosal adenylate cyclase is activated, resulting in increased cyclic AMP, which induces fluid secretion (Ohl and Miller 2001; Zhang *et al.* 2003).



2.4.2.2.5 Growth and survival characteristics

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Salmonellae are resilient microorganisms that easily adapt to extreme environmental conditions. They actively grow at a wide temperature range between 5 and 47ºC, with optimal growth between 35 and 37ºC (Macrae *et al.* 1993). Due to its osmotolerant characteristics, growth of this organism can occur at low water activity (a_w) of 0.93 and in the presence of 4% sodium chloride and around 350ppm sodium nitrite, even though they are not particularly salt-tolerant (Tiganitas *et al.* 2009; Macrae *et al.* 1993). These microorganisms utilize simple carbon compounds as sources of carbon and energy. To satisfy their nitrogen requirements they utilize nitrogenous compounds. *Salmonella* has the ability elicit several acid tolerance responses to avoid or repair damages that are caused by acid stress, growth can therefore occur at pH

ranging between 4.0 and 9.0 with optimal pH for growth of this bacteria ranging between 6.5 and 7.5 (Dunkley *et al* 2009; Dunkley *et al*. 2008; Macrae *et al.* 1993).

2.4.2.3 *Campylobacter*

2.4.2.3.1 General Characteristics and Classifications

The genus *Campylobacter* belongs to the family *Campylobacteraceae* (Corcionivoschi *et al*. 2009). Campylobacters are Gram-negative, non-spore- forming, slender, spiral curved rods with polar flagella (Keener *et al.* 2004; Lehner *et al.* 2005). These microorganisms are fastidious microaerophilic organisms that require an atmosphere containing 10% CO_2 and 5% O_2 (Yan *et al.* 2005; Humphrey *et al.* 2007). Former species in the genus *Campylobacter* are now classified in the genus's *Arcobacter* and *Helicobacter* (*H. cinaedi* and *H. fennelliae*) (Lehner *et al.* 2005; Neubauer and Hess 2006). *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. are L-AlA negative, and can be further identified and differentiated based on biochemical tests such as indoxyl acetate, hippurate, McConkey, aryl sulfatase and rapid H_2S (Figure 2.17) (Lastovica 2006).

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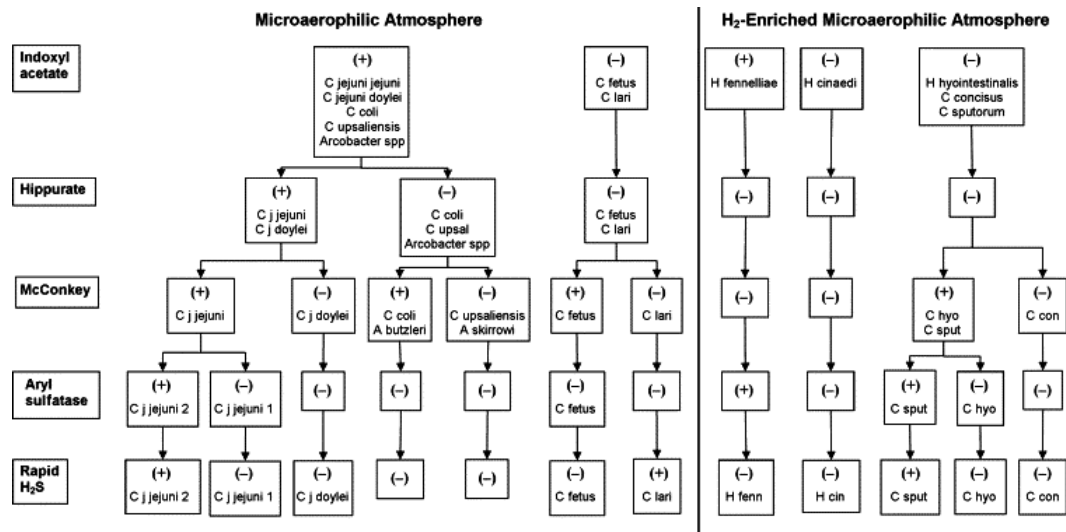

Figure 1.17 Biochemical flowchart for the identification and differentiation of *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. (adapted from Lastovica 2006).

2.4.2.3.2 Viable Non-culturable

Subjection of *Campylobacter* cells to unfavourable conditions such as prolonged periods of air exposure or water (low nutrient status) has been shown to metamorphose cells into a viable but non-culturable (VNC) state (Rowe and Madden 1999). The spiral-curved rods of campylobacters transform to a coccoid morphology (Keener *et al.* 2004). The transitions to a VNC state make the microorganism incapable of cell division in normal media suitable for normal culturable forms (Humphrey *et al.* 2007; Rowe and Madden 1999).

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2.4.2.3.3 Ecology/ Origin

The natural reservoirs of pathogenic *Campylobacter* species are the alimentary tracts of domestic animals, livestock and wild animals and birds (Snelling *et al*. 2005). These microorganisms also harbour rivers, seawater and groundwater, where they are

introduced by sewage discharges or agricultural run-off and faeces from wild animals and birds (Lastovica 2006; Diergaardt *et al.* 2004).

2.4.2.3.4 Importance in food

Campylobacters cause acute enterocolitis in humans and presents 3-5 days of diarrhoea varying from mild, noninflammatory, self-limiting diarrhoea to severe, inflammatory, bloody diarrhoea lasting for several weeks (Dorell *et al.* 2001; Lee and Newell 2006). *Campylobacter* species commonly infecting humans worldwide are *Campylobacter jejuni* and *Campylobacter coli* (Humphrey *et al.* 2007). Complications associated with campylobacteriosis include arthritis, carditis, cholecystitis, colitis, endocarditis, erythemanodosum, Guillain-Barré syndrome, haemolytic-uraemic syndrome, meningitis, pancreatitis and septicaemia (Adams and Motarjemi 1999). The infective dose capable of causing illness in susceptible humans is as low as 500 colony forming units (cfu) (Lee and Newell 2006). The infective dose may vary due to factors such as the vehicle in which it is ingested and the susceptibility of the individual (Anonymous 2002). *Campylobacter* infection is acquired from ingestion or handling of undercooked or contaminated meat products, consuming contaminated or unpasteurized milk and dairy products, drinking water from contaminated supplies, foreign travel and contact with pets (Figure 2.18) (Diergaardt *et al.* 2004; Sahin *et al.* 2002). 90% of all sporadic human cases have implicated the consumption of contaminated chicken meat products and cross-contamination from this source as well as contact with cattle including consumption of beef and milk (Dasti *et al.* 2010).

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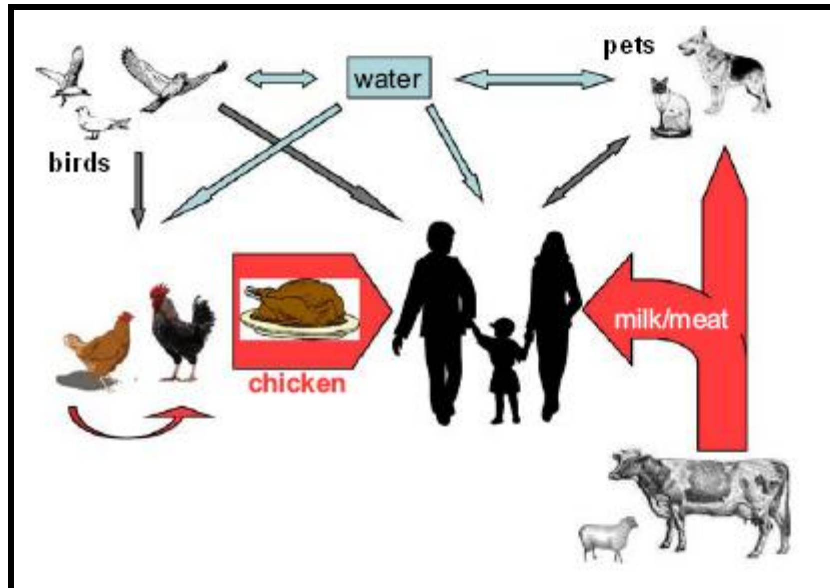

Figure 2.18 Routes of infection by *Campylobacter* spp. (adapted from Dasti et al. 2010).

2.4.2.3.5 Pathogenicity

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Campylobacters can invade directly by invasion of the epithelial cells of the gut and release toxins or indirectly by inducing an inflammatory response (Figure 2.19) (Rowe and Madden 1999). The mechanisms by which *Campylobacter* causes human disease are not yet well defined but are multifactorial (Konkel *et al.* 1999). Factors involved in the pathogenesis of *Campylobacter* infection may include flagellar motility, adherence and invasion through mucosal cells as well as cytotoxic effects (Friis *et al.* 2005; Yan *et al.* 2005). *Campylobacters* must initially evade the mechanical and immunological barriers of the gastrointestinal tract in order to establish an infection. The motility and corkscrew morphology of *Campylobacter* spp. and the relatively short O-sidechain of its lipooligosaccharide are traits which contribute to the ability of *Campylobacters* such as *C. jejuni* to penetrate the mucus layer of the gastrointestinal epithelium, which serves as the first line of defence (Young *et al.* 2007).

Once penetration of the mucus membrane has occurred, the bacterium can interact with the underlying epithelial cells via adherence and adhesion mechanisms and toxin release (Monteville and Konkel 2002). Maximal invasion of host epithelial cells is achieved when *C. jejuni* is metabolically active and secrete proteins from the flagellar type III secretion system (Christensen *et al.* 2009) Several studies on *C. jejuni* have led to the identification of some putative adhesion or binding factors, which include fibronectin-binding outer membrane protein CadF, the *Campylobacter* invasive antigen Cia, the transporter CapA, the periplasmic binding protein PEB1, and the surface exposed lipoprotein JipA (Konkel *et al*. 2004; Monteville and Konkel 2002).

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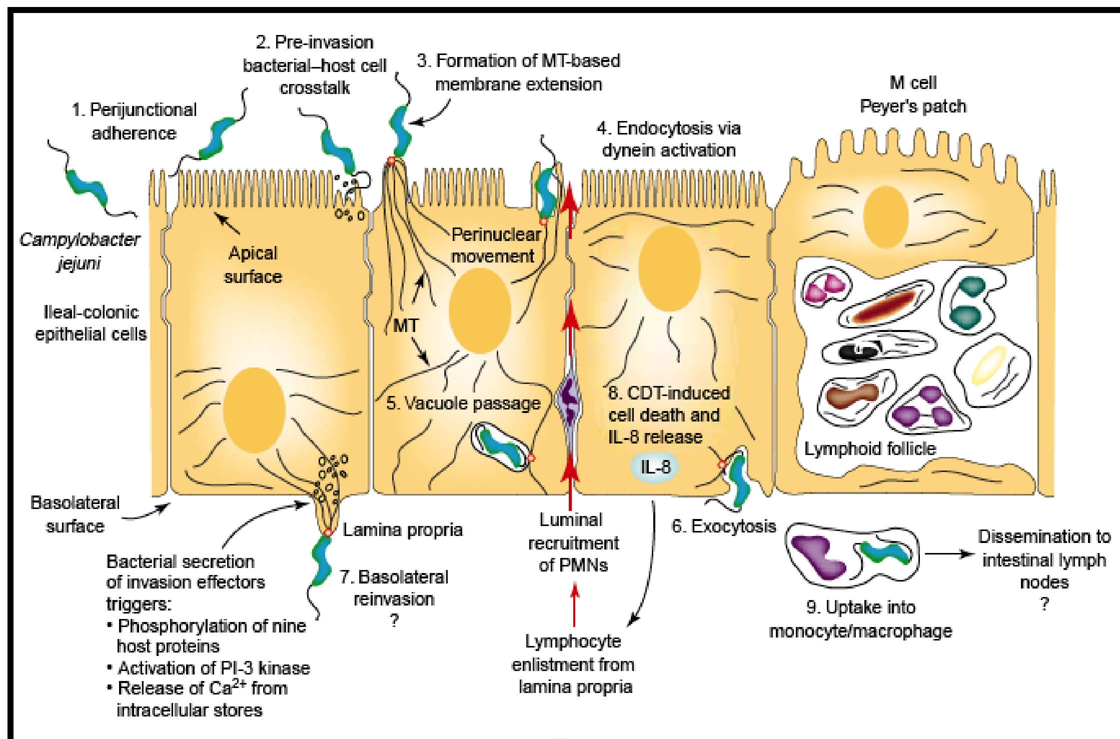

Figure 2.19 The schematic representation of the overall steps in the infection process of host cells by *Campylobacter jejuni*. (1) *C. jejuni* adhere to the apical cell surface at the perijunctional region. (2) The attached *C. jejuni* are upregulated and putative 'invasion' effectors are secreted into the host cell. Phophorylation of host proteins and the release of Ca^{2+} from intracellular stores are triggered by these putative effectors. (3) A localized disruption of cortical actin filaments is triggered by host signalling cascades, followed by the extension of microtubules to form membrane protrusions that meet the adjacent bacterium. (4) The bacterium enters the host cell via membrane invagination brought upon by the interaction of bacterium with putative receptors in membrane caveolae, subsequently triggering endocytosis. Interaction with the microtubule motor protein dynein (red dot) is thought to provide the motive force of endocytosis. (5) The internalised vacuole-bounded *C. jejuni* move via dynein along microtubules to the basolateral surface for exocytosis (6). (7) Released *C. jejuni* are thought to re-enter the epithelium basolaterally. (8) Interleukin-8 is secreted basolaterally from infected cells, subsequently recruiting lymphocytes from the lamina propia. (9) *C.jejuni* is thought to survive in macropahages or monocytes for several days, therefore assisting in their local dissemination. (adapted from Kopecko *et al.* 2001).

CadF is a peptidoglycan-associated protein, which is expressed in all *C. jejuni* and *C. coli* strains and mediates cell adhesion by binding to the cell matrix protein fibronectin (Jin *et al.* 2001). It plays a dual role; it triggers adhesion to host cells by binding specifically to fibronectin, which is located on epithelial cells and it triggers

signalling processes leading to activation of the small GTPases Rac1 and Cdc42, thereby inducing internalisation (Dasti et al. 2010; Monteville and Konkel 2002). CadF is similar to *E. coli* OmpA and forms membrane channels, but the role of this activity has not been established (Mamelli *et al.* 2006).

C. jejuni's invasion is unique as that it enters the host cell with its tip followed by the flagellar end (Figure 2.20) (Dasti *et al*. 2010). The Cia protein's secretion mechanism and role in the invasion mechanism is not apparent, however it is suggested that the production of Cia protein is stimulated early in colonisation in the small intestine by the bile component deoxycholate, but that secretion occurs only after adherence at the site of long-term colonisation (Rivera-Amill *et al.* 2001). Co-cultivation with host cells and in INT 407 (a human intestinal epithelial cell line) cell-condition medium by Konkel *et al.* (1999) has led to the identification of a *C. jejuni* gene termed *Campylobacter* invasion antigen B (CiaB). This study revealed that the CiaB protein amino acid sequence shared similarity with type III secreted proteins associated with the invasion of host cells from other more extensively characterised bacterial pathogens.

Confocal microscopy studies of the *C. jejuni* infected cells revealed that *C.jejuni* CiaB protein was translocated into the cytoplasm host cells. The lipoprotein CapA has also been implicated as a possible adhesion as it is an autotransporter that is homologous to an autotransporter adhesin. Mutation of this adhesin has led to decreased adherence to Caco-2 (a human colonic cell line) cells and decreased colonization and persistence in a chick model (Ashgar *et al.* 2007).

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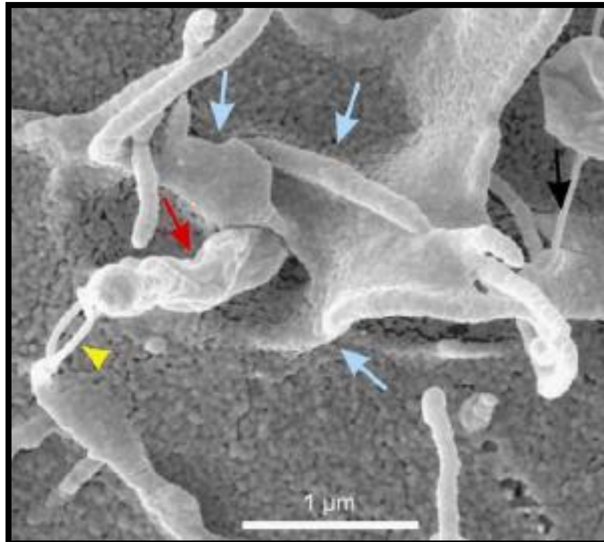

Figure 2.20 Scanning electron microscopy of Int-407 cell invasion by *C. jejuni*. *C. jejuni* 81-176(red arrow) entry into host is associated with membrane ruffles (blue arrow). The bacterium enters into the host cell first with their tip followed by the flagella-containing end (yellow arrow) (adapted from Dasti et al. 2010).

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Leon-Kempis *et al.* (2006) established that the PEB1 adhesin is located in the periplasm and shares homology to the periplasmic-binding proteins of amino acid ATP-binding cassette transporters and is found to be crucial for adherence to HeLa cells. In addition to PEB1 being characterised as an adhesin, the PEB transport system was found to play a key role in the ability of *C. jejuni* to catabolize aspartate and glutamate, two common amino acids likely to be present in gut contents. The surface exposed lipoprotein JipA is another characterised adhesin which is crucial for Hep-2 cell binding as that it binds to Hsp90α, resulting in the activation of NF-κB and p38 mitogen-activated protein kinase, subsequently contributing to proinflammatory responses (Jin *et al.* 2003).

Once internalized, *C. jejuni*-containing vacuoles appear to move along microtubules to the perinuclear region of the cell by interactions with dynein (Hu *et al.* 1999). Interaction with the epithelial cells causes interleukin-8 production which

subsequently recruits dendritic cells, macrophages and neutriphils which interact with *C. jejuni* and eventually results in a massive pro-inflamatory response and increases in the corresponding cytokines (Young *et al.* 2007).

2.4.6.5.1 Cytolethal distending toxin

Cytolethal distending toxin (CDT) activity is encoded by three adjacent genes, CdtA, CdtB and CdtC, which is required for cytotoxity (Figure 2.21) (Eyigor *et al.* 1999). The heterodimeric B subunit consisting of CdtA and CdtC interacts with the enzymatically active (A) subunit CdtB to form a tripartite CDT holotoxin (Lara-Tejero and Galán 2002; Lara-Tara and Galán 2001).

Depending on the type of cell, CDT has the ability to cause DNA lesions, cromatine fragmentation, cytoplasm distension and cell cycle arrest in the G1 or G2/M transition phase, leading to progressive cellular distention (Figure 2.22) and, ultimately death in several cell lines (Lara-Tara and Galán 2001; Eyigor *et al.* 1999; Pickett and Whitehouse 1999). CdtA has some similarity to the ricin B chain, which is responsible for receptor mediated endocytosis of ricin, and CdtC may be responsible for holotoxin binding and transgression through the host cell plasma membrane (Young *et al.* 2007; Hassane *et al*. 2003).

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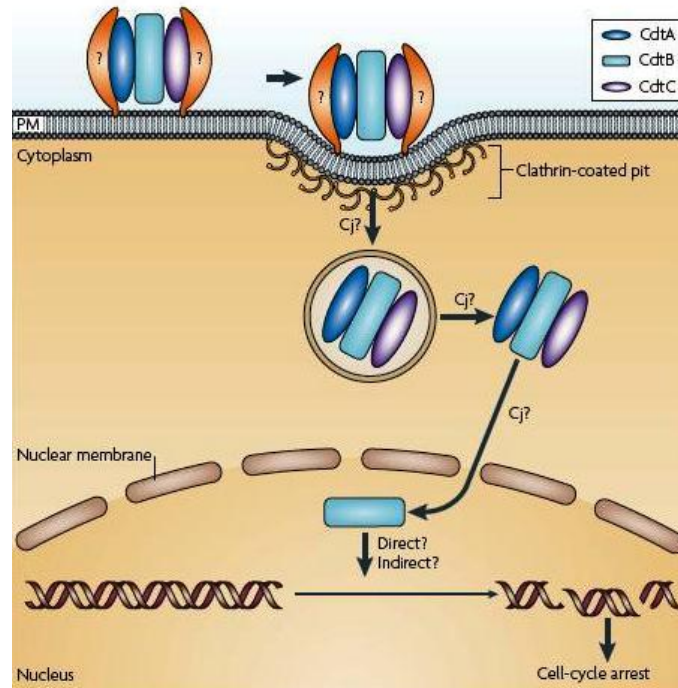

Figure 2.21 Diagrammatic illustration of the uptake and activity of cytolethal distending toxins. The subunits, CdtA, CdtB and CdtC react to form the CDT holotoxin. The holotoxin is suggested to bind to an unknown receptor on the host cell surface. CDT enters the host cells by way of the clathrin-coated pits. CdtB is transported to the nucleus where it enters and causes double–strand DNA breaks and cell-cycle arrest (adapted from Young *et al.* 2007).

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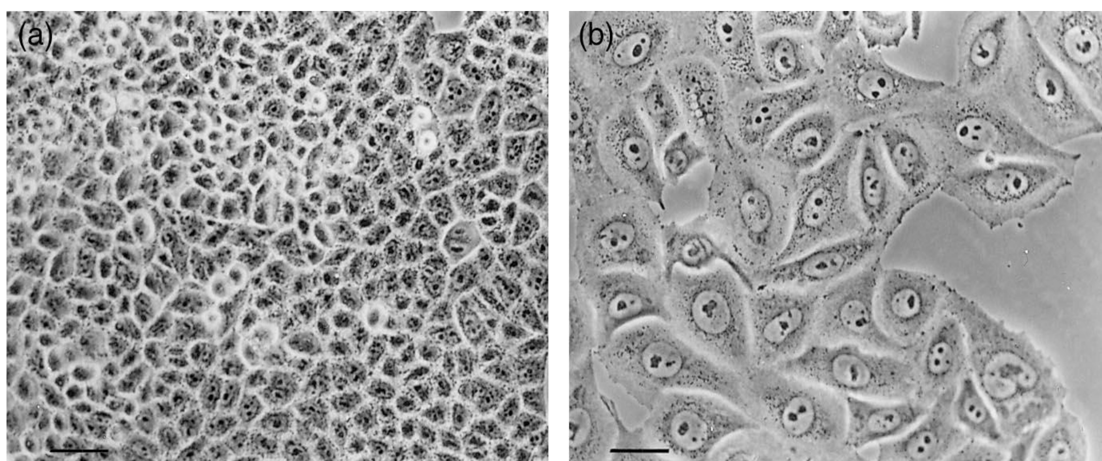

Figure 2.22 Effect of cytolethal distending toxins on HeLa cells. (a)Normal HeLa cells after 72h after addition of control lysates. (b) Distended HeLa 72h after addition of CDT. Distended cells contain nuclei that are beginning to fragment (adapted from Pickett and Whitehouse 1999).

CdtB displays similar activity to the enzyme deoxyribonuclease and appears to degrade plasmid DNA (Figure 2.21) (Dasti *et al.* 2010). It is suggested that CdtB is translocated into the host cell cytoplasm and is transported via the golgi apparatus to the endoplasmic reticulum and reaches the nucleus by a retrograde transport mechanism where it inflicts limited DNA damage, subsequently stimulating a highly coordinated cellular response that leads to the observed cell cycle arrest characteristic of CDT intoxication (Heywood *et al.* 2005).

2.4.2.3.6 Growth and survival characteristics

Campylobacter species are microaerophilic bacteria that require special atmosphere that usually consists of 5% oxygen, 10% carbon dioxide and 85% nitrogen for growth (Duffy *et al.* 2007). They are capable of growth between the pH range of 4.9 and 9.0, but grow optimally at a pH range between 6.5 and 7.5. These microaerophilic microorganisms are thermophilic; being able to grow at a range between 37ºC and 42ºC with optimal growth at the temperature of 42ºC. Campylobacters are easily inactivated by heat since they have a D-value of less than 1min at 60ºC (Keener *et al.* 2004).

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2.4.3 Gram-Positive pathogens

2.4.3.1 *Staphylococcus aureus*

2.4.3.1.1 General characteristics and classifications

Staphylococcus aureus are Gram-positive cocci, which belong to the genus *Staphylococcus.* Cells occur singly, in pairs, tetrads, short chains and irregular grape-like clusters (FDA 2009). These organisms are non-motile, facultative anaerobes. *Staph. aureus* isolates can be identified and confirmed on the bases of their colony morphology and biochemical characterizations (Table 2.6) (Singh and Prakash 2008). Although nonsporeforming, some strains of *Staphylococcus aureus* are capable of producing heat resistant toxins (Bhatia and Zahoor 2007). Staphylococci cells range in diameter from 0.5 to 1.5µm and have a 30 to 40% G+C composition (Dmitriev *et al.* 2004; Jablonski and Bohach 1997).

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Table 2.6 Biochemical characterization of *Staphylococcus aureus* (adapted from Singh and Prakash 2008)

Biochemical test		Reaction
Catalase		+
Oxidase		-
Indole Production		-
Nitrate Reduction		+
Methyl Red		+
Voges-Proskauer		+
Acid from sugar	(a) Glucose	+
	(b) Mannitol	+
	(c) Maltose	+
	(d) Lactose	+
	(e) Raffinose	-
	(f) Sucrose	+
Haemolysis		+
Coagulase		+

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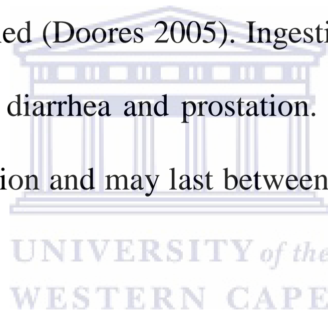
2.4.3.1.2 Ecology/ Origin

Humans and animals are the main reservoirs for *Staphylococcus aureus*. In humans 50% or more individuals harbour *Staph. aureus* in nasal passages, throats and on hair and skin (FDA/CFSAN 2006). The nasopharyngeal region of birds and mammals are especially the primary reservoir of *Staph. aureus* (Atanassova *et al.* 2001) Foods that have been vehicles for *Staph. aureus* in food poisoning incidents include meat and meat products; poultry and egg products; milk and milk products (Balaban and Rasooly 2000). *Staph. aureus* colonise in food equipment, water, dust and environmental surfaces (Le Loir *et al.* 2003).

2.4.3.1.3 Importance in food

Staph. aureus has been considered by many countries to be the second or third most common pathogen causing foodborne illness in humans (Atanassova *et al.* 2001). The microorganism is capable of hiding in pores and hair follicles and is difficult to remove from the skin, and once hands become damp it can be drawn to the surface and transferred to foods (Adams and Motarjeni 1999). Staphylococcal food poisoning is caused by the ingestion of highly heat-stable proteins and is generally, mild and self-limiting (Stiles and Krakauer 2005; Dinges *et al.* 2000). Some *Staph. aureus* strains are capable of producing toxins called enterotoxins when high cell density, estimated at 10^5 cfu/g is reached (Doores 2005). Ingestion of contaminated food leads to nausea, vomiting, cramps, diarrhea and prostation. Symptoms may appear within 30 min to 8 hours after ingestion and may last between 1 to 2 days (Atanassova *et al.* 2001).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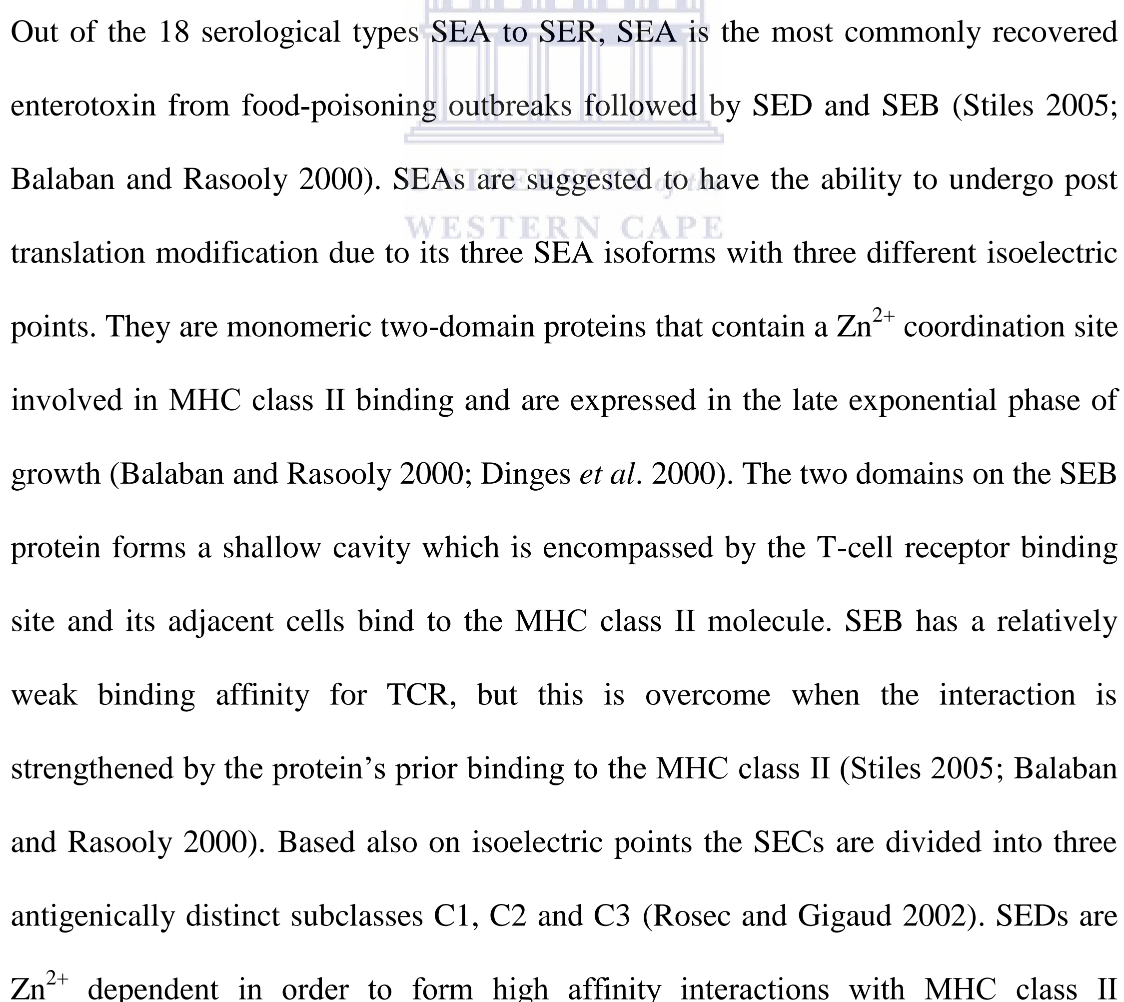
2.4.3.1.4 Pathogenicity

In order to promote its survival and subsequent dissemination, *Staphylococcus aureus* produce numerous virulence factors. These virulence factors include staphylococcal enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1), protein A, coagulases, hemolysins and leukocidins. Out of these virulence factors, SEs has been implicated in staphylococcal food poisoning (Stiles 2005b; Balaban and Rasooly 2000).

2.4.3.1.4.1 Enterotoxins

Staphylococcal enterotoxins (SE's) have been classified into serological types. Serological types SEA, SEB, SEC, SED and SEE have already been discovered in staphylococcal contaminated foods and further extensive analysis of the *Staphylococcus aureus* genome have led to the discovery of enterotoxins homologues (staphylococcal enterotoxin-like superantigens) (Rosec and Gigaud 2002). The percentage of enterotoxigenic strains increase if the newly discovered Staphylococcal enterotoxin-like superantigens are considered together with the serological types SEA to SEE (Bania *et al.* 2006).



Out of the 18 serological types SEA to SER, SEA is the most commonly recovered enterotoxin from food-poisoning outbreaks followed by SED and SEB (Stiles 2005; Balaban and Rasooly 2000). SEAs are suggested to have the ability to undergo post translation modification due to its three SEA isoforms with three different isoelectric points. They are monomeric two-domain proteins that contain a Zn^{2+} coordination site involved in MHC class II binding and are expressed in the late exponential phase of growth (Balaban and Rasooly 2000; Dinges *et al*. 2000). The two domains on the SEB protein forms a shallow cavity which is encompassed by the T-cell receptor binding site and its adjacent cells bind to the MHC class II molecule. SEB has a relatively weak binding affinity for TCR, but this is overcome when the interaction is strengthened by the protein's prior binding to the MHC class II (Stiles 2005; Balaban and Rasooly 2000). Based also on isoelectric points the SECs are divided into three antigenically distinct subclasses C1, C2 and C3 (Rosec and Gigaud 2002). SEDs are Zn^{2+} dependent in order to form high affinity interactions with MHC class II

molecules. Located on the surface of these proteins close to the domain interface of the molecules is a second Zn^{2+} binding site. In the presence of Zn^{2+}, SEDs have the ability to form dimers, which facilitates novel and biologically relevant multimeric interactions with MHC class II molecules (Balaban and Rasooly 2000). According to DNA sequencing identity, SEE is closely related to SEA and SED, sharing an 81% and 53% sequence homology respectively (Stiles 2005a).

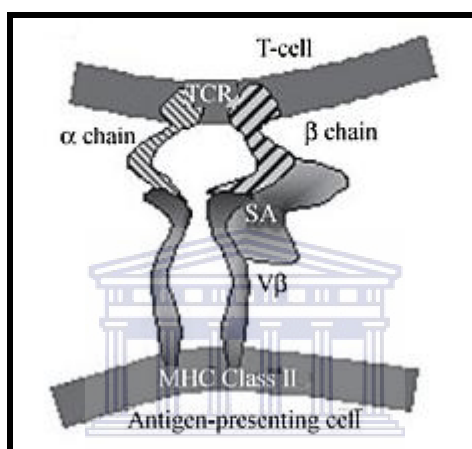


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Figure 2.23 Nonspecific T-cell activation by superantigen (adapted from Le Loir 2003).

Staphylococcal enterotoxins have the ability to function as toxins that cause emetic responses as well as superantigens (SA) that stimulate non-specific T-cell proliferation (Balaban and Rasooly 2000). Their mechanism of action in causing an emetic response is not well understood as the mechanism of SE toxicity. However, these toxins are thought to act as neurotoxins that stimulate vomiting through the vagus nerve. What is known is that putative cellular receptors for SEs responsible for the emetic reflex do exist and is located in the abdominal viscera. These are thought to be the target for SEs. However, to date these receptors have not yet been identified making the early events in pathogenesis an uncertainty (Stiles 2005b; Dinges *et al.* 2000). Immunosuppression and non-specific T-cell proliferation occur when SE's

interact with Vβ chains of T-cell antigen receptors. Non-specific activation (Figure 2.23) and proliferation of T-cells is caused when SAs cross-link the T-cell antigen receptor and the major histocompatibility complex class II of antigen- presenting cells (APC). Activated T-cells secrete massive amounts of cytokines, which lead to toxic shock syndrome (McCormick *et al*. 2001; Bania 2006). SE's activation of T-cells are much larger in magnitude than antigen- specific activation, hence the toxic shock syndrome (Dinges *et al*. 2000; Le Loir *et al*. 2003).

2.4.3.1.5 Growth and survival characteristics

Staphylococcus aureus are able to grow in a wide range of temperatures ranging between 6.5 – 48.5ºC, but grows optimally at 37ºC (Schmitt *et al.* 1990). The microorganism grows well in the presence of oxygen and is capable of growing anaerobically, although in the presence of 80% CO_2 growth is retarded. The minimum and maximum pH limit for the growth of this bacteria species is 4.2 and 9.3 respectively, while optimal pH growth lies in the range between 7 and 7.5 (Bergdoll 1989). *Staph aureus* are resistant to drying due to toleration of low water activity (0.85) and optimal growth at a_w 0.99. High salt concentrations up to 15% NaCl are also tolerable by *Staph. aureus* strains. These characteristics explain the growth of *Staph. aureus* strains in a wide variety of foods and gives them a competitive advantage over other organisms in foods with low a_w and high salt concentrations (Le Loir *et al*. 2003).

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Toxin production occurs when *Staph. aureus* increases to a high cell density. It is estimated that less than 1.0µg toxins are produced when cell levels exceed 100,000

cfu per gram (Doores 2005). Toxins are highly stable and are able to resist digestion by proteolytic enzymes such as pepsin or trypsin. Their ability to prevent digestion allows them to keep their activity in the digestive tract (Le Loir *et al*. 2003). Toxins are optimally produced at temperatures ranging between 10 and 45ºC. The minimum and maximum pH for production are 4, 8 and 9 respectively, optimum pH are between 5, 3 and 7. Toxin production is highest in the presence of oxygen and lowest under anaerobic conditions. The water activity range for production is between 0, 86 and 0, 99, optimum production is $\geq$ 0, 9 a_w. *Staph. aureus* strains and toxins are both resistant to frozen storage (Bobitt 2002).

2.4.3.2 *Listeria monocytogenes*

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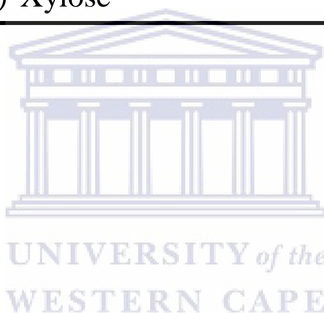
2.4.3.2.1 General Characteristics and Classifications

Listeria monocytogenes are Gram- positive, nonsporeforming, motile rods, which belong to the genus *Listeria* (Lungu *et al.* 2009). These microorganisms are facultative anaerobic and catalase positive with a low DNA composition of 36-42 mol % GC (Hain *et al.* 2007; Doyle *et al.* 1997). *L. monocytogenes* isolates can be identified and confirmed on the bases of their colony morphology and biochemical characterizations (Table 2.7). *L. monocytogenes* have 13 serovars, which have the ability to cause disease, of which serovar 4b strains are responsible for 33 to 50% of sporadic human cases; all foodborne outbreaks (Doyle *et al.* 1997).

Table 2.7 Biochemical characterization of *Listeria monocytogenes* (adapted from Singh and Prakash 2008)

Biochemical test		Reaction
Catalase		+
Oxidase		-
Indole Production		-
Nitrate Reduction		-
Methyl Red		+
Voges-Proskauer		+
Haemolysis		+
Acid from sugar	(a) Rambose	+
	(b) α methyl d mannoside	+
	(c) Xylose	-



2.4.3.2.2 Ecology/ Origin

The microorganism's wide distribution in nature is due to its ability to survive long periods in many different environments and its psychrotrophic behaviour (Lungu *et al.* 2009). *L. monocytogenes* is widely distributed in soil, various aqueous environments, rotting vegetation and wastewater and is frequently carried in the intestinal tract of humans and animals (Macrae *et al.* 1993).

2.4.3.2.3 Importance on food

Infection in humans and animals by *L. monocytogenes* results in listeriosis. Several major outbreaks since 1981 indicate the consumption of contaminated food as the primary vehicle of transmission of listeriosis. The invasive disease infects neonates,

elderly, pregnant woman, immunosuppressed persons, cancer and AIDS patients and those with chronic diseases (Doores 2006). Listeriosis frequently affect pregnant woman in their third trimester. Symptoms for pregnant woman include flu-like illness with fever and headaches. Infection of the fetus is more serious, leading to spontaneous abortion, fetal death, stillbirth, severe neonatal septicemia and meningitis (Hain *et al.* 2007). In nonpregnant adults, infection of the central nervous system results in meningitis and meningoencphalitis (Doyle *et al*. 1997).

2.4.3.2.4 Pathogenicity

Listeriosis involves the internalisation of *L. monocytogenes* into phagocytes (intestinal epithelial cells) and nonphagocytic cells (hepatocytes) and it's spreading from cell to cell using an actin-based motility process (Figure 2.24) (Ireton and Cossart 1997). The invading features of *L. monocytogenes* are termed the "zipper mechanism" which involves the interaction of bacterial surface ligands with respective cellular receptors to create tight apposition of the plasma membrane around the bacterium (Cossart and Toledo-Alejandro 2008). Internalin (InlA) and InlB are proteins that are involved in the internalisation of *L. monocytogenes* and are expressed on the surface of the bacteria. The receptor for InlA is E-cadherin, which is a well characterised member of a family of cell-cell adhesion molecules. Expression of E-cadherin takes place at the adherence junction and on the basolateral face of epithelial cells (Cossart and Toledo-Arana 2008; Orndorff *et al.* 2006).

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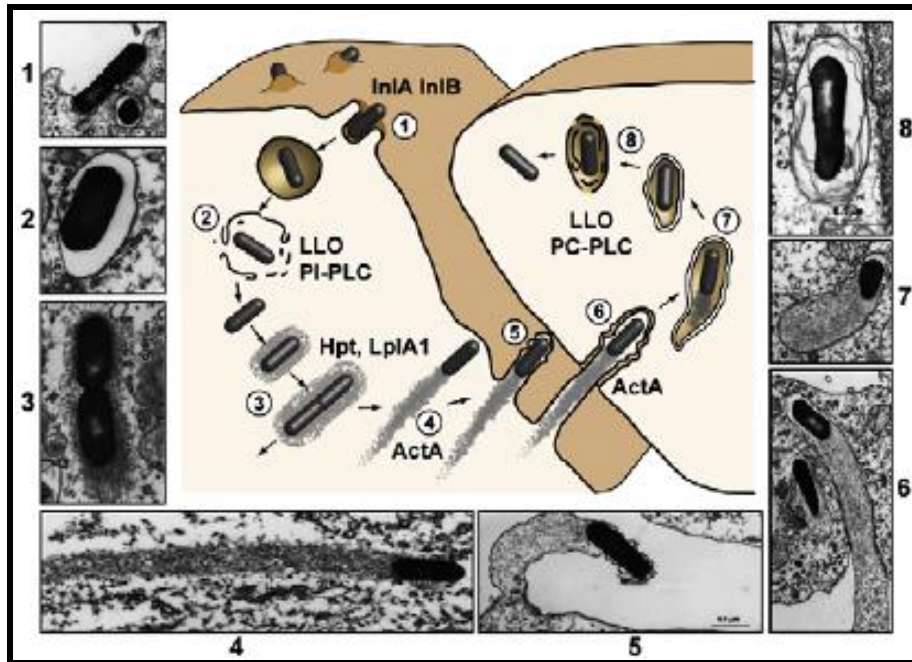

Figure 2.24 Scanning electron micrographs and schematic representation of the infection process of host cells by *Listeria monocytogenes* (adapted from Cossart and Toledo-Alejandro 2008).

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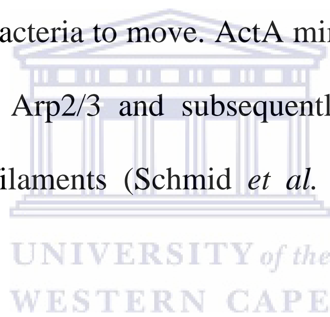
The protein InlB differs from InlA in that it has three receptors and is released partially into the environment due to its loose attachment to the cell wall. gClqR/p32 was the first out of the three receptors to be identified and has the capability of shuttling between mitochondria and the cell surface as well as the nucleus. The second receptor and the most important is Met, which functions as a tyrosine kinase as well as the receptor for the hepatocyte growth factor (HGF). Met activation induces heparin-mediated receptor clustering and potent signalling. Activated heparin induces the detachment of InlB from the cell surface and subsequently induces its oligomerisation. Glycosaminoglycans (GAGs) have been identified as the third receptor, and significantly increase the InlB-dependent activation of Met. GAGs protect detached InlB from proteases at its entry site into the host cell. Local rearrangements in the actin cytoskeleton and other signals that lead to bacterial internalisation are induced by the attachment of these host cell receptors to bacterial ligands Inl A and Inl B (Dussurget *et al.* 2004; Pizarro-Cerdá *et al.* 2004; Cossart *et*

al. 2003). Soon after internalisation, *L. monocytogenes* reside within membrane-bound vacuoles, which unlike the internalisation of *Salmonella* are lysed 30min after entry by listeriolysin O (LLO) released by the bacteria (Cossart and Toledo-Arana 2008). LLO is encoded by the *hly* gene and is not always efficient in some human cells so lysis is mediated by phosphatidylinositol phospholipase C (PlcA) and phosphatidylcholine phospholipase C (PlcB) (Lukowiak *et al.* 2004). PlcA mediates the escape from the primary vacuole, while PlcB mediates the escape from the secondary vacuole (Hain *et al.* 2006). The once vacuole bounded bacteria are released to the cytosol and begin replicating. Free bacteria become enclosed with actin filaments. The release of ActA by the bacteria induces the polymerisation of these actin filaments allowing the bacteria to move. ActA mimic the WASP family proteins by recruiting and activating Arp2/3 and subsequently induce the formation of a network of branched actin filaments (Schmid *et al.* 2005; Vázquez-Boland *et al.* 2001).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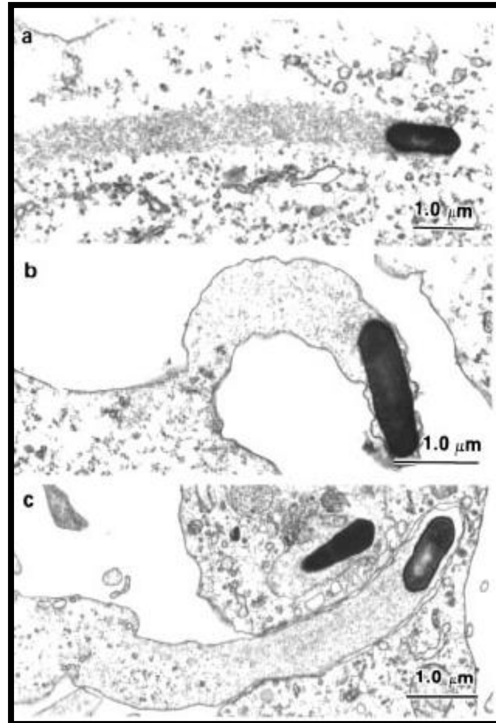

Figure 2.25 *L. monocytogenes* actin-based motility and cell-to-cell infection. (a) *L. monocytogenes* moving with an F-actin comet tail. (b) Contact of the bacterium with the host cell and formation of a characteristic protuberance. (c) Internalisation of the double membrane bounded bacterium into host cell (adapted from Ireton and Cossart 1997).

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Polymerisation of actin filaments, result in an actin comet tail up to 40µm in length in the cytosol (Figure 2.25a), which is left behind by the moving bacteria. When the moving bacteria make contact with the plasma membrane, they induce the formation of intrusions (Figure 2.25b), which are internalised by neighbouring cells. The bacteria are internalised as double membrane bounded vacuoles (Figure 2.25c), which lyse and begin the new cycle of replication, movement and spreading. *L. monocytogenes* direct cell-to-cell spread explains its dissemination within host tissues as well as it circumventing host defences (Dussurget *et al.* 2004; Ireton and Cossart 1997).

2.4.3.2.5 Growth and survival characteristics

Listeria species are resistant to diverse environmental conditions. These microorganisms grow at a wide range of pH, between 5 and 9.5. They are able to grow over a wide range of temperature of 0.5 and 45ºC (Warinner and Namvar 2009; Gandhi and Chikindas 2006). Due to its osmotolerant characteristics, growth can occur at low water activity (a_w) of 0.91 and in the presence of 20% sodium chloride (Lado and Yousef 2007). When stored at 4ºC, *L. monocytogenes* show survival at salt concentrations as high as 25.5%. (Macrae *et al.* 1993; Roscourt and Cossart 1997).

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2.5 SPOILAGE OF MEAT BY MICRORGANISMS

Food becomes a waste to humans when steps are not taken to prevent spoilage. The spoilage process occurs initially when foods are harvested or slaughtered. When food becomes aesthetically unacceptable to the consumer, it's considered to be spoiled by bacteria. Bacterial spoilage in food result in a variety of sensory defects, such as off flavours, formation of slime, colour changes or strong odours (Jackson *et al.* 1997).

2.5.1 Factors affecting spoilage

Highly perishable foods are foods that have been harvested or slaughtered and are processed a little further or none at all. These foods are extremely susceptible to the action of bacteria and are easily spoiled by their metabolic activities (Gram and Dalgaard 2002). Conditions external to the food or factors inherent in the food affect the growth and selection of microorganisms in foods (Gram *et al.* 2002). In order for bacterial spoilage to occur a number of factors need to be in place. These include intrinsic factors, extrinsic factors and implicit parameters. Growth of bacteria and the resultant spoilage in food are influenced by the control of the above factors (McDonald and Sun 1999)

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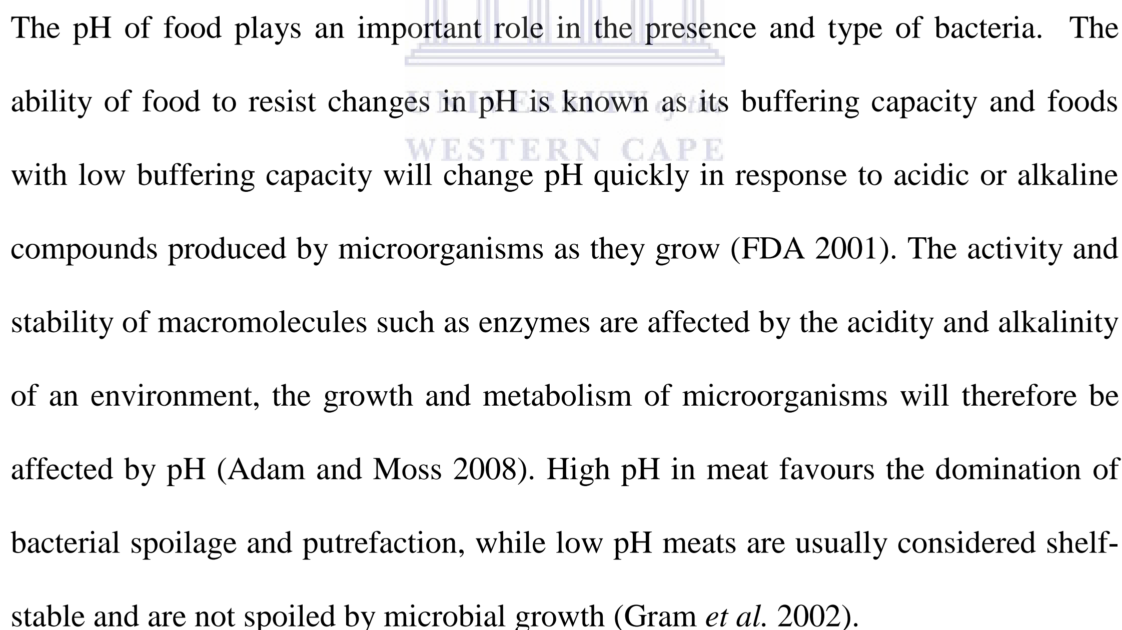
2.5.1.1 Intrinsic factors affecting microbial growth

Intrinsic factors involve properties such as availability of nutrients, acidity, water activity, oxidation-reduction potential and antimicrobial substances inherent in food

(Huis in’t Veld 1996). Food needs to be suitable for the growth of the contaminating bacteria in order for spoilage to occur.

The availability of nutrients in the food product affects the selection and growth of spoilage bacteria. It is the chemical elements that constitute microbial biomass, those molecules that the organism cannot synthesize and is most essential for growth, and a substrate that can be used as an energy source, is derived from foods (Adams and Moss 2008). Meat microflora catabolizes glucose, lactic acid, certain amino acids, nucleotides, urea and water-soluble proteins present in meat (FDA 2001). These compounds serve as essential energy sources and concentration of these compounds affect the type and rate of spoilage (Nychas *et al.* 2008).



The pH of food plays an important role in the presence and type of bacteria. The ability of food to resist changes in pH is known as its buffering capacity and foods with low buffering capacity will change pH quickly in response to acidic or alkaline compounds produced by microorganisms as they grow (FDA 2001). The activity and stability of macromolecules such as enzymes are affected by the acidity and alkalinity of an environment, the growth and metabolism of microorganisms will therefore be affected by pH (Adam and Moss 2008). High pH in meat favours the domination of bacterial spoilage and putrefaction, while low pH meats are usually considered shelf-stable and are not spoiled by microbial growth (Gram *et al.* 2002).

The ability of bacteria to colonize food is affected by the presence and availability of water. The metabolic activity of microorganisms is affected by the foods water activity (a_w), since all chemical reactions of cells require an aqueous environment (Jay

et al. 2005). Elimination of bacterial growth occurs in food when a decrease in a_w arise and only extremophiles and fungi are capable of development (Nychas *et al.* 2008). Gram-negative bacteria are more sensitive to low a_w than Gram-positive bacteria (FDA 2001).

The oxidation-reduction potential of food affects spoilage by microorganisms. The ordered sequence of both electron and hydrogen transfer reactions is essential for the electron transport chain and energy generation by oxidative phosphorylation in living cells (Adams and Moss 2008). Based on their relationship to redox potential (Eh) for growth, the major groups of microorganisms include aerobes with growth at the range +500 to +300mV, anaerobes with growth at the range +100 to less than -250mV, Facultative aerobes with growth at the range +300 to -100mV and microaerophiles (FDA 2001). The metabolic activity of aerobic microorganisms decreases the Eh of food, by depleting O_2 levels present, which subsequently provides anaerobes with a suitable environment in which to grow (Jay *et al.* 2005).

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Most foods naturally contain antimicrobial substances which affect the growth of contaminating bacteria (Prescott *et al.* 2002). These substances differ in their range of activities and potencies, and are present at varying concentrations in the natural food, but are frequently at levels too low to have an effect (Adams and Moss 2008).

2.5.1.2 Extrinsic factors affecting microbial growth

Environmental factors such as temperature, humidity and gaseous atmosphere composition during the storage of raw meat affects the selection of certain bacteria,

and affects their growth rate and activity (McDonald and Sun 1999). Rapid temperature reduction on the carcass surface generally decreases microbial growth and therefore extends the shelf life of the product. Reduction of refrigeration temperature not only affects bacterial growth, but also the composition of the bacterial flora (Nychas *et al.* 2008; Borch *et al.* 1996). Bacterial growth is rapidly initiated at low temperatures once relative humidity increases and becomes high. Microbial growth thus arises when moisture absorption occurs on the food surface in moist environments (Adams and Moss 2001). Moist atmospheric conditions favour a consortium of bacteria which are responsible for spoilage of meat stored at and between -1 and 25˚C (Prescott *et al.* 2002; Ellis and Goodacre 2001). Microorganisms are affected by gases such as carbon dioxide (CO_2), ozone (O_3) and oxygen (O_2), as they have a direct toxic effect that may inhibit growth and proliferation (FDA 2001). A variety of bacteria are able to grow to high final numbers in high oxygen atmospheres, while high CO_2 atmosphere conditions inhibit the growth of Gram-negative bacteria. A modified atmosphere generally affects the structure of microbial community, shifting it from Gram-negative to Gram-positive organisms when lactobacilli begin to dominate (Prescott *et al.* 2002; Borch *et al.* 1996).

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2.5.1.3 Implicit parameters

In meat the development of a microorganism creates interactions which subsequently have an effect on the microbial activity of the other microorganisms present. Microorganisms thus interact and influence the growth of one-another through cell-to-cell communication, antagonistic interactions and metabiosis (Nychas *et al.* 2008; Huis in't Veld 1996). Spoilage bacteria have the ability to produce chemical

communication signals that monitor population size. Gram- negative spoilage bacteria produce these chemical signals in the form of acylated homoserine lactones (AHLs) (Heurlier *et al.* 2006). The concentration of AHLs increases as the growth of Gram-negative bacteria increase and high concentrations of AHLs results in the up-regulation of phenotypic traits (Gobbetti *et al.* 2007). Cell-to-cell communication allows up-regulation or repression of various phenotypic traits such as virulence factors or antibiotic production which subsequently affect the development of other microorganisms (Gobbetti *et al.* 2007; Heurlier *et al.* 2006). Competition for essential nutrients, changes in pH value or redox potential or the formation of antimicrobial substances are the antagonism processes used by spoilage microorganisms to create a selective advantage that subsequently suppress less competitive bacteria (Gram *et al*. 2002; Huis in't Veld 1996). However, development of a certain group of spoilage microorganisms may enhance the growth of other bacteria by creating a favourable environment through production or availability of essential nutrients or removal of oxygen. Anaerobic bacteria rely on metabiosis with Gram-negative microflora to establish themselves in a previously unfavourable environment (Gram *et al*. 2002). These interactions may be the major factor governing the development of spoilage flora and subsequently the type of spoilage in meat.

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2.5.2 Microbial metabolic activities in food spoiling

Meat microflora catabolise glucose, lactic acid, and certain amino acids followed by nucleotides, urea and water-soluble proteins. Fresh meat serves as an ideal substrate since it contains sufficient glucose and simple carbohydrates to support bacterial levels of approximately 10^9 cfu/cm^2 (Ellis and Goodacre 2001). The supply of simple

carbohydrates is exhausted by the time surface microflora increases to 10^{7-8}cfu/cm^2 and is followed by the initial development of 'sensory' spoilage (Nychas *et al.* 2008). Once glucose levels at the surface are depleted bacteria will move on to metabolise secondary substrates such as free amino acids and lactates. The depletion of glucose at surface levels causes some bacterial species to switch from saccharolytic to amino-acid degrading metabolism. Utilisation of free amino acids by bacteria leads to increase levels of ammonia and by-products such as sulphides, indole, scatole and amines. Production of these compounds lead to the characteristic changes associated with spoiled meat (Table 2.8) (Nychas *et al*. 2008; Gram *et al.* 2002; Ellis and Goodacre 2001). These compounds seem to be the precursors of those microbial metabolites that are perceived as spoilage and their concentrations subsequently affect the type and rate of spoilage in fresh meat.

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Table 2.8 Spoilage substrates and metabolites found in microbiologically spoiled meat (Gram *et al.* 2002)

Sensory impression	Spoilage Product	Spoilage substrate	Specific spoilage organism
Ammonia, Putrid	Biogenic amines	Amino acids	Enterobacteriaceae and Lactic Acid Bacteria
Sulphidy off-odours	H_2S	Cysteine	*S. putrefaciens* Enterobacteriaceae *L. sake, L curvatus*
Greening	H_2S	Cysteine	*L. plantarum*
Sulphydryl off-odours	$(CH_3)_2S_2$	Methionine	Pseudomonas spp. Enterobacteriaceae
Acid off-odours	Acetic acid, L,D-lactic acid	Glucose, ribose, other carbohydrates	Lactic Acid Bacteria
Cheesy off-odours	Acetoin, diacetyl, 3-methylbutanoyl	glucose	*B. thermosphacta* Enterobacteriaceae Homofermentative Lactic Acid Bacteria

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2.5.3 Specific spoilage organisms

Fresh meat is a highly nutritious substrate that possesses a neutral to slightly acidic pH (Beef 5.1- 6.2, Chicken 6.2-6.4 and veal 6.0) and high moisture content (Jay *et al.* 2005). It therefore provides the ideal environment, permitting the growth of a wide range of microorganisms. Specific spoilage microorganisms (SSO) generally constitute a fraction of the fresh meats' natural microflora and are primarily only present in low quantities (Nychas *et al.* 2007). Once meat is stored under refrigerated

temperatures, SSO growth increases more rapidly than the remaining microflora (Huis in't Veld 1996). The initial microflora inherent in meat stored aerobically under refrigerated temperatures are varying levels of Gram-positive genera usually represented by micrococci, then lactic acid bacteria (such as *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* spp.) and *Bronchothrix thermosphacta* (Liu *et al.* 2006; Gram *et al.* 2002; Ellis and Goodacre 2001). Other componants of spoilage microflora include Gram-negative bacteria such as *Acinetobacter* spp., *Alcaligenes* spp., *Moraxella* spp. and *Enterobacteriaceae* (Paramithiotis *et al.* 2009; Liu *et al.* 2006). *Pseudomonas* species are however, considered to be the main spoilage microflora responsible for spoilage in fresh meat during aerobic storage (Lebert *et al.* 1998).

2.5.3.1 *Pseudomonas* spp.

The activity of motile and non-motile Gram-negative, psychrotrophic, aerobic rods dominated by *Pseudomonas* spp. are responsible for meat spoilage in air with low temperatures (between 2°C and 10°C) (Crowley *et al.* 2010; Rodríguez-Calleja *et al.* 2005). Species within the genus are strictly aerobic, but in some cases can utilise nitrate as an electron acceptor and grow anaerobically (Lebert *et al.* 1998). *Pseudomonas* species are a heterogeneous group; this is suggested by their moles % G+C content of their DNA (50-70) (Cousin 1999). *Pseudomonas* flora is widely distributed in the environment and may contaminate foods from many sources (Huis in't Veld 1996). These bacteria have been observed to attach more rapidly and can readily form biofilms (Figure 2.26) in any environment beneficial for growth, therefore dominating proteinaceous foods stored aerobically at low temperatures

(Gram *et al.* 2002; Ellis and Goodacre 2001). Biofilms created by pseudomonads are a growth form rather than a fixed property and as a complex community are highly heterogenous in behaviour (Garcia-Medina *et al.* 2005). The community of microorganisms begins to form once attachment of *Pseudomonas* spp. to the meat substrate occurs and the bacteria produces and accumulates extracellular polymers which result in the community being surrounded by a hydrated exopolymer matrix (Clutterbuck *et al*. 2007). Once engulfed in this glycocalyx matrix, bacteria are immobilized and in close proximity to one another with surrounding open water channels or voids, which act as gene pools for genetic acquisition and permits nutrients, metabolic products and oxygen to circulate and exchange with the base fluid layer, enabling the growth and maintenance of the biofilm (Wuertz *et al.* 2004; Davey and O'Toole 2000).

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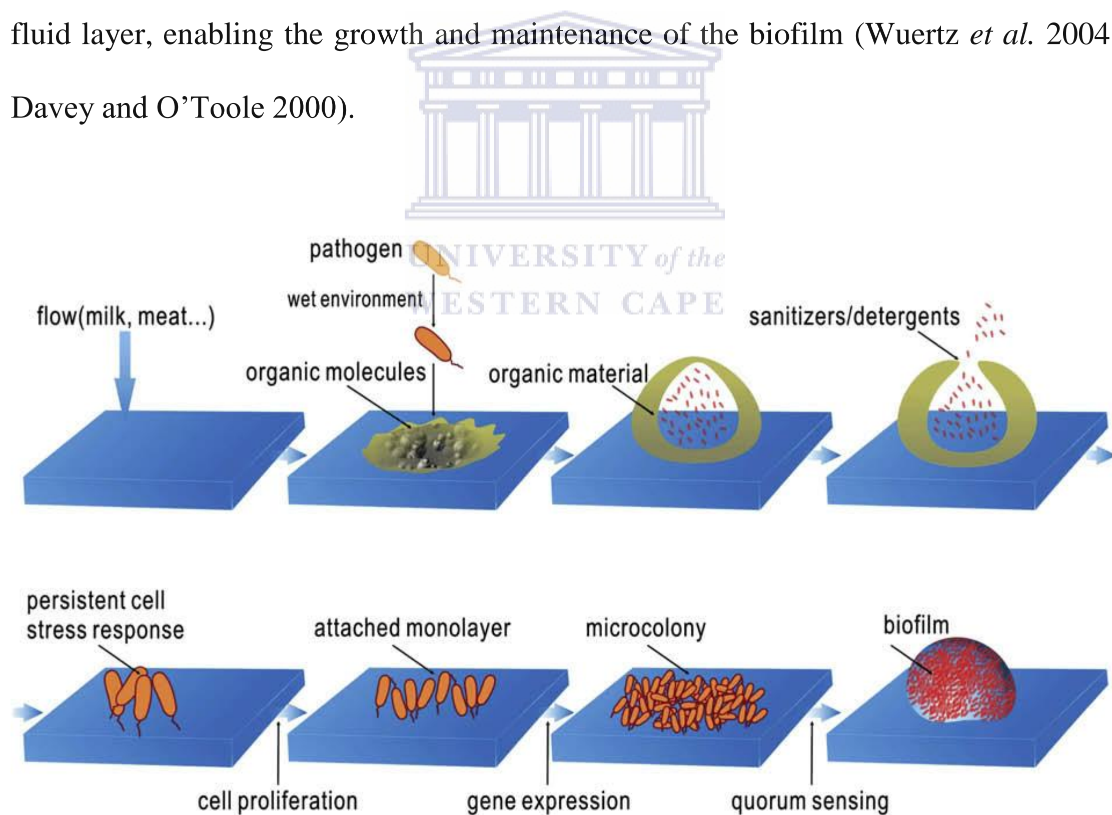
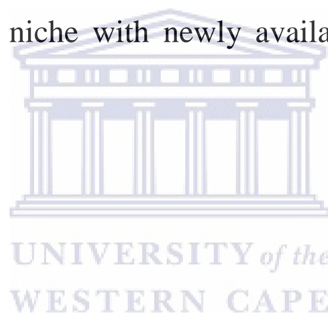


Figure 2.26 The formation of a biofilm on a meat substratum. *Pseudomonas* spp. are initially attracted to organic molecules in the meat substratum. Growth initiation and subsequent biofilm formation follows with expression of cellular genes and quorum sensing (Shi and Zhu 2009).

A wide range of materials as substrates for growth are utilised by pseudomonads. Once glucose and lactate present in meat are exhausted pseudomonads begin to metabolise nitrogenous compounds such as amino acids which result in off-odours, off-flavours and rancidity (Nychas *et al.* 2008; Ellis and Goodacre 2001). *Pseudomonas* spp. competitive ability contributes to their success in dominating fresh meat during aerobic storage. Due to their ability to produce quorum sensing molecules of the N-Acyl Homoserine Lactones (AHLs) family which controls the production of phenotypic traits, *Pseudomonas* spp. can outgrow less competitive bacteria (Heurlier *et al.* 2006). Another ecological advantage is gained by this flora when their proteolytic activity leads to their penetration into meat subsequently resulting in access to a new niche with newly available resources for exploitation (Nychas *et al*. 2008).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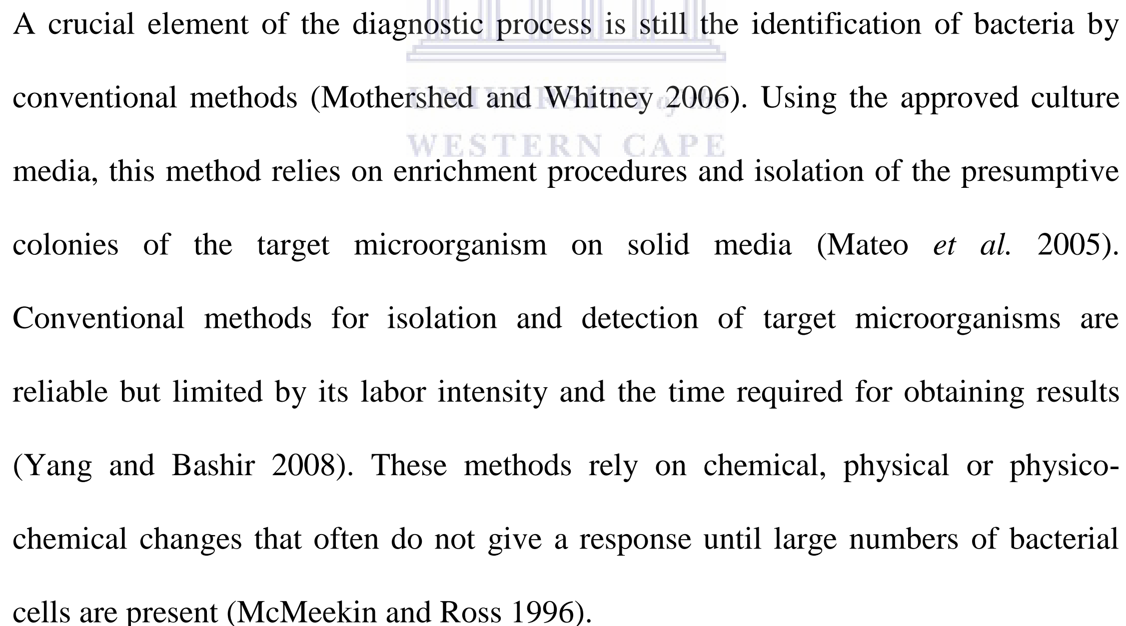
2.6 ISOLATION AND DETECTION METHODS FOR MICROORGANISMS IN RAW MEAT

The isolation and detection of pathogens and spoilage bacteria is necessary, as it allows for, surveying of microbial conditions of raw meat, deciding between acceptance or rejection of batches of meat product, or for purposes related to the implementation and maintenance of control systems such as the Hazard Analysis Critical Control Point (HACCP) system (Brown *et al.* 2000).

2.6.1 Conventional methods



A crucial element of the diagnostic process is still the identification of bacteria by conventional methods (Mothershed and Whitney 2006). Using the approved culture media, this method relies on enrichment procedures and isolation of the presumptive colonies of the target microorganism on solid media (Mateo *et al.* 2005). Conventional methods for isolation and detection of target microorganisms are reliable but limited by its labor intensity and the time required for obtaining results (Yang and Bashir 2008). These methods rely on chemical, physical or physico-chemical changes that often do not give a response until large numbers of bacterial cells are present (McMeekin and Ross 1996).

2.6.1.1 Pre-enrichment

It is necessary to apply techniques that selectively encourage the growth of bacteria in the detection in raw foods. An intrinsic difficulty in the detection of foodborne pathogens is that the bacteria are usually present in low numbers of <100 CFU/g (Naravaneni and Jamil 2005).

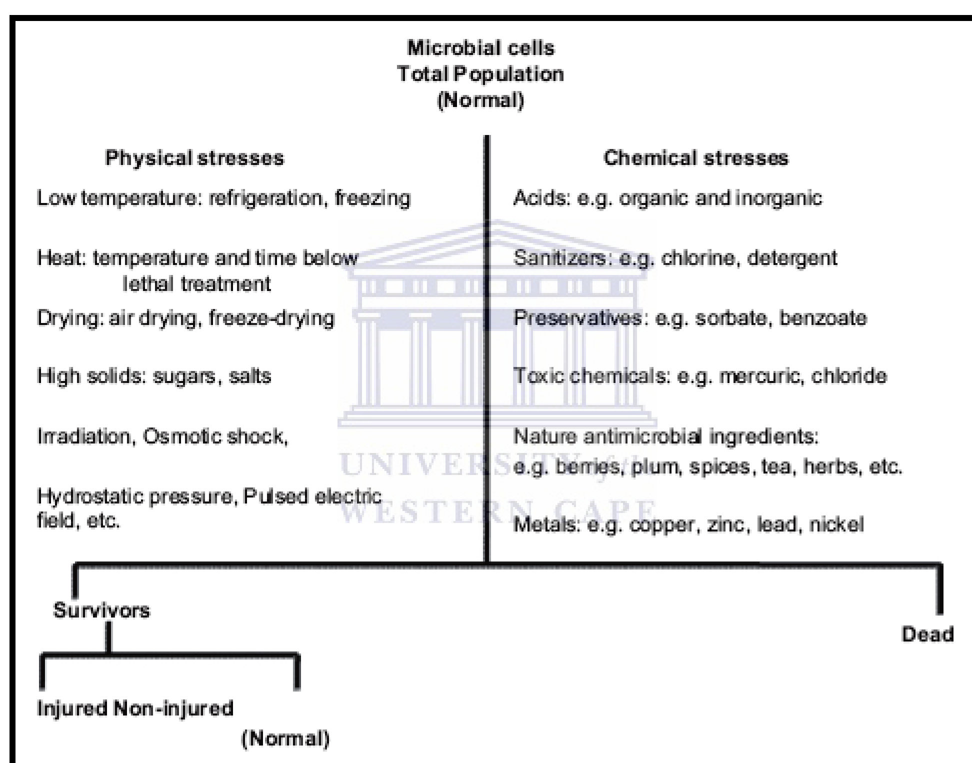


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Figure 2.27 Effects of unfavourable conditions on microbial cells in food products (adapted from Wu 2008).

Microorganisms are exposed to unfavourable conditions (Figure 2.27) during food processing and handling procedures. These conditions may cause injury of pathogens and spoilage microorganisms (Table 2.9). Sublethally injured microorganisms have damaged structural and functional components which renders them susceptible to

either selective agents or conditions to which normal cells are resistant. Bacteria might not be able to grow on selective media at elevated incubation temperatures when sublethally injured. The outer membrane of the bacterial cell is the initial site of injury and changes to its permeability allows the access of selective agents that are generally excluded (Wu 2008).

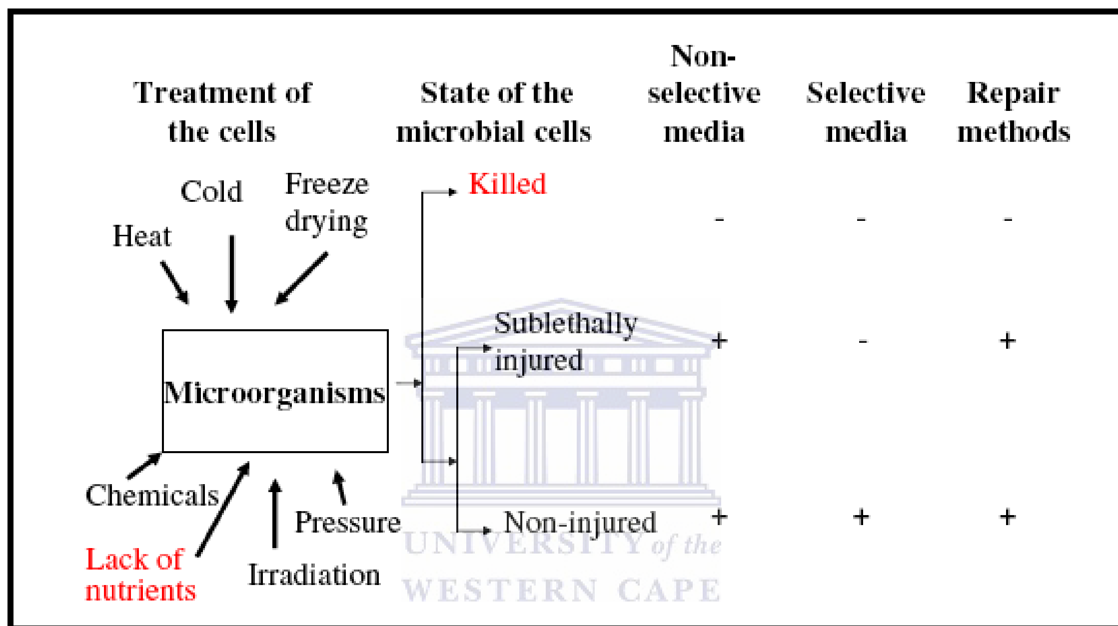

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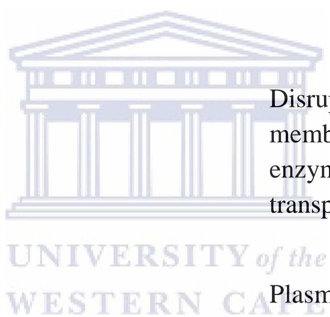
Figure 2.28 Resuscitation of sublethally injured and non-injured cells by various medium systems (adapted from Wu 2008).

Incubation in non-selective (Figure 2.28) media such as Buffered peptone water (BPW) and/or Brain heart infusion (BHI) and/ or Tryptone soy broth at 37ºC allows the sublethally injured cells to repair themselves and return to a normal physiological state with commencement of growth and cell division. BPW has an advantage over other mediums due to its buffering capacity. These media are non-selective and permit the growth and proliferation of the majority of aerobic and facultative organisms present in samples (Macrae *et al*. 1993).

Table 2.9 Effect of unfavourable conditions on a microbial cell (adapted from Prescott *et al*. 2002)

Unfavourable conditions	Effect on microbial cell
Low Temperature	Decreases enzyme activity and at very low temperatures the membranes solidify.
High Temperature	Denaturing of enzymes, transport carriers and other proteins. Melting and disintegration of the lipid bilayer in membranes.
Drying (Lyophilisation)	Dessication
pH	Disruption of plasma membrane, inhibition of enzyme and membrane transport protein activity.
Osmotic Pressure (addition of salt and sugar)	Plasmolysis
UV Radiation	DNA damage (formation of thymine dimers, which inhibits DNA replication and function)
X-Ray Radiation	Breaks hydrogen bonds, oxidizes double bonds, destroys ring structures, and polymerization of some molecules. DNA damage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2.6.1.2 Selective and differential detection

Detection is often complicated by low numbers of target pathogens and spoilage bacteria compared with background microflora present in food products. Selective media are formulated to suppress the growth and proliferation of competing microflora to enable the favourable growth of target bacteria. To achieve a degree of selectivity in solid and liquid media, selective compounds such as surface-active agents, salts, antibiotics, sulfanilamides, acids and dyes are added. (Wu 2008; Prescott *et al.* 2002). Selective media do not permit the repair of sublethally injured microorganisms and thus fail to detect them, but have a greatly improved isolation rate when inoculated with an actively growing population (Macrae *et al.* 1993). Differential detection involve the use of media that differentiate between different groups of bacteria and based on their biological characteristics allow tentative identification of these microorganisms (Prescott *et al.* 2002).

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2.6.1.2.1 Gram-negative microorganisms

2.6.1.2.1.1 *E. coli* and Coliform selectivity

Violet red agar (VRBA) with dye 4-methyl-umbelliferyl-β-D-glucuronic acid (MUG) is a selective medium for the detection and enumeration of coliforms (Figure 2.29) in water, food and dairy products (Beloti *et al.* 2002). Coliforms attack lactose present in the agar, producing purple colonies surrounded by purple haloes. Pale colonies with greenish zones on VRBA with MUG are the result of non-lactose or late-lactose fermenters. Mug is a non-fluorescent dye that produces a fluorophore when

hydrolysed by β-glucuronidase produced by *E. coli* (Cakir *et al.* 2002). A hand held UV lamp under 366nm can detect MUG hydrolysis (Bridson 2006; Dogan-Halkman *et al.* 2003; Batt 1999; Macrae *et al*. 1993).

2.6.1.2.1.2 *Salmonella* selectivity

Rappaport-Vassiliadis (RV) (Figure 2.29) broth is more efficient than other enrichment medium for the isolation of *Salmonella* spp. from faeces, water and foodstuffs (Boer 1998). RV contains high concentrations of magnesium chloride, which raises osmotic pressure, and in combination with malachite green inhibits bacteria other than *Salmonella* (Aspinall *et al.* 1992). The broth exploits four characteristics of *Salmonella* spp. compared with other Enterobacteriaceae. These characteristics include the ability of salmonellae to survive at high osmotic pressures, to multiply at low pH values, to be resistant to malachite green and to have less demanding nutritional requirements (Bridson 2006; Rybolt *et al.* 2004; Oliveira *et al.* 2003; Busse 1995).

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The development of Xylose lysine deoxycholate (XLD) agar (Figure 2.29) in 1965 by Taylor was originally for the isolation and identification of *Shigella* species, it is currently used for the isolation and presumptive identification of both *Salmonella* spp. and *Shigella* spp. Primary differentiation of shigellae and salmonellae from non-pathogenic bacteria relies on xylose fermentation, lysine decarboxylation and the production of hydrogen sulphide (Nye *et al.* 2002; Mallinson *et al.* 2000). XLD agar is perfect for the screening of samples containing mixed flora and suspected of harbouring enteric pathogens because its recovery of *Salmonella* species and *Shigella*

species is not obscure by profuse growth of other species. Salmonellae produce hydrogen sulphide, which reacts with ferric ammonium citrate in the medium and is therefore distinguished from shigellae by red colonies with black centres (Health Protection Agency 2008; Bridson 2006; Cox and Berrang 2000; Busse 1995).

2.6.1.2.1.3 *Pseudomonas* selectivity

Pseudomonas agar base supplemented with a selective agent that consists of centrimide, fucidin and cephalosporin is a selective medium for the isolation of *Pseudomonas* species (Figure 2.29). The base medium contains magnesium chloride and potassium sulphate to enhance the production of the pigment pyocyanin (Ramalho *et al.* 2002). Pseudomonas CFC supplements is recommended for the selection isolation of pseudomonades generally from chilled foods and processing plants. The presence of blue-green or brown pigmentation or the fluorescence may be taken as presumptive evidence of pseudomonads (Bridson 2006; Tryfinopoulou *et al.* 2001).

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2.6.1.2.2 Gram-positive microorganisms

2.6.1.2.2.1 *Staphylococcus aureus* selectivity

Baird Parker agar base enriched with Egg Yolk Tellurite emulsion is a selective medium (Figure 2.29) for the isolation and enumeration of coagulase-positive staphylococci from food, skin, soil, air and other materials. Baird Parker agar base contains peptone beef extract and yeast extract as sources of nitrogenous compounds,

carbon and sulphur, vitamins and trace minerals. Without destroying the selectivity, sodium pyruvate is incorporated in order to protect damaged cells and stimulate the growth of *Staph. aureus* (Silva *et al.* 2000). The tellurite additive is toxic to egg yolk-clearing strains other than *Staph. aureus* and imparts a black colour to the colonies. The egg yolk additive, in addition to being enrichment, aids in the identification process by demonstrating lecithinase activity. Glycine and lithium chloride have inhibits flora other than *Staph. aureus* (Bridson 2006; Capita *et al.* 2001).

2.6.1.2.2.2 *Listeria* selectivity

Fraser broth contains a high nutrient content and a large buffer capacity that creates optimum growth conditions for *Listeria* (Figure 2.29). The growth of accompanying bacteria is largely inhibited by supplements that contain lithium chloride, nalidixic aid and acriflavin hydrochloride, and with the addition of esculin and ammonium iron (Ш) citrate facilitate the detection of the β-glucosidase activity of *Listeria* (Capita *et al.* 2000). The glucose esculin is hydrolysed by β-glucosidase into esculetin and glucose. The esculetin then forms an olive-green to black complex with the iron (Ш) ions, therefore growth of *Listeria* in Fraser broth is observed by the blackening of the broth (Bridson 2006; Duarte *et al.* 1999).

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Listeria selective agar base (Oxford formulation) is a selective and diagnostic medium for the detection of *Listeria monocytogenes* (Figure2.29). This medium consists of Columbia blood agar as the basis and selective agents that consists of lithium chloride, acriflavin, colistin, cycloheximide, cefotetan and fosfomycin. Incorporation of esculin and ferric salt, results in the differentiation of *Listeria* species. This

bacterium hydrolyses esculin, producing black zones around the colonies due to the formation of black iron phenolic compounds derived from the aglucon. It has been reported that Oxford agar has good inhibition of interfering flora such as enterococci (Bridson 2006; Gouws and Liedemann 2005; Leclercq 2004; Scotter 2001; Macrae *et al*. 1993).

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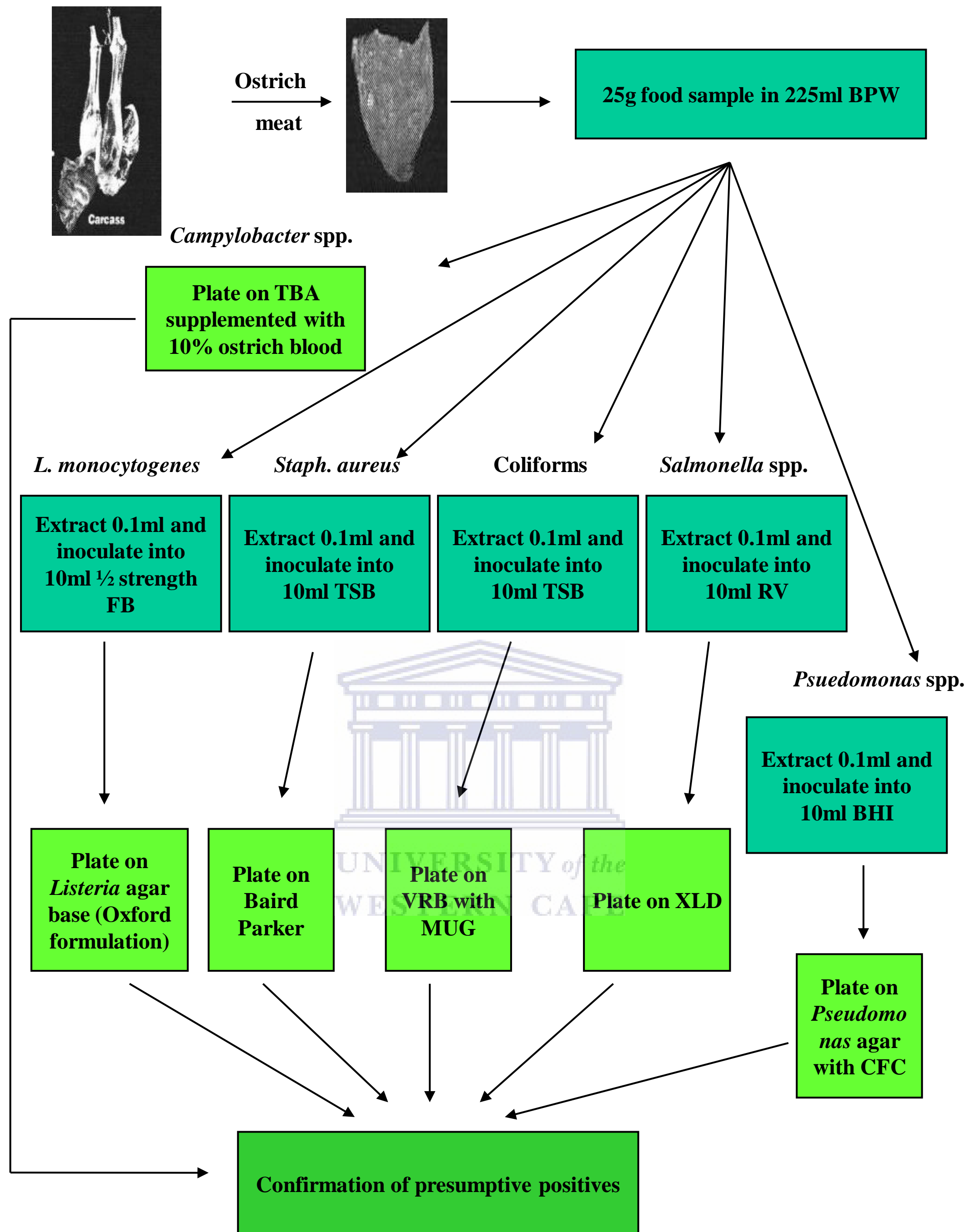

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Figure 2.29 Methods for the isolation and detection of microorganisms in ostrich meat.

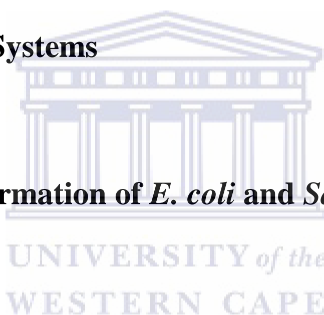
2.6.1.3 Confirmation of presumptive positives

2.6.1.3.1 The Staphylase test

The Staphylase test is a commercially available kit from Oxoid. The agglutination test detects bound coagulase on a disposable reaction card. This kit establishes agglutination of sheep red blood cells sensitised with fibrinogen. Fewer false-negatives are produced, since they are more specific for *Staph. aureus* (Weist *et al.* 2006; Zschock *et al.* 2005; Duguid 1989).

2.6.1.3.2 API Identification Systems

2.6.1.3.2.1 API 20E for confirmation of *E. coli* and *Salmonella* isolates



The API 20E (BioMérieux, Inc, SA) system facilitates the 24-hour identification of Enterobacteriaceae as well as 24 or 48-hour identification of other Gram-negative bacteria. The API 20E strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate fermentation (O'Hara *et al.* 1992). The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents (Popovic *et al.* 2004). Carbohydrate fermentation is detected by colour change in the pH indicator (BioMerieux). The API 20E consists of the following tests. ONPG tests for the presence or absence of beta-gactosidase, ADH tests for the hydrolysis of arginine, LDC tests for the hydrolysis of lysine, ODC tests for the hydrolysis of ornithine, CIT tests for the utilization of citrate, H_2S tests

for the production of H_2S, URE tests for the production of urease, TDA tests for the hydrolysis of tryptophane, IND tests for the production of indole, VP tests for the production of acetoin, GEL tests for the hydrolysis of gelatin, GLU tests for the fermentation or oxidation of glucose, MAN tests for the fermentation or oxidation of mannitol, INO tests for the fermentation or oxidation of inositol, SOR tests for the fermentation or oxidation of sorbitol, RHA tests for the fermentation or oxidation of rhamnose, SAC tests for the fermentation or oxidation of sucrose, MEL tests for the fermentation or oxidation of melibiose, AMY tests for the fermentation or oxidation of amygdalin and ARA tests for the fermentation or oxidation of arabinose (Beauchamp *et al.* 2006; BioMerieux, Inc SA information catalogue 2006; Nucera *et al.* 2006).

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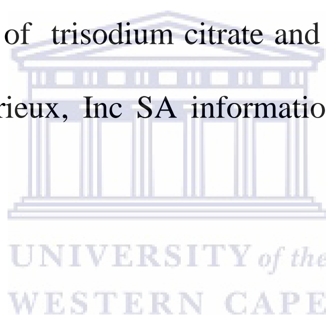
2.6.1.3.2.2 API 20NE for confirmation of *Pseudomonas* spp.

The API 20NE is a modification of the API 20E strip, as the substrates have been changed to include 8 conventional tests and 12 assimulation tests that are based on the observation of microbial growth in the presence of a single source of carbon (Winn *et al.* 2006). It facilitates the identification of non-fastidious, non-enteric Gram-negative rods within 24 hours. The system consists of 20 microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests are inoculated with a bacterial suspension, which reconstitutes the media. After incubation, the metabolic end products are detected by indicator systems or the addition of reagents. The substrate microtubes contain assimilation tests and are inoculated with minimal medium. If the bacteria are capable of utilizing the corresponding substrate, then they will grow. The API 20NE consists of the following

twenty tests. NO_3 tests for the presence of nitrites or nitrogen, TRP tests for production of indole, GLU tests for the fermentation of glucose, ADH tests for the production of arginine dihydrolase, URE tests for the production of urease ESC tests for the hydrolysis of esculin, GEL tests for the hydrolysis of gelatin, PNPG tests for the production of β-galactosidase, GLU tests for the assimilation of glucose, ARA tests for the assimilation of arabinose, MNE tests for the assimilation of mannose, MAN tests for the assimilation of mannitol, NAG tests for the assimilation of n-acetyl-glucosamine, MAL tests for the assimilation of maltose, GNT tests for the assimilation of potassium gluconate, CAP tests for the assimilation of capric acid, ADI tests for the assimilation of adipic acid, MLT tests for the assimilation of malate, CIT tests for the assimilation of trisodium citrate and PAC tests for the assimilation of phenylacetic acid (BioMerieux, Inc SA information catalogue 2006; Truu *et al.* 1999; Barr *et al.* 1989).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2.6.1.3.2.3 API *Listeria*

API Listeria (BioMérieux, Inc. SA) facilitates the 24-hour identification of *Listeria.* The API Listeria strip consists of 10 microtubes containing dehydrated substrates for the demonstration of enzymatic activity and carbohydrate fermentation. The substrates are reconstituted by adding a bacterial suspension. After incubation, the metabolic end products are detected by indicator system or addition of reagents. Carbohydrate fermentation is detected by colour change in the pH indicator. The API 20NE consists of the following ten tests. DIM tests for the presence or absence of acrylamidase, ESC tests for the hydrolyses of esculin, αMAN tests for the presence of α-mannosidase, DARL tests the acidification of D-arabitol, XYL tests the

acidification of xylose, RHA tests the acidification of rhamnose, MDG tests the acidification of methyl- αD-glucopyranoside, RIB tests the acidification of ribose, G1P tests the acidification glucose-1-phosphate, TAG tests the acidification of tagatose (Abdelgadir *et al.* 2009; BioMerieux, Inc SA information catalogue 2006; Gouws and Liedemann 2005; McLauchlin 1997).

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2.6.2 Molecular based detection method

2.6.2.1 Polymerase Chain Reaction

The study of bacterial communities in a wide range of environments is currently achievable due to the development of DNA amplification by the polymerase chain reaction (PCR). PCR is a molecular based method that allows rapid, sensitive and specific identification of microorganisms either from cultured isolates or directly from clinical, environmental or plant samples (Figure 2.31) (Albuquerque *et al.* 2009). It proves to be a powerful diagnostic tool for the analysis of microbial infections as well as for the analysis of microorganisms in food samples; and the availability of such a reliable, rapid and accepted test system to detect the presence or absence, or even the degree of contamination of pathogens, becomes increasingly important for the agricultural and food industry (Malorny *et al.* 2003). The method involves the amplification of specific DNA fragments from genomic DNA in an *in vitro* enzymatic reaction (Yang and Rothman 2004).

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2.6.2.1.1 PCR Process

In order to synthesize a target DNA sequence from genomic DNA, a specific DNA polymerase and two oligonucleotide primers are utilized. A single PCR cycle comprises of three steps (Figure 2.30) (Barken *et al.* 2007):

- Denaturation separates the complementary strands of double-stranded DNA into single-stranded DNA.

- Annealing allows the primers to hybridise to their 3` hydroxyl end is directed towards the target sequence ready for amplification.
- Extension of the primers on the target DNA template sequence is achieved with a thermostable DNA polymerase.

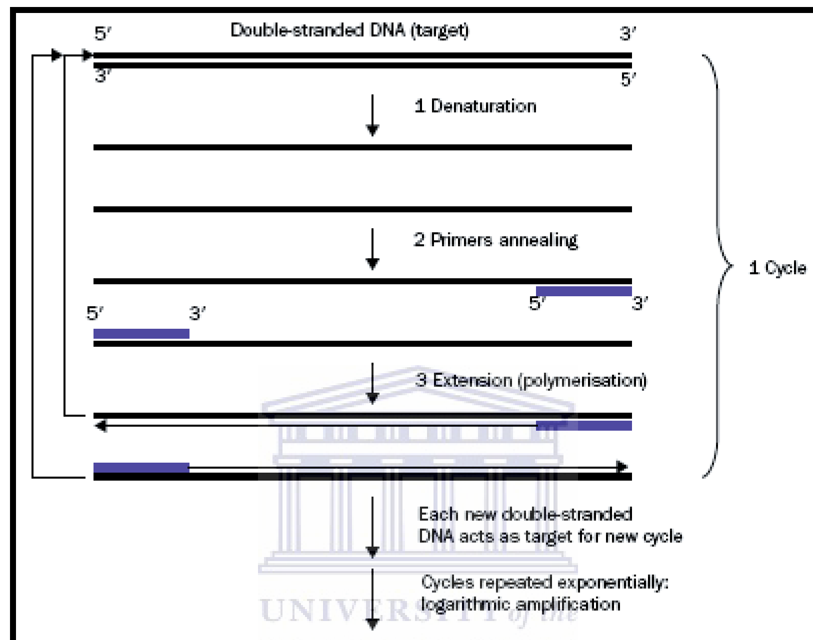

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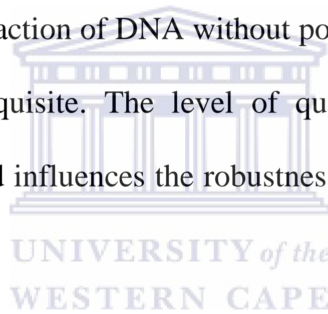
Figure 2.30 Three step cycle of the polymerase chain reaction (adapted from Yang and Rothman 2004).

Repeating the three-step cycle consecutively leads to an exponential accumulation of target DNA copies. These PCR products can be visualised as single ethidium bromide-stained bands on electrophoretic agarose gels (Mothershed and Whitney 2006; Yang and Rothman 2004). Species and group specific identification and identification of subspecies are achievable by PCR. This method is superior in sensitivity and has a very low detection limit of 10^1 cfu/ml to 10^6 cfu/ml per reaction compared to culture methods, whose detection limits are such that they require an enrichment stage, prior to testing, to allow the target organism to multiply (Yang and Bashir 2008).

2.6.2.1.2 Limitations of PCR

The inability to quantitate the cell number of a bacterial species and the inability to discriminate between viable and non-viable cell in a sample is a few of the drawbacks of PCR (Bertram- Drogatz *et al*. 1999). Others include False-negative and False-positive test results. False-negative test results stem from inadequate removal of PCR inhibitors, ineffective release of microbial DNA content from the cells and poor DNA recovery after extraction and purification steps. Contamination may serve as substrates for amplification and result in false-positive results (Yang and Rothman 2004). Purity of the target nucleic acids is of utmost importance since it influences the performance of PCR and extraction of DNA without polymerase inhibiting substances present is therefore a prerequisite. The level of quality assurance conducted on reagents and instruments used influences the robustness of this method (Barken *et al.* 2007).



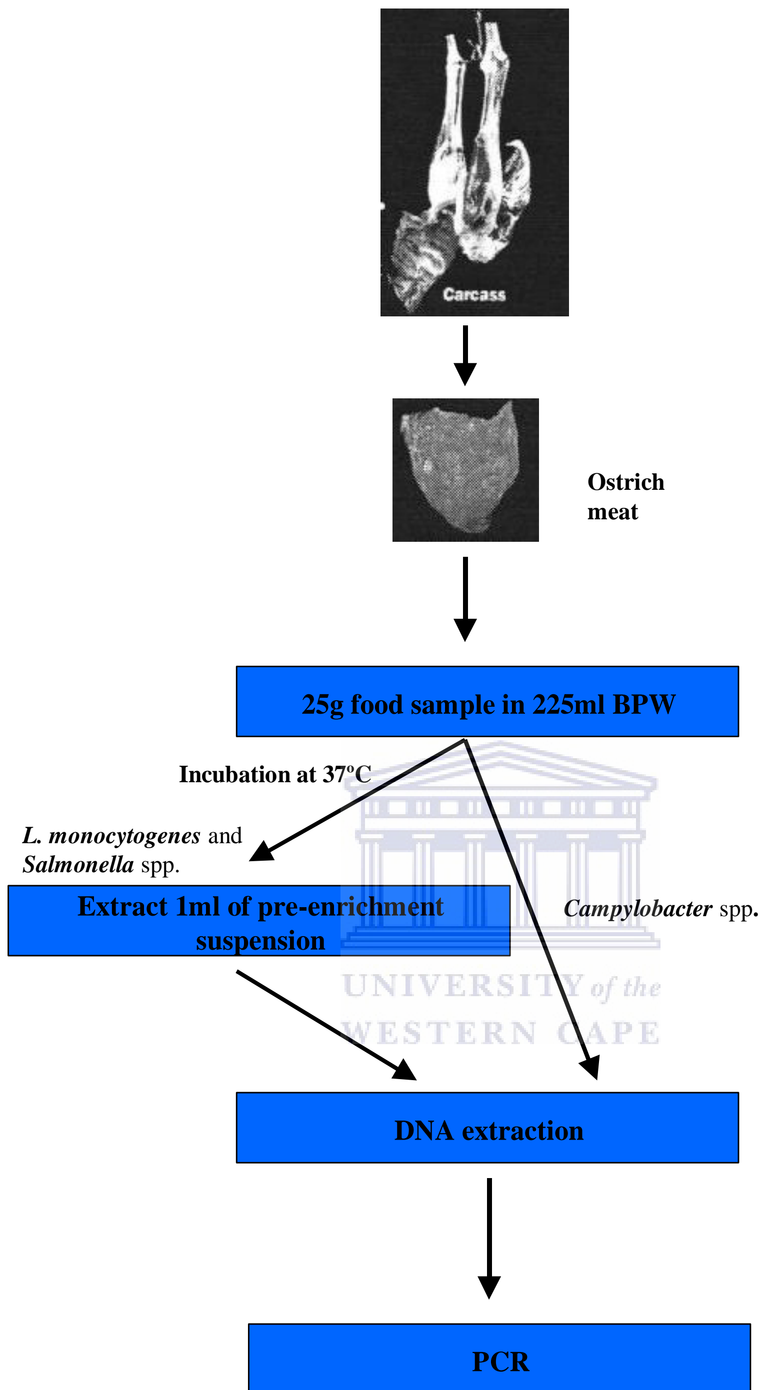


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Figure 2.31 Method for the detection of *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacter* spp. in ostrich meat.

2.7 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance in food-borne pathogens becomes an important public health concern when treatment fails if food-borne pathogens become resistant to an antimicrobial agent used for treatment. The use of antibiotics has increased largely due to its enhancement rates of weight gain in food animals. Antimicrobials are routinely administered by way of feeds and water in subtherapeutic doses to treat infections, increase the feeding efficiency and rate of weight gain in cattle, pigs and poultry (Gouws and Brözel 2000; Manie *et al*. 1999). Antimicrobials administered to animals through feed and water is administered as feed additives, and is usually highly concentrated when used for therapy and prophylaxis (Wegener 2003). As a result, selective pressure has been created for the emergence and dissemination of antimicrobial- resistant animal pathogens, human pathogens that have food animal reservoirs and commensal bacteria that are present in food animals (Angulo *et al.* 2004).

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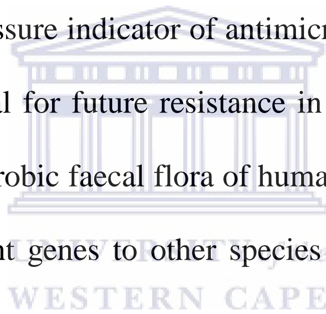
The number of antimicrobial resistant bacteria is rapidly increasing because of extended use and abuse of antibiotics. Human infection of resistant bacteria from food-producing animals is evident in human bacterial pathogens that have food animal sources. These sources include *Campylobacter*, which has reservoirs in chickens and turkeys, *Salmonella*, which has important reservoirs in cattle, chickens, pigs and turkeys, and *Staphylococcus aureus*, which has reservoirs in poultry, pork and beef (Pesavento *et al.* 2007; Angulo *et al.* 2004). In the last decade, isolates from food have shown an increase in resistance against most antibiotics (Pesavento *et al.* 2007). Chloramphenicol resistant *Salmonella* species isolated from infected cows on a

farm have been traced through a slaughterhouse to a meat packaging company. Multiresistant *E. coli* have been isolated from chickens dosed with spectomycin via their drinking water. Bacteria in young turkeys given virginiamycin developed resistance to both quiniupristin and dalfopristin (Bower and Daeschel 1999).

Bacteria become resistant to antimicrobials either through mutations in chromosomal loci or by acquiring genetic elements such as plasmids, phages, transposons and integrons through horizontal transference (Zhang *et al.* 2006). Antimicrobial resistant commensal bacteria create reservoirs of resistant genes that could be transferred to pathogenic bacteria. Incidence of these antibiotic resistant naturally occurring host floras serve as a selective pressure indicator of antimicrobial agent use. This indicator consequently reflects potential for future resistance in future pathogens. *E. coli* as a predominant species in the aerobic faecal flora of humans and animals has established the ability to transfer resistant genes to other species including pathogenic bacteria (Angulo *et al*. 2004).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2.8 CONCLUSION

Food safety and shelf-life are both important microbial concerns in relation to meat production. The challenges in providing a safe ostrich supply are complex because all aspects of ostrich production (from farm to fork) need to be considered. Pathogenic microorganisms such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp. and *Campylobacter* spp.; or/and spoilage microorganisms such as *Pseudomonas* spp. can be part of the ostrich's natural miroflora or may be contaminants. Cross- contamination of carcasses with pathogens and spoilage bacteria can occur at virtually every stage of the slaughter process. The safety of ostrich meat depends on the application of effective control measures at all stages of production, including feed manufacture, it is therefore essential that everybody working with food animals, meat or meat products is adequately trained and kept up to date with developments in food safety so that they can play their part in the production and preparation of safe and quality meat and meat products. Food safety aspects of ostrich meat production should be mainly controlled at farm level, so that during the slaughter process, uncontaminated conditions can be maintained. Each stage presents unique foodborne disease hazards and control opportunities; developement of rapid microbiological test procedures for enumeration and presence / absence of pathogens and spoilage bacteria at these stages provides information, useful for development of tools in the control of foodborne hazards. PCR may be a powerful diagnostic tool for the analysis of microorganisms in food samples, but will not replace conventional culture methods for microbial detection, until it can be elaborated to further characterise detected microorganisms. Antibiotic resistance in bacteria isolated from

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food animals is a concern to public health and lack of information concerning antimicrobial usage in ostriches warrants further research.

CHAPTER 3

Microbial quality of ostrich meat produced at two export-approved abattoirs in the Klein Karoo, South Africa

3.1. ABSTRACT

Aim: The aim of this study was to determine the quality of ostrich meat and to evaluate the ostrich slaughter process.

Materials and Results: Fresh and vacuum-packed ostrich meat was collected from four processing points of two South African export-approved abattoirs in the Klein Karoo. Over the seven-month period of the study, a total of 120 ostrich meat samples were tested, of these, 32 were from pre-chill, 33 were from post-chill, 34 were from pre-packaging and 21 were from vacuum-packed samples. The ostrich meat samples were evaluated for the presence of *Salmonella* species, *Campylobacter* species and *Listeria monocytogenes* with molecular and conventional methods. Total viable counts on pre-chill, post-chill, pre-packaging and vacuum-packed samples were 1.25, 2.11, 7.37 and 3.15 $\log_{10}$ CFU/cm^2, respectively. There was no significant difference in mean TVC of all samples over the seven-month period. Of the meat samples 99.17% were found to be positive for coliforms, indicating a high rate of faecal contamination. Of the 120 ostrich meat samples tested, 33.33% were positive for *E. coli.* 11.67% of the meat samples tested was found to be positive for *Staphylococcus aureus.* Vacuum-packed samples had the highest prevalence of *Staph. aureus* among

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the four processing stages. All meat samples from the four processing stages tested negative for *L. monocytogenes* and *Campylobacter.* Of the 120 samples analysed, one was found to be both positive for *Salmonella* spp. on XLD and PCR. *Campylobacter* and *L. monocytogenes* was not detected in this study.

Conclusion: The results indicate that the slaughter process contributes significantly to the microbial quality of ostrich meat. Results obtained, suggests that current intervention strategies used in the abattoirs are relatively effective to some extent in reducing *Salmonella*, *Campylobacter* and *Listeria monocytogenes* contamination in ostrich meat, while new interventions should be developed in order to ensure the overall safety of ostrich meat, especially for the faecal contamination of the meat. Complementation of culture-based methodologies with culture-independent (PCR) techniques still remains a better chance of detection of pathogens in meat samples. The low detection rate for *Salmonella* spp. and the absence of *L. monocytogenes* and *Campylobacter* spp. positives in samples in this study indicate that current in-plant process practices appear to uphold the quality of ostrich meat.

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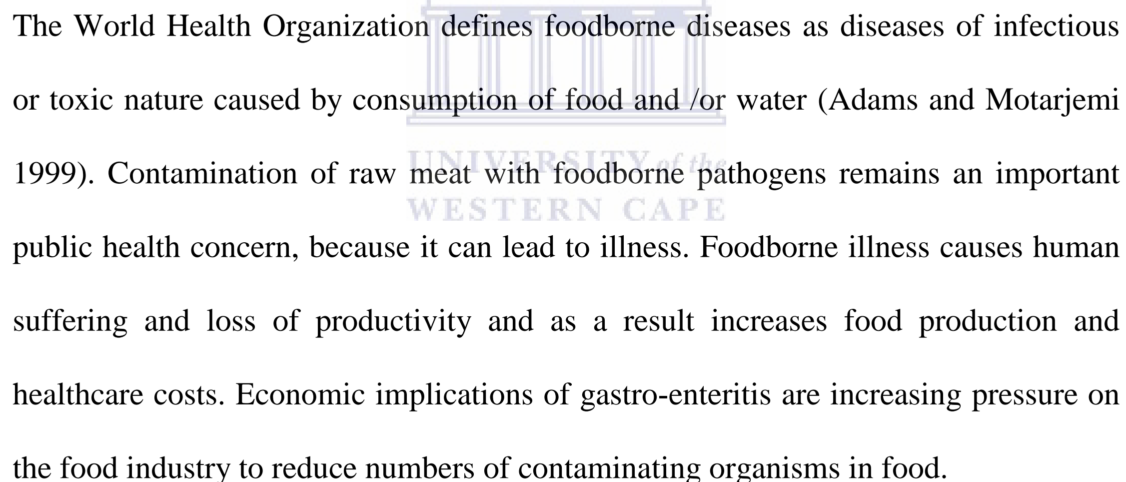
Significance and impact of the study: Ostrich meat was not a source of *Campylobacter* and *L. monocytogenes.* Contamination of ostrich meat was mostly by enteric pathogens. Data obtained from this study provides some baseline information that could be used in future studies on ostrich meat. It will also allow for the implementation of food quality control measures of food-borne pathogens that were detected. This study provides useful baseline information for future research. It will also allow for the implementation of food quality control measures of food-borne

pathogens that are detected. Other benefits include the provision of rapid, sensitive and economic techniques for detection of the leading food-borne pathogens.

3.2. INTRODUCTION

Ostrich farming started 146 years ago in South Africa in 1864. The focus many years ago was on slaughtering the ostrich only for its feathers and skin (SAOBC 2005a). It was estimated that in 2002, South Africa slaughtered 310 000 ostriches compared to the rest of the world's 80 000 ostriches (SAOBC 2005b). 70% of the total ostrich meat produced annually worldwide is contributed by South Africa; this amounted to 950 million kg of ostrich meat (Hoffman 2005). The nutritional value of ostrich meat has led to its increasing popularity in western society and has made it a swift competitor in the red meat market (Alonso-Calleja *et al*. 2004).

The World Health Organization defines foodborne diseases as diseases of infectious or toxic nature caused by consumption of food and /or water (Adams and Motarjemi 1999). Contamination of raw meat with foodborne pathogens remains an important public health concern, because it can lead to illness. Foodborne illness causes human suffering and loss of productivity and as a result increases food production and healthcare costs. Economic implications of gastro-enteritis are increasing pressure on the food industry to reduce numbers of contaminating organisms in food.



E. coli is naturally found in the intestinal tract of animals. Entrance into food occurs through contamination of foods with faecal material, which commonly occurs during the slaughter process of animals. Presence of *E. coli* in food and water indicates the heightened risk of the presence of other faecal-borne bacteria. Meat and meat products, fish, poultry, milk and milk products, vegetables and water have been implicated in *E. coli* diarrhoeal outbreaks (Macrea *et al.* 1993). In 1982, the first

recognition of pathogenic *E. coli* that produce Shiga toxins was given when *E. coli* serotype 0157: H7 was associated with two outbreaks of bloody diarrhea (Doyle *et al.* 2001).

The pathogen *Salmonella* has been recognised as the causative agent of salmonellosis. *S. enteritidis* emerged during the 1980s as a major cause of foodborne disease in Europe and North America. Poultry, pigs and cattle serve as major reservoirs of salmonellae. Red meats tend to be less frequently contaminated with salmonellae than poultry. However, this situation is influenced by the carrier rate in the live animal. Dissemination of this microorganism is favoured by modern conditions of intensive rearing (Macrea *et al.* 1993; Ohl and Miller 2001). Between the period 1992 and 2003 in England and Wales, 910 general outbreaks of salmonellae were reported to the Communicable Disease Surveillance Centre (CDSC) (Hughes *et al.* 2006). Individuals infected with *Salmonella* spp. exhibit abdominal cramps, nausea, vomiting and diarrhea (Li *et al*. 2004)

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Thermophillic campylobacters are the principle cause of human campylobacterioses. *Campylobacter* strains are invasive and can penetrate internal organs or deep tissues of animals (Rowe and Madden 1999). The most common host of this microorganism are the avian species, since they have higher body temperatures. A study revealed that *Campylobacter* was found on the skin of poultry carcasses in the early stages of slaughtering with no contamination from the internal organs (Keener *et al.* 2004). Campylobacters may persist as contaminants of poultry products throughout the entire supply chain (Mead 2004).

Staphylococcus aureus have the ability to produce enterotoxins and are therefore the causative agents in Staphylococcal food-poisonings. *Staphylococcus aureus* can be found on the skin and hair of mammals. Raw meats and poultry can be contaminated with *Staphylococcus aureus* by humans' and/ or animals. Since this pathogen can contaminate food products during preparation and processing, it remains a major cause of foodborne diseases in humans. Infection is exhibited by abdominal cramps, nausea and vomiting followed by diarrhea. (Le Loir *et al*. 2003; Macrea *et al.* 1993).

The pathogen *Listeria monocytogenes* is the causative agent of listeriosis. Listeriosis is associated with the highest case fatality rate (30%) (Gouws and Liedemann 2005). Studies in the 1980s from the US Centre of Disease Control and Prevention (CDC) revealed an annual estimation of 1965 cases of listeriosis, with 481 deaths (Doyle *et al.* 2001). *L. monocytogenes* has been isolated from more than 37 species of mammal and 17 species of birds. Since the 1970s, the incidence of listeriosis observed in both humans and animals have increased, this has been attributed to changes in agriculture practices. The environmental contamination of *L. monocytogenes* on the farm, abattoir and at the processing plant is a more significant route in the dissemination of this pathogen than contamination via the intestinal tract (Macrea *et al.* 1993). Infection with *L. monocytogenes* is characterized by the symptoms: encephalitis, meningitis and stillbirth (Ireton and Cossart 1997).

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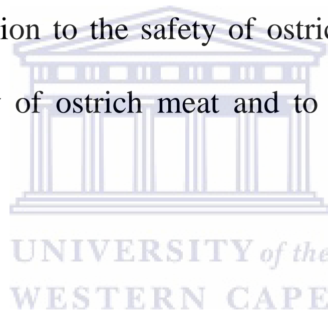
Conventional culture analyses for pathogens in food rely on enrichment procedures and isolation of the presumptive colonies of the target microorganism on solid media, using approved culture media. These analyses require several days before results are obtained. Molecular methods such as polymerase chain reaction (PCR) amplification

are reliable alternatives to culture for the detection of pathogens in food samples and are characterized by its selectivity, sensitivity and speed (Yang and Rothman 2004). PCR provides improved sensitivity for the detection of foodborne pathogens (Gouws and Liedemann 2005).

Overall, raw meat has been implicated as significant sources of all of the pathogens described above. Information in scientific literature regarding the microbiological safety of ostrich meat destined for human consumption is currently lacking due to ostrich being a relatively new emerging meat species. Taking into account the progressive increase of the production and consumption of this meat type and to address the questions in relation to the safety of ostrich meat, the aim of this study was to determine the quality of ostrich meat and to evaluate the ostrich slaughter process.



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3.3. MATERIALS AND METHODS

3.3.1. Ostrich meat sample collection and preparation

Ostrich meat (120 samples) was collected over a seven-month period from two South African export-approved abattoirs in the Klein Karoo. Samples of fresh and vacuum-packed ostrich meat were collected from four processing points, which included pre-chill (post-evisceration), post-chill (post-cold storage), pre-packaging (post-debonning) and vacuum-packed (post-packaging). Pre-chill samples were collected the same day as slaughter, while the post-chill and pre-packaging were collected the second day (after the debonning process). On arrival at the lab, all samples were logged and a sample number assigned. Ostrich meat samples (25g) were homogenized in 225ml buffered peptone water (Oxoid LP0034) and stomached in a Seward stomacher 400 at normal speed for 1min. Incubation at 37ºC for 24h followed for the detection of specific microorganisms. This served as the pre-enrichment suspension for the conventional detection and the primary enrichment phase for the molecular detection of pathogens.

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3.3.2. Conventional detection of microorganisms

3.3.2.1 Total Viable Count

For the Total Viable Count (TVC), serial dilutions were carried out on the homogenized suspension using buffered peptone water (Oxoid LP0034) and pour plated with plate count agar (Merk C6). The results were initially expressed as $\log_{10}$

CFU/g meat. Results were transformed into CFU/cm^2 to facilitate the comparison with data found in other studies. The following equation (Alonso-Calleja *et al.* 2004) was used: $\log_{10}$ CFU/cm^2 ostrich samples = $\log_{10}$ CFU/g ostrich samples – 0.633.

3.3.2.2 Detection of indicator microorganisms

The presence of *E. coli* and coliforms were detected by transferring 0.1ml of pre-enrichment suspension to 10ml of tryptic soy broth. Duplicate violet red bile agar (VRBA) with Mug (Oxoid CM0978) was spread with 0.1ml of the overnight broth culture and incubated at 37°C for 18-24h. Presumptive positive colonies for *E. coli* were confirmed under UV light (Villari *et al.* 1996).

3.3.2.3 Detection of *Salmonella*

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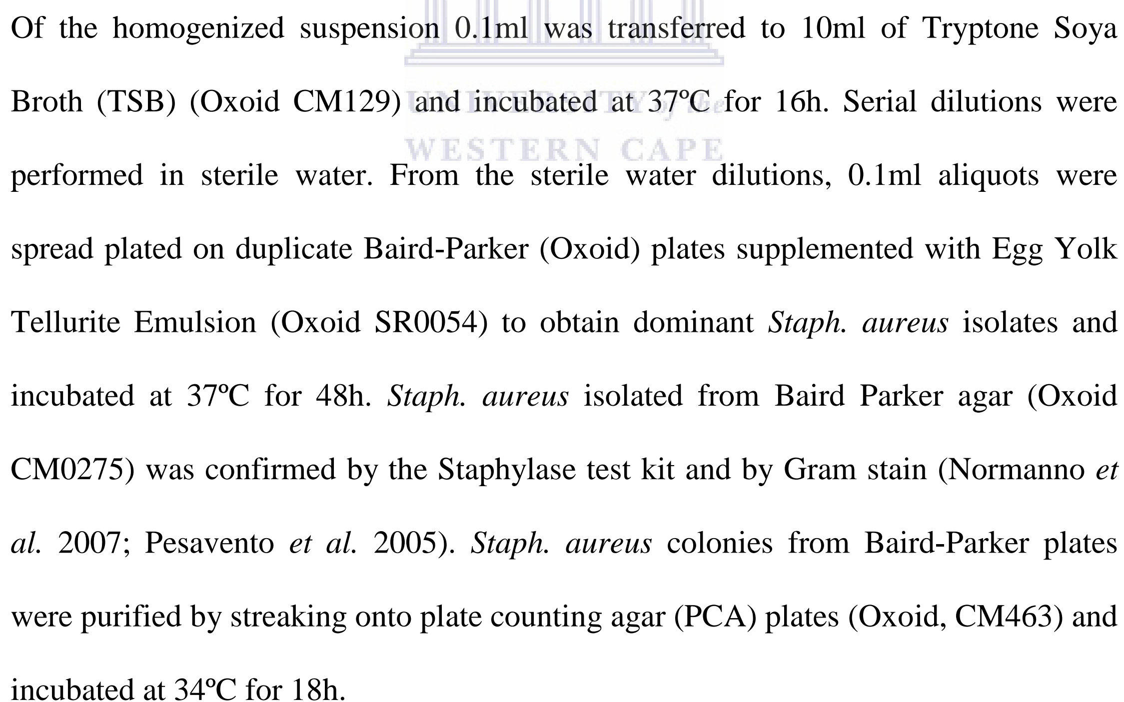
Salmonella species were detected by transference of 0.1ml of pre-enrichment suspension to 10ml of Rappaport-Vassiliadis (RV) medium (Oxoid CM 669) and incubated at 42ºC for 18-24 h (Whyte *et al.* 2002). The broth cultures (0.1ml) were spread plated on duplicate xylose lysine deoxcholate (XLD) agar (Oxoid CM469) plates and incubated at 37ºC for 18-24h. Presumptively positive samples were confirmed with API 20E (BioMereux) (Mokgatla *et al.* 1998).

3.3.2.4 Detection of *Campylobacter*

Ostrich meat samples (25g) were homogenized in 225ml buffered peptone water (Oxoid LP0034). The samples was stomached in a Seward stomacher 400 at normal

speed for 1min. Detection of *Campylobacter* spp. were carried out by slowly transferring 0.5ml of the homogenised suspension onto sterile cellulose acetate membranes (0.65µm & 8 µm pore size) which were placed onto the surface of tryptose blood agar (TBA) (CM0233) plates enriched with 10% ostrich blood for 10-15 min. Following filtration, the cellulose membranes were removed and TBA plates incubated anaerobically at 37ºC for 48h in an anaerobic jar under microaerophilic conditions generated by *Campylobacter* gas generating kits (Oxoid BR038B) (Diergaardt *et al.* 2003). Presumptively positive samples were confirmed with RapID CB Plus (Remel).

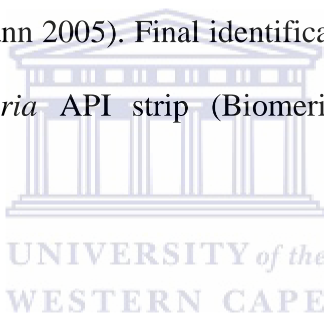
3.3.2.5 Detection of *Staphylococcus aureus*



Of the homogenized suspension 0.1ml was transferred to 10ml of Tryptone Soya Broth (TSB) (Oxoid CM129) and incubated at 37ºC for 16h. Serial dilutions were performed in sterile water. From the sterile water dilutions, 0.1ml aliquots were spread plated on duplicate Baird-Parker (Oxoid) plates supplemented with Egg Yolk Tellurite Emulsion (Oxoid SR0054) to obtain dominant *Staph. aureus* isolates and incubated at 37ºC for 48h. *Staph. aureus* isolated from Baird Parker agar (Oxoid CM0275) was confirmed by the Staphylase test kit and by Gram stain (Normanno *et al.* 2007; Pesavento *et al.* 2005). *Staph. aureus* colonies from Baird-Parker plates were purified by streaking onto plate counting agar (PCA) plates (Oxoid, CM463) and incubated at 34ºC for 18h.

3.3.2.6 Detection of *L. monocytogenes*

Listeria monocytogenes strains were detected using a two-step enrichment procedure. Of the pre-enrichment suspension 0.1ml was inoculated into 10ml of ½ strength Fraser broth (Oxoid CM895) and incubated at 37 ºC for 18-24h. This served as the secondary enrichment. The secondary enrichment (0.1ml) was spread in duplicate on *Listeria* selective agar base (Oxford formulation) (Oxoid CM856) and incubated at 37ºC for 18-24h. Suspect colonies of typical morphology (black/grey with a dimpled centre) was picked and streaked onto Tryptic Soy Agar (TSA), (Oxoid CM131) to obtain pure cultures. The suspect colonies were then screened by PCR and Gram stain reaction (Gouws and Liedemann 2005). Final identification of confirmed isolates was carried out using the *Listeria* API strip (Biomerieux, Inc. SA) according to manufacturer's instructions.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3.3.3 Molecular detection of pathogens

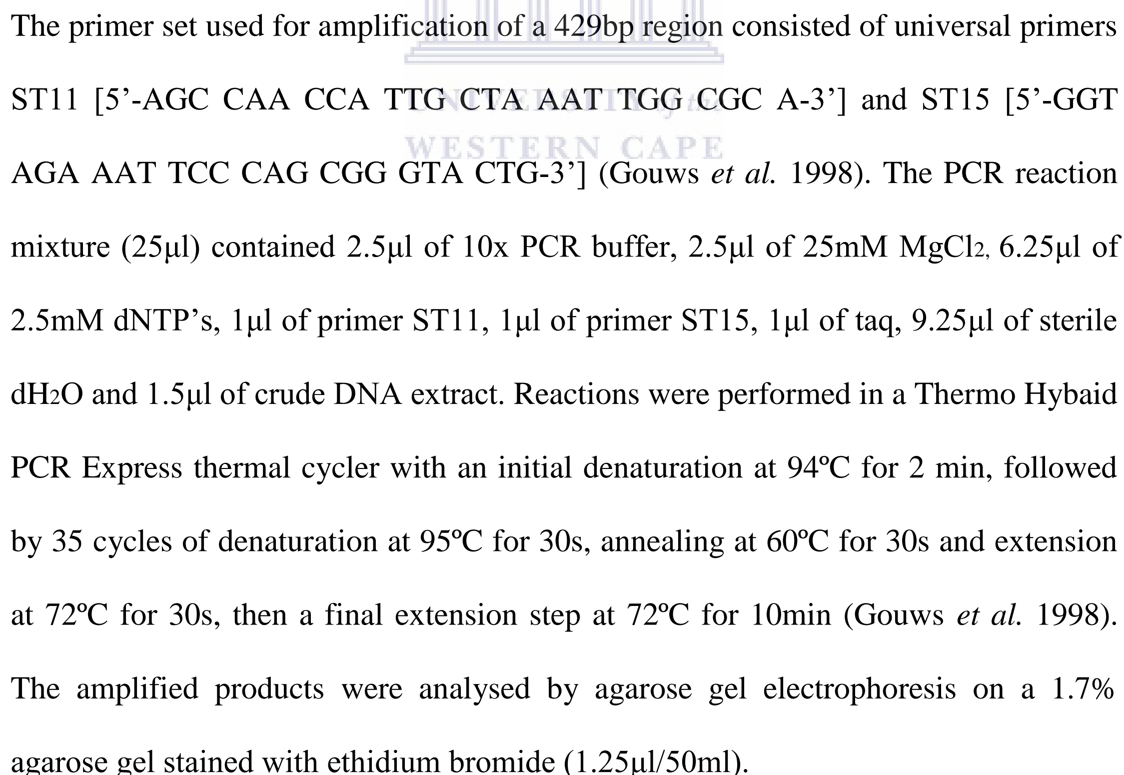
3.3.3.1 Preparation for PCR

DNA was extracted from the pre-enrichment suspension of the ostrich meat samples for *L. monocytogenes* and *Salmonella*. DNA for the detection of *Campylobacter* spp. was extracted from the homogenised suspension before it was incubated at 37ºC for 18-24h. The suspensions (1.5ml) were transferred to an eppendorf tube. The 1.5ml samples were centrifuged at low-speed (1000g, 2min) to pellet meat particles. The supernatant was removed and centrifuged at 13000g for 10min. The recovered pellets were subjected to a modified extraction procedure based on a protocol previously

described for the detection of *L. monocytogenes* in food products by using colonies (Gouws and Liedemann 2005). The pellets were resuspended in 50µl of 1x PCR buffer followed by the addition of a 2% Triton X solution (BDH Chemicals Ltd). The cell suspension was thoroughly mixed and incubated at 100ºC in a water-bath for 15min. The mixture was allowed to cool to room temperature before 1,5µl of the crude DNA extract was used for PCR amplification. This crude DNA extraction protocol was the same for *L. monocytogenes, Salmonella* and *Campylobacter* spp.

3.3.3.2. PCR conditions and DNA analysis

3.3.3.2.1 *Salmonella*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The primer set used for amplification of a 429bp region consisted of universal primers ST11 [5'-AGC CAA CCA TTG CTA AAT TGG CGC A-3'] and ST15 [5'-GGT AGA AAT TCC CAG CGG GTA CTG-3'] (Gouws *et al.* 1998). The PCR reaction mixture (25µl) contained 2.5µl of 10x PCR buffer, 2.5µl of 25mM $MgCl_2$, 6.25µl of 2.5mM dNTP's, 1µl of primer ST11, 1µl of primer ST15, 1µl of taq, 9.25µl of sterile dH_2O and 1.5µl of crude DNA extract. Reactions were performed in a Thermo Hybaid PCR Express thermal cycler with an initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 30s, annealing at 60ºC for 30s and extension at 72ºC for 30s, then a final extension step at 72ºC for 10min (Gouws *et al.* 1998). The amplified products were analysed by agarose gel electrophoresis on a 1.7% agarose gel stained with ethidium bromide (1.25µl/50ml).

3.3.3.2.2 *Campylobacter*

The multiplex PCR amplification (Table 3.1) is based on a method that distinguishes between *Campylobacter, Arcobacter* and *Helicobacter* in poultry samples (Neubauer and Hess 2006). The PCR reaction proposed by Neubauer and Hess, (2006) was adapted and optimised for detection in ostrich meat.

Table 3.1 Primers used in the multiplex PCR (Neubauer and Hess 2006)

Primer	Sequence	Fragment size (bp)
REVERS	5' –GTG GAG TAC AAG ACC CGG GAA- 3'	-
HELIP2	5' –CCA AGG GCT ATG ACG GGT GTA TCC- 3'	1107
CAMPCJLI	5' –ACT CCT TTT CTT AGG GAA GAA TTC - 3'	946
ARCOB1	5'-TGT AGG CGG ATT GAT AAG TTT GAA-3'	822

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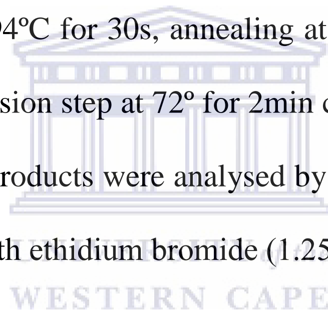
The PCR reaction mixture (50µl) contained 10µl of 5x PCR buffer, 3µl of 25mM $MgCl_2$, 4µl of 10mM dNTP's, 1µl of Reverse primer, 1µl of ArcoB1, 1µl of CampCJL, 1µl of HeliP2, 0.25µl taq, 9.25µl sterile dH_2O and 1.5µl crude DNA extract. Reactions were performed in a Thermo Hybaid PCR Express thermal cycler with an initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 2min, annealing at 65ºC for 1min and extension at 72ºC for 1min, then a final extension step at 72ºC for 10min (Neubauer and Hess 2006). The amplified products were analysed by agarose gel electrophoresis on a 1.7% agarose gel stained with ethidium bromide (1.25µl/50ml). PCR products were analysed by agarose gel electrophoresis on a 0.8% agarose gel stained with ethidium bromide (1.25µl/50ml).

3.3.3.2.3 *Listeria monocytogenes*

PCR assays were performed in 25µl volumes. The extracted DNA (1.5ml) was added to a reaction mixture of 23.5µl. The reaction mixture (23.5µl) consisted of 2.5µl of 10x PCR buffer, 2.5µl of 25mM $MgCl_2$, 2µl of 2.5mM dNTP's, 1µl of primer A, 1µl of primer B, and 1µl of taq and 13.5µl of dH2O. Primer A [5'-CAT TAG TGG AAA GAT GGA ATG- 3'] and primer B [5'-GTA TCC TCC AGA GTG ATC GA- 3'] was used to amplify a 730bp region of the hly gene. The PCR reactions were performed in a Thermo Hybaid PCR Express thermal cycler with a pre-initial denaturation of 10min at 80ºC followed by the initial denaturation of 3min at 94ºC then followed by 30 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 30s and extension at 72ºC for 30s and a final extension step at 72º for 2min completed the reaction (Gouws and Liedemann 2005). PCR products were analysed by agarose gel electrophoresis on a 0.8% agarose gel stained with ethidium bromide (1.25µl/50ml).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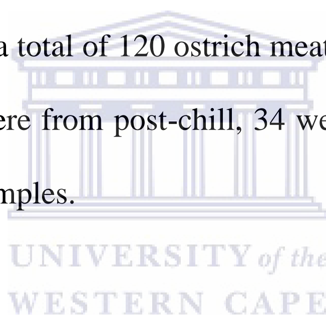
3.4. RESULTS AND DISCUSSION

The microbiological condition of meat obtained from large birds depends on the types of microorganisms carried on the skin, feathers, in the gastro-intestinal tract, or in the muscle tissue itself. Circumstances, such as the type of farming system, in which the birds are reared, as well as the conditions under which the carcass is dressed and slaughtered also play a role in the microbial conditions of the meat. Microbial numbers tend to be high when attention to the prevention of the contamination of meat is less when the concern in high speed processing large plants is focussed on the assurance of undamaged valuable skin (Gill 2007). Over the seven-month period (May through to November), a total of 120 ostrich meat samples were tested, of these, 32 were from pre-chill, 33 were from post-chill, 34 were from pre-packaging and 21 were from vacuum-packed samples.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Total viable count (Table 3.2) on pre-chill, post-chill, pre-packaging and vacuum-packed samples was, respectively, 1.25, 2.11, 7.37 and 3.15 $\log_{10}$ CFU/cm^2. This study found that TVC counts do not agree with previous findings of Karama *et al.* (2003) who had higher TVC counts at post-skinning (4.32 $\log_{10}$ CFU/cm^2), post-evisceration (4.21 $\log_{10}$ CFU/cm^2) and post-chilling (4.57 $\log_{10}$ CFU/cm^2). These results were found to have no change of practical significance on ostrich carcases at different processing points during the slaughter process. Pre-chill (1.25 $\log_{10}$ CFU/cm^2) samples in this study contained the least TVC of the four processed points, followed by post-chill (2.11 $\log_{10}$ CFU/cm^2) samples. The TVC was significantly the highest on pre-packaging (7.37 $\log_{10}$ CFU/cm^2) samples. This result may be due to the fact that the pre-packaging samples are collected after debonning where potential

for microbial contamination is highest. Small abattoirs have reported carcasses to carry total aerobes at mean numbers of 10^3 CFU/cm^2 (Gill 2007).

Table 3.2 Total viable counts (log_{10}) in raw ostrich meat at various processing points over a period of 7 months

	May	June	July	August	September	October	November	Average TVC at processing point
Pre-chill	0.74[a] (0.11)[b]	0.89 (0.26)	1.80 (1.17)	0.65 (0.02)	0.50 (-0.13)	1.34 (0.71)	1.30 (0.67)	1.88 (1.25)
Post-chill	2.65 (2.02)	2.13 (1.50)	2.69 (2.06)	1.92 (1.29)	1.66 (1.03)	2.41 (1.78)	1.98 (1.35)	2.74 (2.11)
Pre-packaging	2.08 (1.45)	1.86 (1.23)	3.09 (2.46)	2.85 (2.22)	3.28 (2.65)	3.68 (3.05)	3.01 (2.38)	8.00 (7.37)
Vacuum-packed	3.36 (2.73)	3.70 (3.07)	3.35 (2.72)	3.21 (2.58)	0 (0)	0 (0)	2.49 (1.86)	3.78 (3.15)
Average TVC for month	2.18 (1.55)	2.19 (1.56)	2.71 (2.08)	1.69 (1.06)	1.84 (1.21)	2.00 (1.37)	1.98 (1.35)	

a Log_{10} CFU/g

b Log_{10} CFU/cm^2

The results in this study show a significant difference in TVC in ostrich meat at the various processing points. As expected, log_{10} mean counts increased with succession stages of the ostrich slaughtering process. This was however not in agreement with vacuum-packed meat, which has decreased log_{10} mean counts (3.15 log_{10} CFU/cm^2), as the oxygen concentration in vacuum packaged meat decreases while that of carbon dioxide increases by metabolism, creating an unfavourable environment for most Gram negative bacteria and selecting towards CO_2-tolerant bacteria (Borch *et al.* 1996

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and Gram and Huss 1996). TVC's found in vacuum-packed (3.78 log_{10} CFU/g) meat (Table 3.2) were found to be lower than those found by Capita *et al*. (2006) and Alonso-Calleja *et al.* (2004), where TVC's were 4.9 log_{10} CFU/g and 7.09 log_{10} CFU/g respectively. The initial microbial load on ostrich meat before vacuum packaging and the period between vacuum packaging of ostrich meat and microbial testing may account for these differences in results.

The prevalence of coliforms and *E. coli* (Table 3.3) was much higher than that of any other microorganism tested. Of the 120 ostrich meat samples tested, 40 (33.33%) were positive for *E. coli and* the microorganism was detected at every processing stage at which meat samples where collected. The highest prevalence of *E. coli* was found on pre-chill (10%) samples, followed by post-chill and pre-packaging samples which produced the same results of 11 (9.17%) *E. coli*-positive samples. In vacuum-packed samples the prevalence of *E. coli* was observed to be lower than the prevalence in pre-chill, post-chill and pre-packaging samples. Significant differences in the proportion of ostrich meat samples positive were noted between pre-chill and vacuum-packed samples. No significant differences were observed between pre-chill, post-chill and pre-packaging samples. Karama *et al.* (2003) reported an incidence of 18.8% for *E. coli* on ostrich carcasses and that the majority of the samples testing positive were collected after evisceration. The high prevalence of *E. coli* on ostrich meat after evisceration may be accounted for by the cross-contamination between clean and contaminated flesh (e.g. infected air sacs, abscesses), and/ or faecal spillage when intestines and cloacae are removed.

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A significantly high number of samples positive for coliforms were observed. Of the meat samples 119 (99.17%) were positive for coliforms, indicating a high rate of possible faecal contamination. Prevalence rates did not differ much among the pre-chill, post-chill and pre-packaging meat samples. However, differences in the prevalence rate of coliforms on vacuum-packed meat samples were observed (Figure 3.3). Compared to prevalence's observed in pre-chill (26.67%), post-chill (27.50%) and pre-packaging samples (28.33%), vacuum-packed samples displayed the lowest (16.67%) prevalence rate.

Table 3.3 The prevalence of coliforms, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* and *Campylobacter* on ostrich meat samples tested

Microorganism	Pre Chill	Post Chill	Pre-packaging	Vacuum-Packed	Total
Coliforms	26.67%[1] (32/120) [2]	27.50% (33/120)	28.33% (34/120)	16.67% (20/120)	99.17% (119/120)
E. coli	10.00% (12/120)	9.17% (11/120)	9.17% (11/120)	5.00% (6/120)	33.33% (40/120)
Staphylococcus aureus	0.83% (1/120)	2.50% (3/120)	3.33% (4/120)	5.00% (6/120)	11.67% (14/120)
Listeria monocytogenes	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)
Salmonella	0.00% (0/120)	0.83% (1/120)	0.00% (0/120)	0.00% (0/120)	0.83% (1/120)
Campylobacter	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)	0.00% (0/120)

[1] Percentage of positive samples at each point.

[2] Number of samples tested at each point that was positive.

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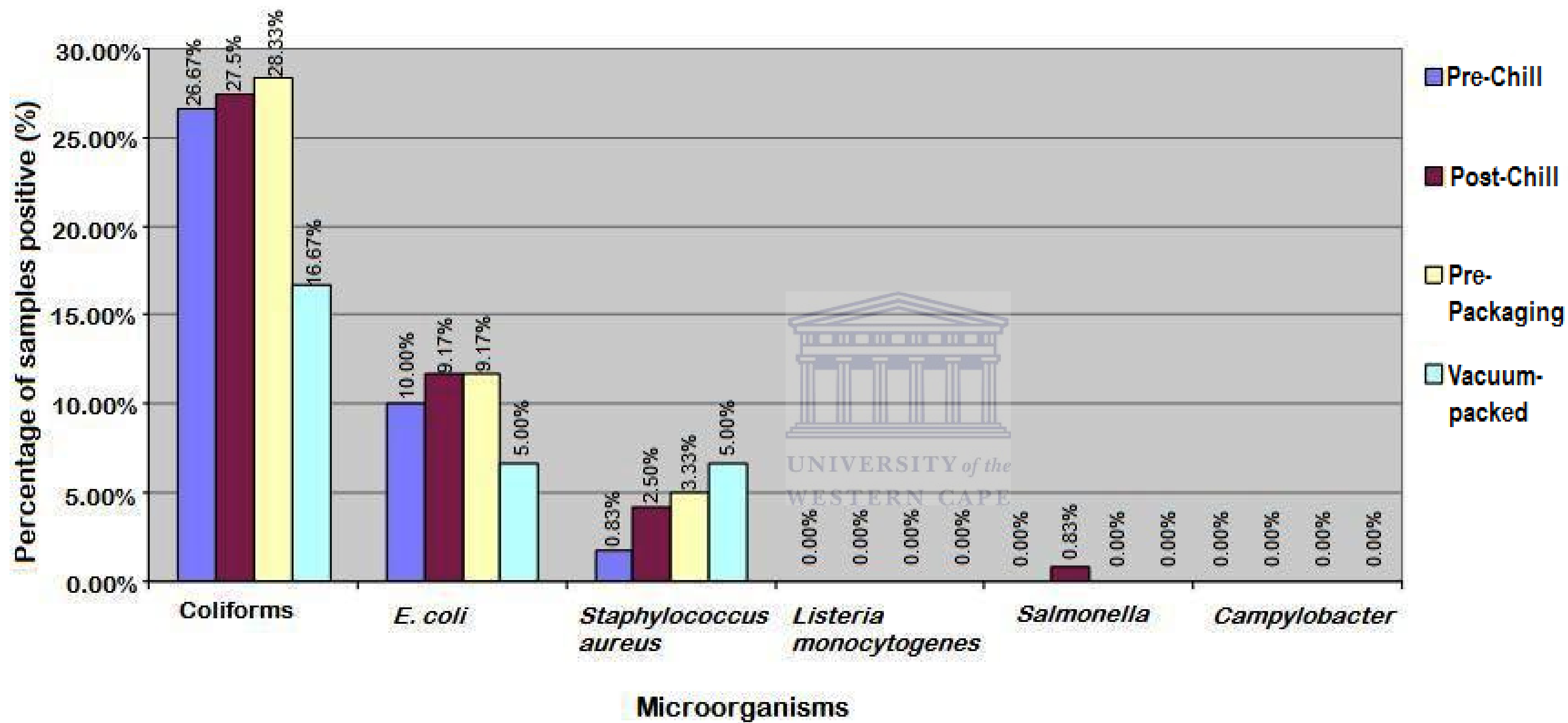

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Figure 3.1 Prevalence of positive samples for microorganisms at four processing points.

Fourteen (11.67%) of the 120 meat samples tested were positive for *Staphylococcus aureus* (Table 3.2). *Staph. aureus* was found in pre-chill (0.83%), post-chill (2.50%), pre-packaging (3.33%) and vacuum-packed samples (5%). In another study *Staph. aureus* was found in post-skinning (2.89 $\log_{10}$ CFU/cm^2), post-evisceration (2.90 $\log_{10}$ CFU/cm^2) and post-chilling (2.38 $\log_{10}$ CFU/cm^2) samples from ostrich carcasses (Karama *et al*. 2003). Vacuum-packed samples had the highest prevalence (5 %) of *Staph. aureus* among the four processing stages (Figure 3.2). This observation is significant since the prevalence of other microorganisms (coliforms, *E. coli* and *Salmonella*) tested in vacuum-packed samples were found to be less than prevalences observed in samples from the other three processing stages (pre-chill, post-chill and pre-packaging). This does not agree with that of Alonso-Calleja *et al.* (2004), who reported the absence of *Staph. aureus* in vacuum-packed retail ostrich meat. The difference in the *Staph. aureus* results between the two studies may be the fact that the vacuum-packed retail ostrich meat was purchased between 3 and 7 days after packaging, whereas vacuum-packed samples in this study were examined 1 to 2 days after packaging, and since the presence of 80% carbon dioxide and pH4 consequently retards the growth of *Staph. aureus*, the shelf life of vacuum-packed meat is significantly extending. Meats that are vacuum-packed still contain trapped oxygen that are utilised by microorganisms resulting in the production of metmyoglobin (Aberle *et al.* 2001). These metabolic activities result in the production of carbon dioxide and the increase in acidity, and subsequently the flourishing of facultative anaerobic bacteria that produce lactic acid (Nychas *et al*. 2008; Gram *et al.* 2002). This may suggest that the microbiological contamination experienced might be overcome with vacuum-packaging. *Staph. aureus* is a major component of the skin

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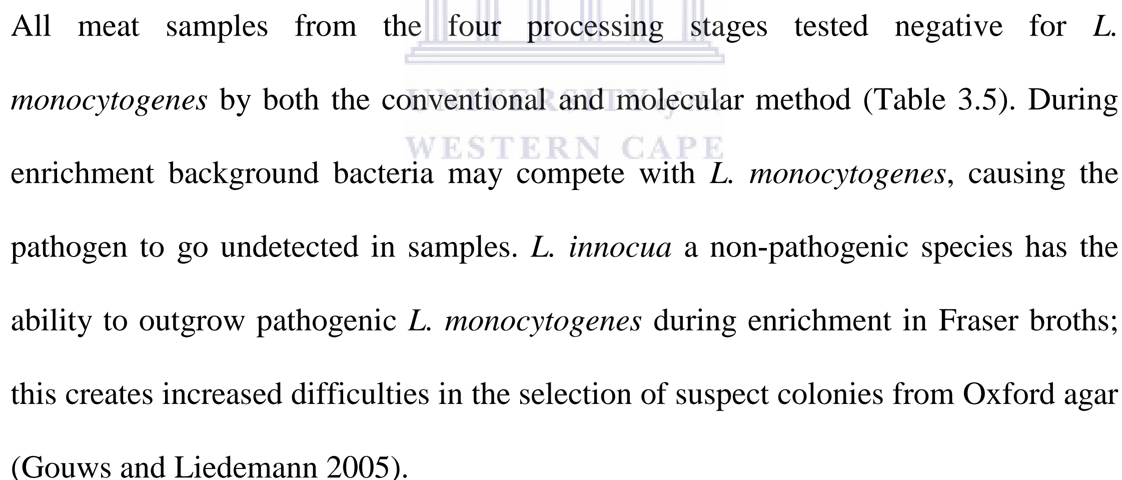
and mucous membranes of mammals, especially on the nasopharyngeal region of birds (Atanassova *et al.* 2001), its occurrence on raw ostrich meat would be expected.

Only one (0.83%) out of 120 samples tested positive for *Salmonella*. *Salmonella* spp. was detected in the same meat sample from a post-chilling point by both conventional and molecular method. In the molecular method a 429pb band (Figure 3.4) was observed for this positive post-chill sample in the *Salmonella* PCR reaction. The extensive handling and exposure of meat to surfaces that are more susceptible to contamination during the deboning process may account for it's presence in the meat sample. Since *Salmonella* is found in the gastrointestinal tracts of many animals and birds, recovery of these organisms from ostrich carcasses is not at all surprising.



All meat samples from the four processing stages tested negative for *L. monocytogenes* by both the conventional and molecular method (Table 3.5). During enrichment background bacteria may compete with *L. monocytogenes*, causing the pathogen to go undetected in samples. *L. innocua* a non-pathogenic species has the ability to outgrow pathogenic *L. monocytogenes* during enrichment in Fraser broths; this creates increased difficulties in the selection of suspect colonies from Oxford agar (Gouws and Liedemann 2005).

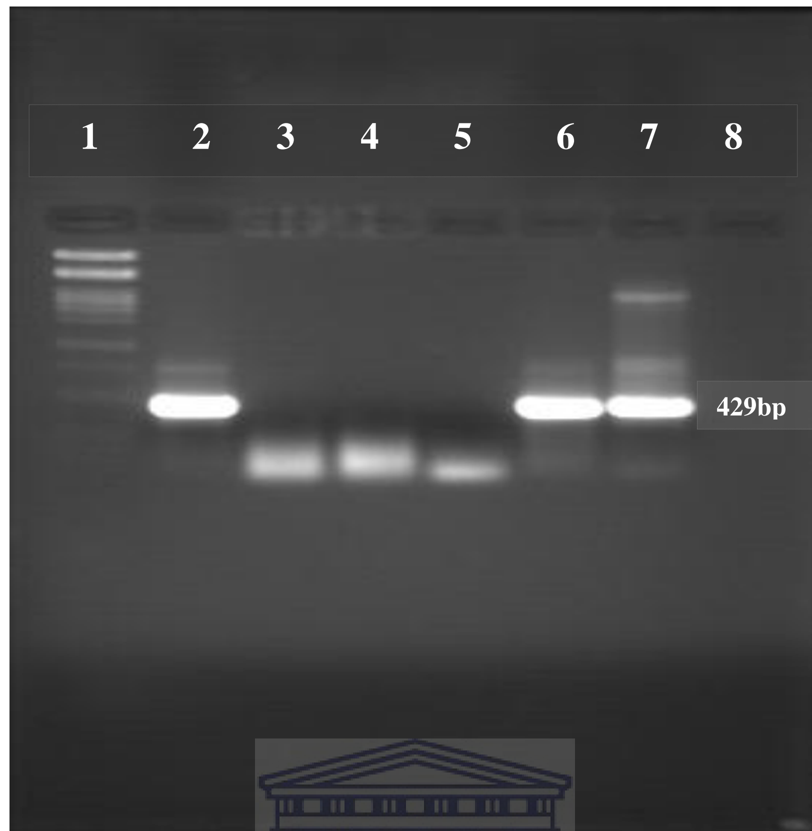


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Figure 3.2 Agarose gel electrophoresis of PCR amplified product of *Salmonella* from ostrich meat samples. Lanes 2 to 5 represents ostrich meat samples; Lanes 7 and 8 represent *Salmonella enterica* (positive control); Lane 1 carries the molecular size marker (lambda DNA cut with restriction enzyme Pst1).

From the 120 ostrich meat samples, no isolates of *Campylobacter* spp. were detected by the conventional or molecular method. *Arcobacter* and *Helicobacter* were not detected in any of the samples tested in PCR. These findings does not correspond with results found by Ley *et al.* (2001), in which 10% (19/191) of ostrich carcasses positive for *Campylobacter* was reported. Due to prolonged exposure to unfavourable conditions, bacteria that are naturally present in food samples usually have reduced viability (Gouws *et al.* 1998). This may also account for absence of contamination found in this study. *Campylobacter* spp. may go into a viable but nonculturable state,

which is not detected by culture methods. This too might explain its absence on TBA plates. Conventional methods for the detection of *Campylobacter* spp. by culture present serious difficulties for standard selection. There is no general agreement regarding the determination of the standard for the detection of this foodborne pathogen.

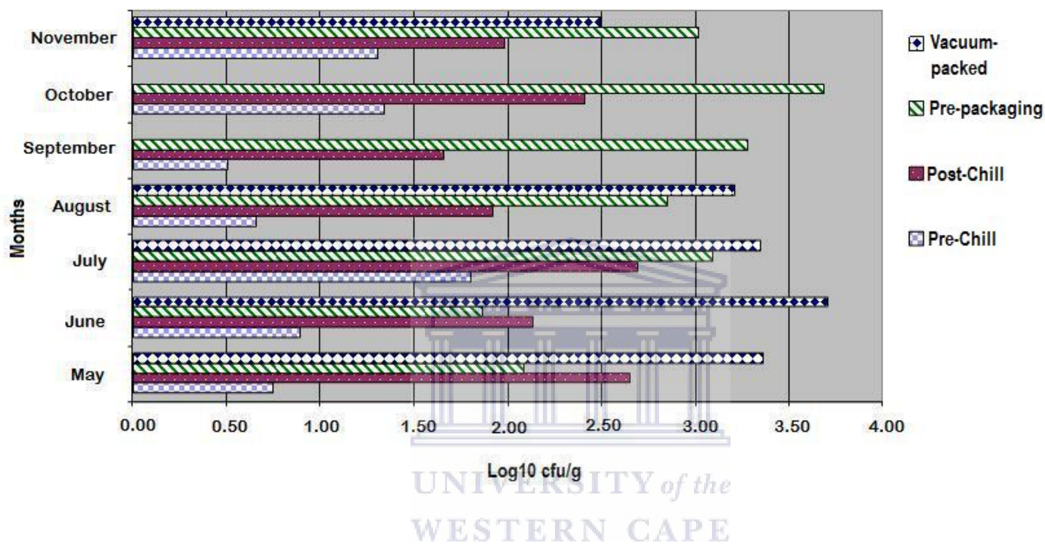

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Figure 3.3 Monthly comparison between May and November of the Total Viable Count in Log_{10} cfu/g at four processing points on the ostrich slaughter line.

There was no significant difference in mean TVC of all samples over the seven-month period (Fig. 3.1). TVC counts were all above mean 1.69 log_{10} CFU/g (Table 3.2). Pre-chill samples (Fig. 3.1) contained the highest TVC in July and the lowest in September. Post-chill samples contained the highest TVC in May and July, with the lowest TVC in September. TVC in pre-packaging samples were the highest in October and the lowest in June. Vacuum-packed samples were not collected in September and October, but of the months in which samples were collected; June produced the highest TVC's.

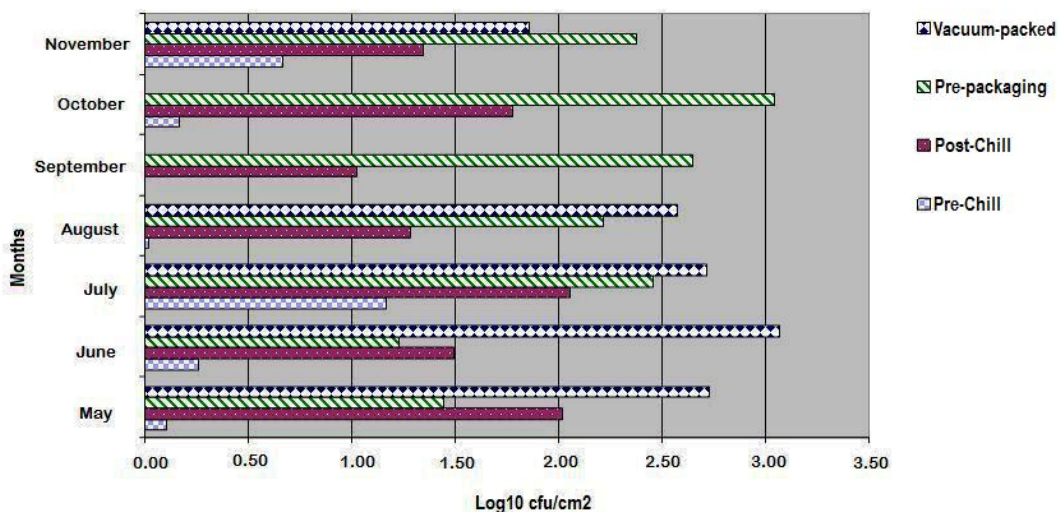

Figure 3.4 Monthly comparison between May and November of the Total Viable Count in Log_{10} cfu/cm^2 at four processing points on the ostrich slaughter line.

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The prevalence or absence of *L. monocytogenes*, *Campylobacter* spp., *Salmonella* spp., *Staph. aureus*, *E. coli* and coliforms on ostrich meat samples is demonstrated in figure 3.3 and 3.4 during the 7-month period. The prevalence of *E. coli* exhibited a multi-seasonal distribution with the highest incidence (Table 3.3) of *E. coli* (66.67%) occurring in July, followed by November (56.67%) and August (40%). *E. coli* failed to be detected in September and the prevalence of coliforms was found to be coherent over the 7-month period. The highest incidence of *Staph. aureus* occurred in June (33.33 %), while a second peak incidence was noted in May (21.88%) and July (16.67%). The organism failed to be detected in August, September, October and November. *Salmonella* spp. was only detected in September with a prevalence of 10%. No *L. monocytogenes* or *Campylobacter* spp. was detected over the 7-month period. The difference in distribution found in this study may be influenced by factors

such as the source of animals, the plant environment and seasonal factors (climate, temperature and relative humidity).

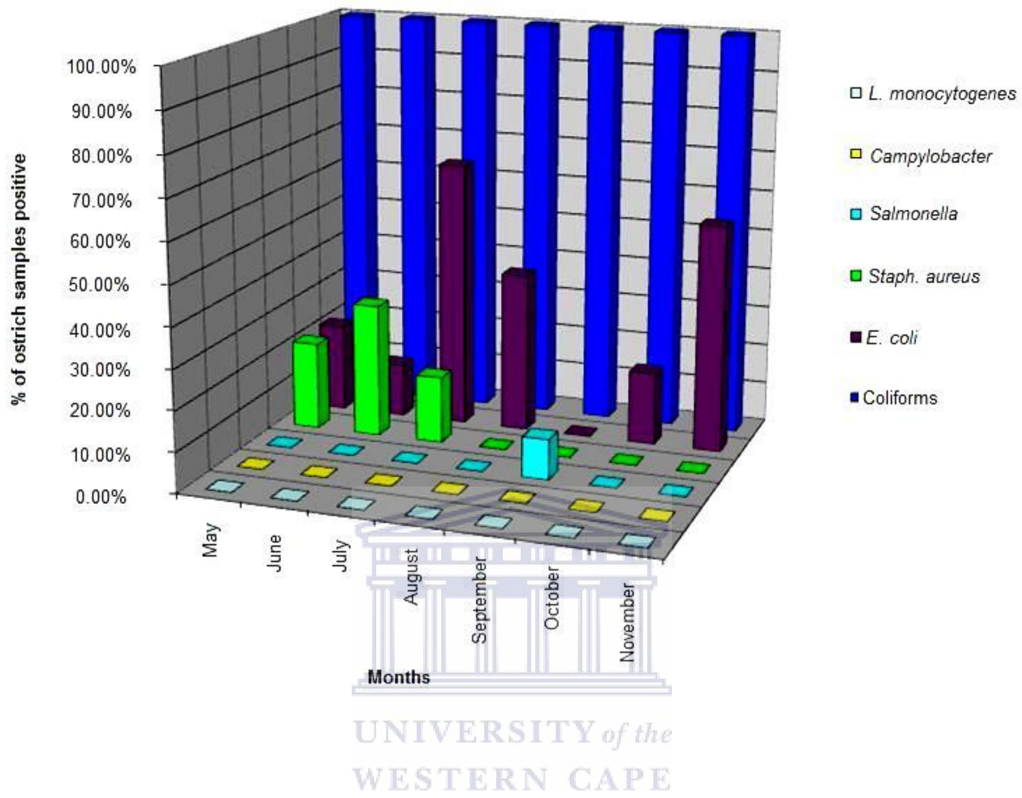

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Figure 3.5 Incidence of foodborne pathogens on ostrich meat.

Table 3.4 Prevalence of pathogenic microorganisms over a period of seven months (May-Nov)

Months	*L. monocytogenes*	*Campylobacter spp.*	*Salmonella spp.*	*Staph. aureus*	*E. coli*	Coliforms
May	0%	0%	0%	21.88%	21.88%	100%
June	0%	0%	0%	33.33%	13.33%	100%
July	0%	0%	0%	16.67%	66.67%	100%
August	0%	0%	0%	0%	40%	100%
September	0%	0%	10%	0%	0%	100%
October	0%	0%	0%	0%	18.18%	100%
November	0%	0%	0%	0%	56.67%	100%

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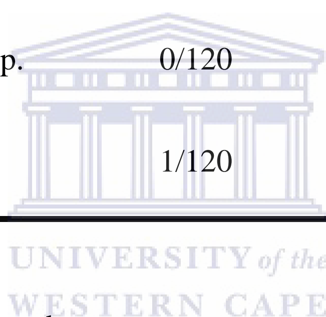
To determine the accuracy of molecular detection, results from PCR were compared to those of the culture method. As shown in Table 3.5 the detection of pathogens by both PCR and conventional method reflect each other. Naturally contaminated samples are complex, and the initial numbers of target bacteria are unknown. Combining pre-enrichment and PCR enhances the sensitivity of the PCR reactions by increasing the number of microorganisms (Mateo *et al*. 2005). In the current study samples was not pre-enriched at 37ºC at 18-24h for the detection of *Campylobacter* spp. Applying pre-enrichment in the protocol for detection of *Campylobacter* spp. may increase the number of positive results. Polysaccharides, urea, humic acids or haemoglobin exhibit similar solubility to DNA, these substances appear to be abundant in complex samples such as food and may inhibit the amplification of nucleic acids by PCR (Moreira 1998). DNA extraction was performed in this study to avoid the inhibition of the PCR by organic and inorganic compounds. Centrifugation

and washing was applied in the extraction of the DNA to remove contaminants and as a result enhance the specificity of the PCR (Lantz *et al.*1994).

Table 3.5 Results of PCR and conventional methods for the detection of *Listeria monocytogenes*, *Campylobacter* spp. and *Salmonella* spp. in raw ostrich samples

	Conventional method (Culture-based)	Molecular method (PCR)
L. monocytogenes	0/120	0/120
Campylobacter spp.	0/120	0/120
Salmonella spp.	1/120	1/120



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Microbial levels present in the raw meat can increase during processing, transportation and storage. Cutting of meat presents an increase of surface area and as a result greater potential for exposure to microorganisms, these facts could account for the prevalence and loads of microorganisms found in this study. Variations in microbial prevalence's among studies are contributed to factors such as the improvement of enrichment and isolation procedures, the differences in numbers of collected samples, the types of samples and the manner in which they were collected, and the season in which the samples were collected (Li *et al.* 2004).

There is a lack of scientific literature regarding the detection of pathogens such as *L. monocytogenes*, *Campylobacter* spp. and *Salmonella* spp. on fresh ostrich raw meat

destined for human consumption. The low detection rate for *Salmonella* spp. and the absence of *L. monocytogenes* and *Campylobacter* spp. positives in samples in this study indicate that current in-plant process practices appear to uphold the quality and safety of ostrich meat. Low numbers of samples found positive for pathogens in this study prevents the comparison of detection by the conventional and molecular techniques.

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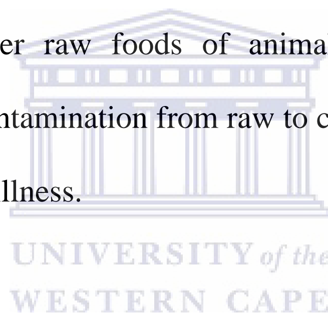
3.5 CONCLUSION

The microbiological quality and safety of ostrich meat is a true reflection of the bacterial load associated with the live bird and the precautions taken to control the spread of microorganisms during the slaughter process. The data from this study shows that the incidence of bacteria does tend to increase in succession with the slaughter processing chain. This study also indicates that ostrich meat may not be a significant source of the foodborne pathogens (*L. monocytogenes*, *Campylobacter* spp. and *Salmonella* spp.) that are seen in other meat industries. The recovery of foodborne pathogens from the ostrich meat indicates that the same considerations must be taken as with other raw foods of animal origin. Temperature abuse, underprocessing and cross-contamination from raw to cooked products can make such foods vehicles for foodborne illness.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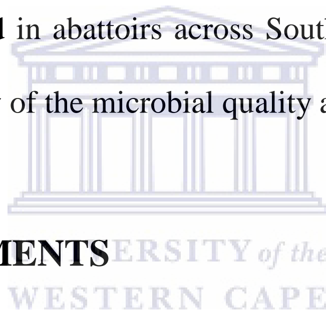
Complementation of culture-based methodologies with culture-independent (PCR) techniques still remains a better chance of detection of pathogens in meat samples. PCR is a valuable tool in detecting pathogens in a less time-consuming and tedious way. This technique may more appropriately be used as a rapid preliminary method of assessing ostrich meat in quality control in the ostrich meat industry. More results of the microbial quality and safety of ostrich meat are needed for comparison.

The lack of information in scientific literature regarding the microbiological status of these foodborne pathogens in ostrich meat makes it difficult to distinguish whether contamination could be due to the fact that the environments, where the meat is slaughtered and processed are more probable sources of the organism than the animal

itself. The contamination problems found in the slaughtering process may result from animal husbandry and farm management practices which fail to recognise the requirements for safe meat production.

The results obtained provide baseline data on the microbial quality of ostrich meat in South Africa, which may help in the maintenance and fine tuning of good practices of slaughter hygiene in ostrich meat production since it is of essential importance for the prevention of microbial meat contamination in the concern of ensuring both health protection and meat quality. Samples of ostrich meat at various processing points in the abattoir need to be analysed to indicate which processes contribute to microbial levels. Ostrich meat produced in abattoirs across South Africa need to be studied in order to get a proper overview of the microbial quality and safety of ostrich meat.



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3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

Evaluation of raw fresh and vacuum-packed ostrich meat for the presence of *Pseudomonas* spp.

4.1 ABSTRACT

Aim: The aim of this study was to determine the presence of pseudomonads on ostrich meat by molecular and conventional methods

Materials and Methods: Raw fresh and vacuum-packed ostrich meat (120 samples) were collected from four processing points of two South African export-approved abattoirs in the Klein Karoo. The ostrich meat samples were evaluated for the presence of pseudomonads by molecular and conventional methods. In this study 19.17% of the ostrich samples tested positive by means of PCR, while only 5.8% were confirmed to be positive on Pseudomonas agar supplemented with CFC. Significant in this study was that samples found positive by PCR increased in succession with the slaughter processing chain.

Conclusion: *Pseudomonas* species are present in ostrich meat. The presence of these microorganisms indicates contamination and temperature abuse in the slaughter process. The slaughter processing of ostriches contributes significantly to the quality of the meat.

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Significance and impact of the study: *Pseudomonas* spp. has been detected in ostrich meat.

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4.2 INTRODUCTION

Slaughter processes amplify microorganisms by allowing contamination of carcasses and of cross-contamination between infected and uninfected carcasses (Anonymous 2000). In order to create conditions where the microorganisms of slaughtered animals can be controlled, hygienic conditions at the slaughterhouse have to be maintained (Bolder 2007). As soon as the food is harvested or slaughtered the spoilage process begins, and if steps are not taken to prevent spoilage the food will be rendered useless (Macrae *et al.* 1993).

Meat spoilage at low temperature in air is mainly the result of the metabolic activity of motile and non-motile psychotrophic, aerobic rods dominated by *Pseudomonas* spp. (Rodríguez-Calleja *et al.* 2005) *Pseudomonas* species are Gram-negative bacteria belonging to the Pseudomonadaceae family. There association with water, soil and vegetation makes these bacteria common in fresh foods. Growth of pseudomonads in food results in spoilage, lowering the quality of the food (Arnaut-Rollier *et al.* 1999; Cousin 1999). These bacteria have the ability to grow slowly at low temperatures and can spoil food at higher temperatures. Growth of pseudomonads on foods result in a variety of sensory defects, which include off-flavours, formation of slime, colour changes and strong odours (Nychas *et al.* 2008; Ellis and Goodacre 2001).

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Detection of *Pseudomonas* spp. is important in the food industry since it causes serious spoilage problems. In order to prolong the shelf life of ostrich meat; it is necessary to know its microbial status. Improving the quality of ostrich meat allows for competitive marketing for the ostrich industry. The aim of this study was to

determine the presence of *Pseudomonas* spp. on fresh and vacuum-packed raw ostrich meat in the process of final product.

4.3 MATERIALS AND METHODS

4.3.1 Ostrich meat sample collection and preparation

Ostrich meat was collected from two South African export-approved abattoirs in the Klein Karoo. Samples of fresh and vacuum-packed ostrich meat were collected from four processing points, which included pre-chill (post-evisceration), post-chill (post-cold storage), pre-packaging (post-debonning) and vacuum-packed (post-packaging). Pre-chill samples were collected the same day as slaughter, while the post-chill and pre-packaging samples were collected the second day (after the debonning process). On arrival at the lab, all samples were logged and a sample number assigned. Ostrich meat samples (25g) were homogenized in 225ml buffered peptone water (Oxoid LP0034). The samples was stomached in a Seward stomacher 400 (Seward Ltd) at normal speed for 1min and then incubated at 37ºC for 24h.

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4.3.2 Conventional detection of Pathogens

***Pseudomonas* spp**

Pre-enrichment suspension (0.1ml) was transferred to 10ml of brain heart infusion (BHI) broth (Oxoid CM375). The BHI broth was then incubated at 30ºC for 18-24h. Duplicate Pseudomonas agar (Oxoid CM559) with CFC (cephaloridine, fucidin, centrimide) supplement (Oxoid SR103) plates were spread with 0.1ml of broth culture. The plates were incubated at 30ºC for 18-24h (Salvat *et al.* 2003).

Pseudomonas presumptively positive colonies on *Pseudomonas* agar (Oxoid CM559) with CFC supplement (Oxoid SR103) were confirmed with API 20NE.

4.3.3 Molecular detection of pathogens

Preparation for PCR

DNA was extracted from the pre-enrichment suspension of the ostrich meat samples for *Pseudomonas spp.* The suspensions (1.5ml) were transferred to an eppendorf tube. The 1.5ml samples were centrifuged at low-speed (1000g, 2min) to pellet meat particles. The supernatant was removed and centrifuged at 13000g for 10min. The recovered pellets were subjected to a modified extraction procedure based on a protocol previously described for the detection of *L. monocytogenes* in food products by using colonies (Gouws and Liedemann 2005). The basic boiling extraction procedure described by Gouws and Liedemann (2005) was enhanced by the addition of Triton X solution (BDH Chemicals Ltd). The pellets were resuspended in 50µl of 1x PCR buffer followed by the addition of a 2% Triton X solution (BDH Chemicals Ltd) (50µl). The cell suspension was thoroughly mixed and incubated at 100ºC in a water-bath for 15min. The mixture was allowed to cool to room temperature before 1.5µl of the crude DNA extract was used for PCR amplification.

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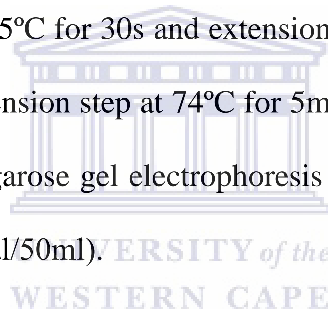
Pseudomonas

The primer set used for PCR amplification of the Internal Transcripted Spacer (ITS1) region consisted of primer fPs16S [5'-ACT GAC ACT GAG GTG CGA AAG CG-3'] and rPs23S [5' -ACC GTA TGC GCT TCT TCA CTT GAC C- 3'] (Locatelli *et al.* 2002). The 25µl reaction mixture consisted of the following 2.5µl of 10x PCR buffer, 3µl of 25mM $MgCl_2$, 2.5µl of 2.5mM dNTP's , 1µl of primer fPs16s, 1µl of primer rPs23s, 1µl of taq, 13µl of sterile dH_2O and 1.5µl of crude DNA extract. The PCR reactions were performed in a Thermo Hybaid PCR Express thermal cycler with an initial denaturation at 94ºC for 3 min, followed by 40 cycles of denaturation at 94ºC for 1min, annealing at 55ºC for 30s and extension at 74ºC for 1min. the reaction was completed by a final extension step at 74ºC for 5min (Locatelli *et al.* 2002). PCR products were analysed by agarose gel electrophoresis on a 1.3% agarose gel stained with ethidium bromide (1.25µl/50ml).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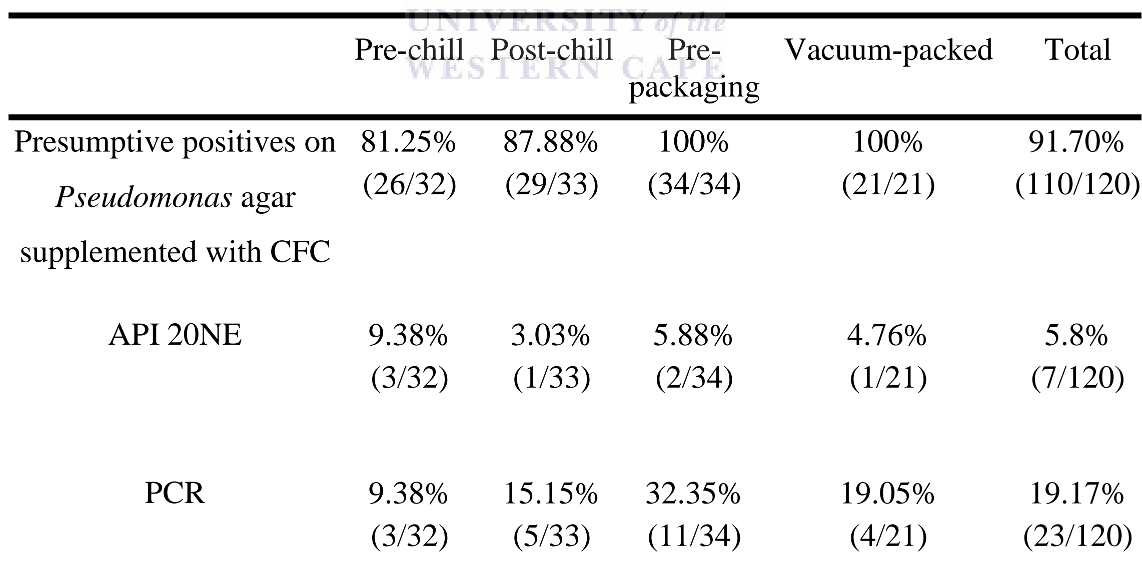
4.4 RESULTS AND DISCUSSION

Out of 120 ostrich meat samples tested, 110 (91.7%) were presumptively positive for pseudomonads on *Pseudomonas* agar (Table 4.1). Samples confirmed to be positive for *Pseudomonas* spp. by means of API 20NE totalled to 5.8%. The differences in presumptive and confirmed positives were therefore 85.9%. Samples confirmed to be positive for pseudomonads by means of PCR totalled to 19.17% (Results not shown). The difference in confirmed and PCR positives were 13.37%. The *Pseudomonas* agar did not detect all of the positive samples, with *Pseudomonas* agar detecting 7/23 for pseudomonads.

Table 4.1 The prevalence of ostrich meat samples positive for *Pseudomonas* spp.



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	Pre-chill	Post-chill	Pre-packaging	Vacuum-packed	Total
Presumptive positives on *Pseudomonas* agar supplemented with CFC	81.25% (26/32)	87.88% (29/33)	100% (34/34)	100% (21/21)	91.70% (110/120)
API 20NE	9.38% (3/32)	3.03% (1/33)	5.88% (2/34)	4.76% (1/21)	5.8% (7/120)
PCR	9.38% (3/32)	15.15% (5/33)	32.35% (11/34)	19.05% (4/21)	19.17% (23/120)

Confirmed positives from *Pseudomonas* agar were highest in pre-chill samples. There were not much significant differences in the samples from the various process points. The highest prevalence for samples tested by PCR was observed in pre-packaging

(32.35%) samples. The samples found positive by PCR increases in succession with the slaughter processing chain (Figure 4.2), except for vacuum-packed samples. This indicates that contamination and temperature abuse in the slaughtering process plays a part in the quality of ostrich meat. The low prevalence found in vacuum-packed samples are expected since, vacuum packaging inhibits respiratory pseudomonads (Alonso-Calleja *et al.* 2004). Although in some cases pseudomonads have the ability to grow anaerobically by utilizing nitrate as an alternate electron acceptor (Lebert *et al.* 1998).

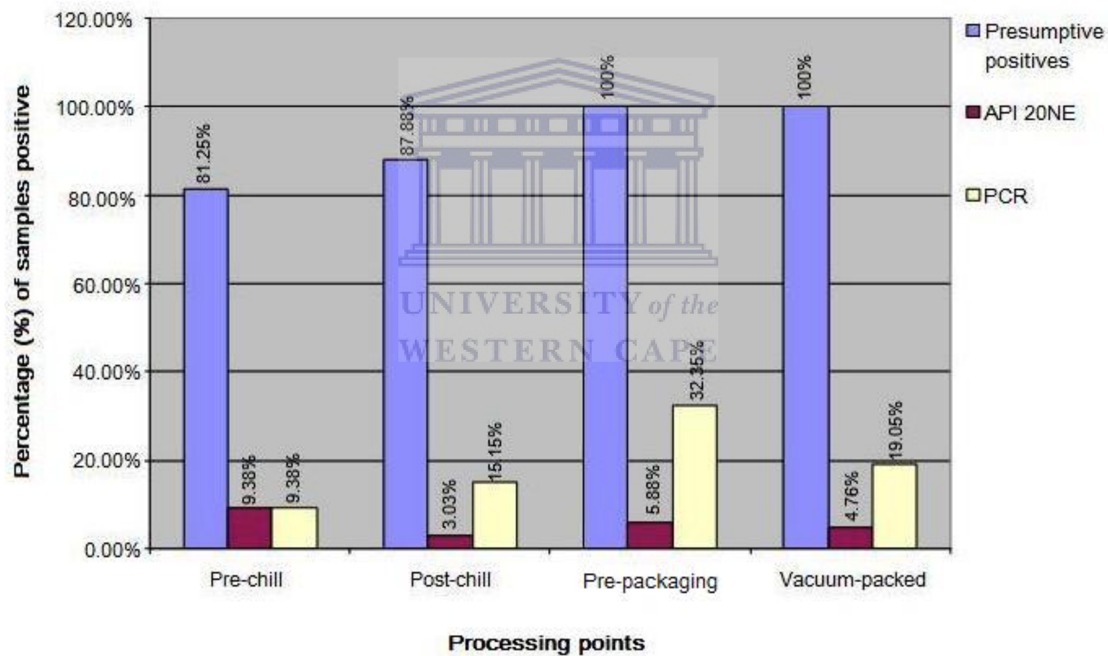

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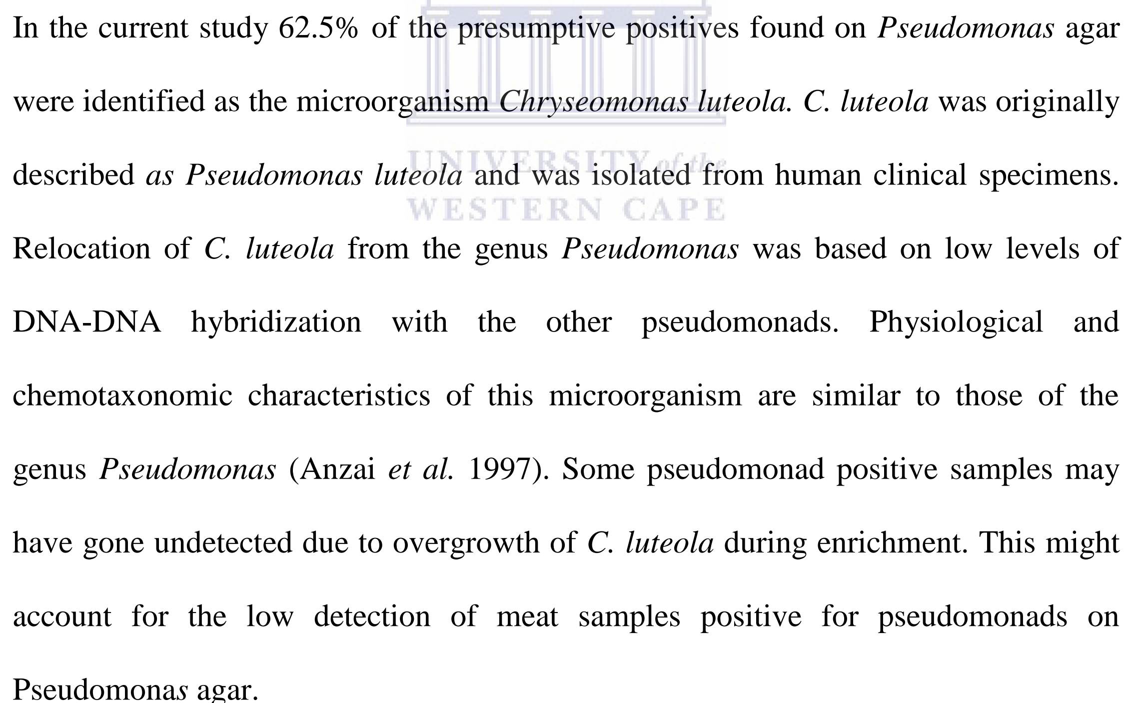
Figure 4.2. The diagrammatic comparison of the prevalence of samples positive for *Pseudomonas* species at four processing points.

Karama *et al.* (2003) reported 2.89, 2.86, and 2.38 log CFU/cm² for post-skinning, post-evisceration and post-chilling respectively. In the study, significant differences were observed between the counts for the post-skinning and post-chilling processing

points and between the counts for the post-evisceration and post-chilling process points. The increase in *Pseudomonas* spp. counts on carcass samples after chilling was also noted. In another study from Spain (Capita *et al*. 2006) *Pseudomonas* spp. counts at 0 days were both 4.1 $\log_{10}$ CFU/g for vacuum-packed ostrich steaks at 4ºC and 10ºC. After 3days it was noted that *Pseudomonas* spp. counts increased, with counts being 4.9 and 6.8 $\log_{10}$ CFU/g for 4ºC and 10ºC respectively. *Pseudomonas* spp.counts were found to be higher in packed ostrich steaks at 10ºC than at 4ºC after 3 days. This expresses the importance of temperature during storage and that interruption of the refrigeration chains can accelerate growth of spoilage microbes (Bolder 2007).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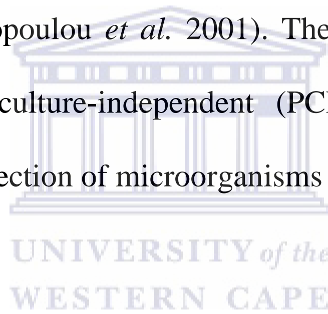
In the current study 62.5% of the presumptive positives found on *Pseudomonas* agar were identified as the microorganism *Chryseomonas luteola. C. luteola* was originally described *as Pseudomonas luteola* and was isolated from human clinical specimens. Relocation of *C. luteola* from the genus *Pseudomonas* was based on low levels of DNA-DNA hybridization with the other pseudomonads. Physiological and chemotaxonomic characteristics of this microorganism are similar to those of the genus *Pseudomonas* (Anzai *et al.* 1997). Some pseudomonad positive samples may have gone undetected due to overgrowth of *C. luteola* during enrichment. This might account for the low detection of meat samples positive for pseudomonads on Pseudomona*s* agar.

In this study *Pseudomonas* spp have been detected on ostrich meat samples by both conventional and PCR method. Detection by PCR was successful as it detected more positive samples than culturing. Microbial populations of interest do not need to be

viable or culturable as long as their nucleic acids are sufficiently intact to facilitate molecular detection strategies (Apajalahti *et al.* 2003). However, a possible drawback of PCR is that it may yield positive results for *Pseudomonas* spp., but confirmation by culturing cannot be obtained. This could be due to stressed bacteria or because certain serotypes are difficult to culture, but also to false positive results of the PCR-based method. If a negative PCR result is obtained, the analysis can be terminated, and further confirmation is unnecessary. The selectivity of the medium may possibly decrease as the quantity of interfering bacteria increases and almost impossible when these background microfloras are more fastidious, which subsequently results in false presumptive positive detection or false negative confirmed results when *pseudomonas* spp. is not detected, (Tryfinopoulou *et al.* 2001). The Complementation of culture-based methodologies with culture-independent (PCR) techniques will therefore provide a better chance of detection of microorganisms in meat samples.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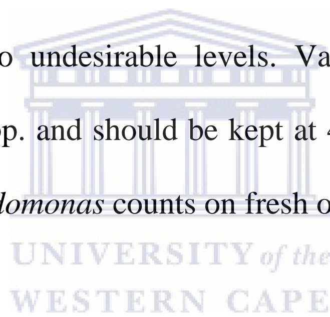
4.5 CONCLUSION

Pseudomonas spp. was detected on ostrich meat samples and was found to increase in succession with the stages of the slaughter processing chain. Detection of pseudomonads on meat indicates that contamination and temperature abuse does play an important role in the slaughter process and proves that the slaughter process does contribute to the final quality of the meat. Collaboration of culture-based methodologies with culture-independent (PCR) techniques provided a better chance of detection of the spoilage microorganism in ostrich meat samples. Detection of pseudomonads is important because it dictates the period required for the microorganism to increase to undesirable levels. Vacuum packaging reduced the incidence of *Pseudomonas* spp. and should be kept at 4°C. Further studies should be undertaken, to establish *Pseudomonas* counts on fresh ostrich meat samples.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4.6 ACKNOWLEDGEMENTS

This work was funded by the National Research Foundation (NRF). Sincere gratitude is extended to the members of the Klein Karoo Laboratory for their support.

CHAPTER 5

Antimicrobial susceptibility of *Staphylococcus aureus* isolated from ostrich meat in South Africa

5.1 ABSTRACT

Aim: The aim in this study was to investigate the antibiotic resistant pattern of *Staphylococcus aureus* in ostrich meat.

Materials and Results: The antibiotic agar disc diffusion method was used to investigate *Staphylococcus aureus* isolated from ostrich meat samples. *Staph. aureus* was present in 14 of the 120 samples (11.67%) collected in a 7-month survey from two South African export-approved abattoirs in the Klein Karoo. Each isolate was tested against ampicillin, cephalothin, clindamycine, erythromycin, gentamicin, methicillin, oxacillin, penicillin G (benzylpenicillin), teicoplanin, tetracycline, trimethoprim-sulfamethoxazole and vancomycin. No evidence of cephalothin and vancomycin resistance was observed, although a significant 21.43% of isolates was found to be resistant to methicilin. All *Staph. aureus* isolated from ostrich meat were resistant to the antibiotic teicoplanin and a large number were multiresistant, i.e. resistant to three or more antibiotics.

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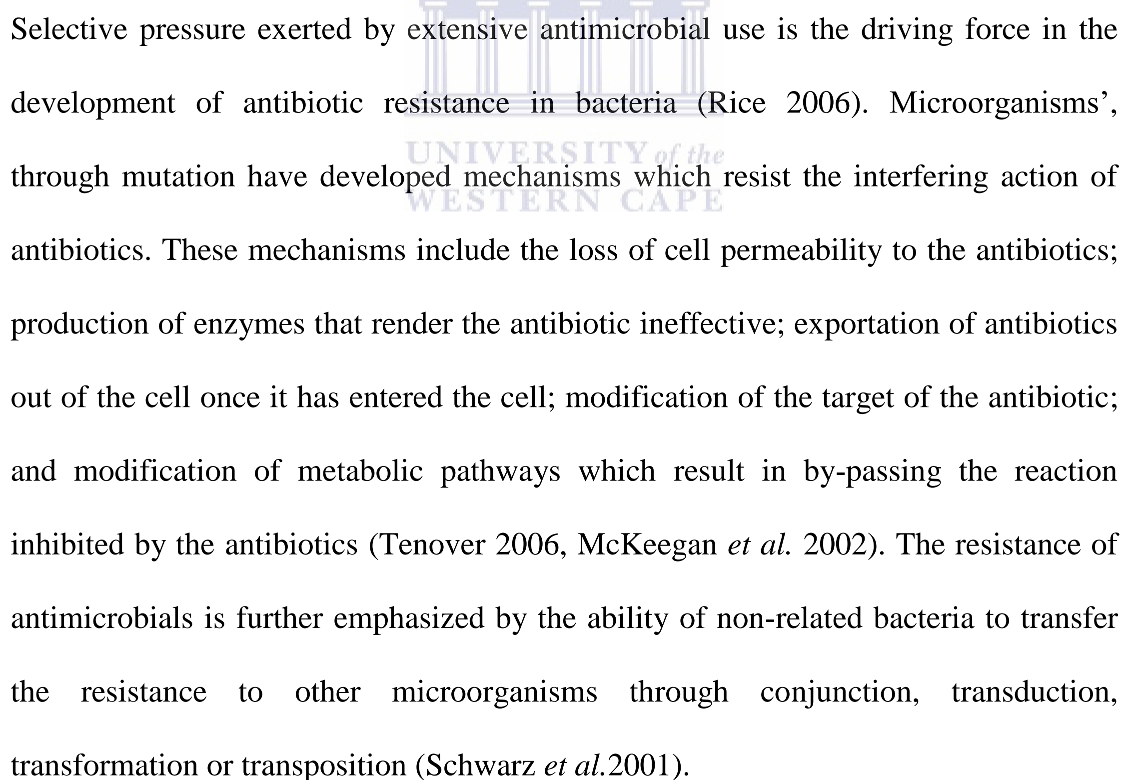
Conclusion: *Staph. aureus* isolated from ostrich meat shows a varied resistance pattern. Antimicrobial resistant *Staph. aureus* isolated from ostrich meat may pose a serious problem and should be addressed in terms of their potentially negative effect on the safety of the food supply and on consumer health.

Significance and impact of study: *Staph. aureus* isolates showed resistance to methicillin. A large number of isolates was resistant to ampicillin, penicillin and teicoplanin. Antimicrobials used in the treatment of humans being eliminated from ostrich husbandry practices to reduce the resistance of foodborne pathogens, therefore ensuring the health of the consumer.

5.2 INTRODUCTION

As early as the 1950's, antimicrobial substances have been used in livestock and poultry agricultures. Antimicrobial substances interfere with the metabolic machinery of microbial cells. This interference compromises the microorganisms' ability to survive and reproduce (Bower and Daeschel 1999). For this purpose the use of antibiotics has increased significantly. Antimicrobials are routinely administered by way of feeds and water in low nontherapeutic doses over prolonged periods to promote growth and increase the feeding efficiency and rate of weight gain in cattle, pigs and poultry (Shea 2004; Gouws and Brözel 2000; Manie *et al.* 1999).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Selective pressure exerted by extensive antimicrobial use is the driving force in the development of antibiotic resistance in bacteria (Rice 2006). Microorganisms', through mutation have developed mechanisms which resist the interfering action of antibiotics. These mechanisms include the loss of cell permeability to the antibiotics; production of enzymes that render the antibiotic ineffective; exportation of antibiotics out of the cell once it has entered the cell; modification of the target of the antibiotic; and modification of metabolic pathways which result in by-passing the reaction inhibited by the antibiotics (Tenover 2006, McKeegan *et al.* 2002). The resistance of antimicrobials is further emphasized by the ability of non-related bacteria to transfer the resistance to other microorganisms through conjunction, transduction, transformation or transposition (Schwarz *et al.*2001).

Two years after the introduction of penicillin in the 1940's in clinical therapy, the first evidence of *Staphylococcus aureus* penicillin resistant strains appeared. At present

nearly all strains of *Staph. aureus* are resistant to natural penicillins, aminopenicillins, and antipseudomonal penicillins (Rice, 2006).The introduction of methicillin in the 1960's led to outbreaks involving methicillin-resistant strains of *Staphylococcus aureus* (MRSA). Twenty years later fluoroquinolones were introduced and was effective against 95% of MRSA, but within twelve months the majority of the strains became resistant. Patients colonized with MRSA and/or reduced vancomycin-sensitive *Staph. aureus* strains are critical factors in health care (Bower and Daeschel 1999; Pesavento *et al*. 2007).

The development of antimicrobial resistance in pathogenic bacteria is a matter of increasing public health concern (Rice 2006, Rice 2009). Antimicrobial substances that are presently being utilised to treat or prevent bacterial infections in animals are fundamentally the same classes of compounds that are used in human clinical therapy (Schwarz *et al.*2001). Antimicrobial resistant bacteria may infect humans either through the food supply or through direct contact with animals. Spread of the antimicrobial resistant bacteria between diverse hosts can occur directly through skin to skin contact; contact with bacteria-containing material (saliva, faeces, etc.); or by the uptake of contaminated food, feed, air or water (Schwarz *et al.*2001).

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As a direct consequence of increasing antimicrobial resistance in foodborne pathogens is the increase of foodborne illnesses by decreasing treatment options for patients with bacterial infections. Resistance in foodborne pathogens result in treatment failures in humans, when the foodborne pathogen exhibits resistance to antimicrobial agents used for treatment (Angulo *et al.* 2004).

Since meat and meat products appear to be the major sources of foodborne infections and the most important link between food-producing animals and humans, the aim of this study was to investigate 120 samples of ostrich meat for the presence of *Staph. aureus* and the *Staph. aureus* isolates analysed for antimicrobial resistance.

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5.3 MATERIAL AND METHODS

5.3.1 Sample collection

Strains of *Staph. aureus* were obtained from a seven-month microbiological survey of raw fresh and vacuum- packed ostrich meat collected from four processing points in two South African export-approved abattoirs in the Klein Karoo.

5.3.2 Isolation of *Staphylococcus aureus*

Ostrich meat samples (25g) were homogenized in 225ml buffered peptone water (Oxoid LP0034). The samples was stomached in a Seward stomacher 400 at normal speed for 1min and incubated at 37ºC for 24h. Of the homogenized suspension 0.1ml was transferred to 10ml of Tryptone Soya Broth (TSB) (Oxoid CM129) and incubated at 37ºC for 16-18h. Serial dilutions were performed in sterile water. From the sterile water dilutions, 0.1ml aliquots were spread plated on duplicate Baird-Parker (Oxoid) plates supplemented with Egg Yolk Tellurite Emulsion (Oxoid SR0054) to obtain dominant *Staph. aureus* isolates and incubated at 37ºC for 48h. *Staph. aureus* isolated from Baird Parker agar (Oxoid CM0275) was confirmed by the Staphylase test kit and by Gram stain test. *Staph. aureus* colonies from Baird-Parker plates were purified by streaking onto plate counting agar (PCA) plates (Oxoid, CM463) and incubated at 34ºC for 18h.

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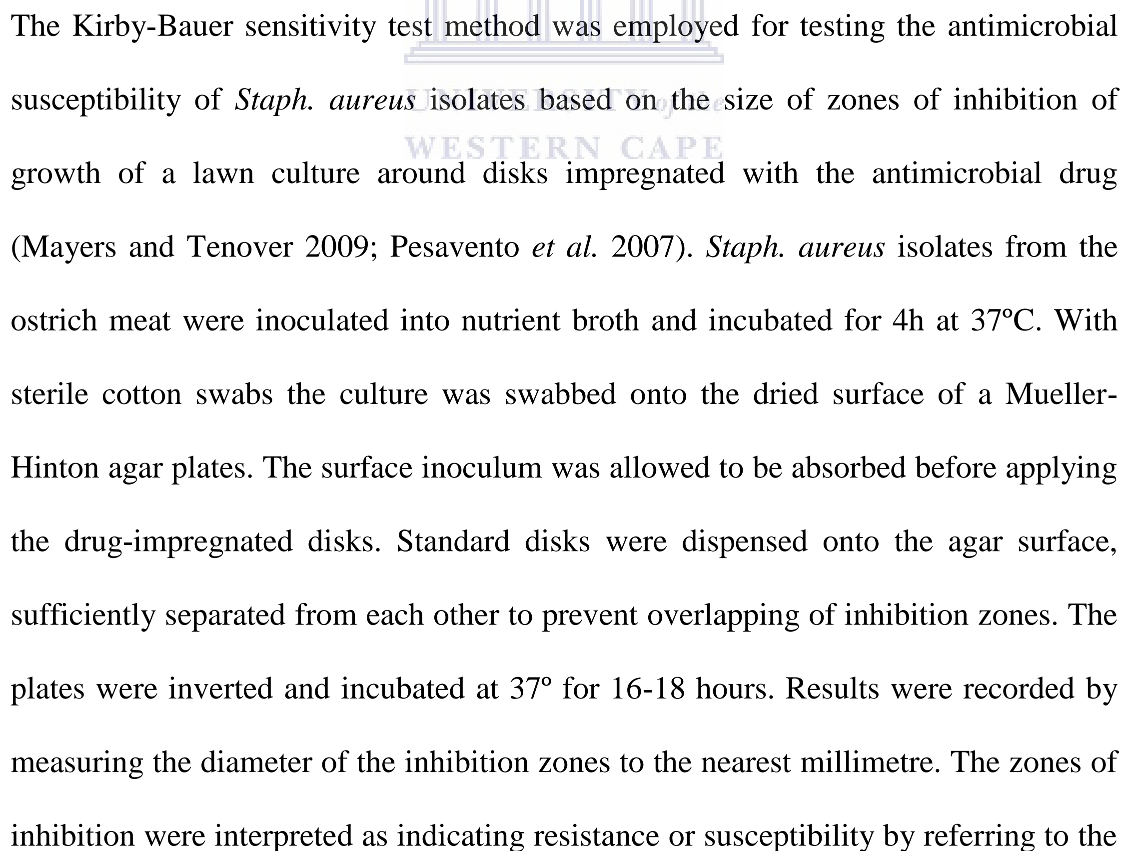
5.3.3 Antimicrobial agents

Antimicrobial susceptibility testing was done using the agar disk diffusion method (Pesavento *et al.* 2007). The antibiotics tested include ampicillin (10µg), cephalothin (30µg), clindamycine (2µg), erythromycin (15µg), gentamicin (10µg), methicillin (5µg), oxacillin (1µg), penicillin G (benzylpenicillin) (10u), teicoplanin (30µg), tetracycline (15µg), trimethoprim-sulfamethoxazole (25µg) and vancomycin (30µg). Only 14 isolates identified as *Staph. aureus* was tested for susceptibility.

5.3.4 Determination of susceptibility to antibiotics by the Kirby-Bauer sensitivity test method.



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The Kirby-Bauer sensitivity test method was employed for testing the antimicrobial susceptibility of *Staph. aureus* isolates based on the size of zones of inhibition of growth of a lawn culture around disks impregnated with the antimicrobial drug (Mayers and Tenover 2009; Pesavento *et al.* 2007). *Staph. aureus* isolates from the ostrich meat were inoculated into nutrient broth and incubated for 4h at 37ºC. With sterile cotton swabs the culture was swabbed onto the dried surface of a Mueller-Hinton agar plates. The surface inoculum was allowed to be absorbed before applying the drug-impregnated disks. Standard disks were dispensed onto the agar surface, sufficiently separated from each other to prevent overlapping of inhibition zones. The plates were inverted and incubated at 37º for 16-18 hours. Results were recorded by measuring the diameter of the inhibition zones to the nearest millimetre. The zones of inhibition were interpreted as indicating resistance or susceptibility by referring to the

interpretive table of the Kirby-Bauer sensitivity test method (WHO 2006). Strains exhibiting intermediate resistance were reported as resistant.

5.4 RESULTS

During the seven-month survey, 120 samples of raw fresh and vacuum-packed meat from two export abattoirs in the Klein Karoo were analysed. *Staph. aureus* was present in 14 of the 120 samples (11.67%). The percentage of the isolates resistant to the fourteen various antibiotics tested are represented in table 6.

All strains tested for antimicrobial resistance were susceptible to cephalothin and vancomycin. This result is in accordance with the resistant rates obtained in a study made in Florence (Italy) (Pesavento *et al.* 2005) from raw meat, which included poultry, pork and beef. All their *Staph. aureus* isolates were susceptible to vancomycin with only one (10%) isolate from pork displaying resistance to cephalothin. It was found that *Staph. aureus* strains in this study presented higher levels of resistance to ampicillin (92.86%) and penicillin (92.86%) compared to isolates found in a pigeon slaughterhouse in Italy (15.2%) (Losito *et al.* 2005) and isolates originating from cow foremilk in Botswana (Guta *et al.* 2002).

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The highest resistance rate (100%) was seen for the antibiotic teicoplanin (30µg). This result is in total disagreement with results found in another study where all isolates from poultry, pork and beef were susceptible to teicoplanin (Pesavento *et al.* 2005). Of the *Staph. aureus* isolates 14.29% was resistant to oxacillin, tetracycline and trimethoprim-sulfamethoxazole. Resistance rate for oxacillin in this study was found to be lower than rates found in retail (50%) and freshly slaughtered (64.2%) beef studied in South Africa (Manie *et al*, 1999).

Clindamycine, gentamicin and methicillin displayed a higher percentage of resistant isolates (21.43%). Half of the isolates exhibited resistance to erythromycin and similar results (57.3%) for erythromycin were seen for *Staph. aureus* strains isolated from milk and meat in a study done in Nairobi (Kenya) (Ombui *et al*. 2000).

Looking at the resistance pattern of the isolates, it can be seen that all isolates were multiresistant to more than three antimicrobial substances tested. One isolate exhibited multiresistance to as much as seven antibiotics tested. Methicillin-resistant *Staph. aureus* strains have always shown multiresistance in studies (Pesavento *et al*. 2005). This is found to be true in this study where isolates resistant to methicillin displayed resistance to more than four antibiotics.

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Table 5.1 Results of the susceptibility testing of *Staphylococcus aureus* isolates from fresh and vacuum- packed ostrich meat.

Sample	Antibiotics											
	AMP	**KF**	**DA**	**E**	**CN**	**OX**	**P**	**TEC**	**TE**	**SXT**	**M**	**V**
Staph. aureus 0527-1	**R**	S	S	S	S	S	**R**	**R**	S	S	S	S
Staph. aureus 0527-2	**R**	S	S	S	S	**R**	**R**	**R**	**R**	S	S	S
Staph. aureus 0527-3	**R**	S	S	S	S	**R**	**R**	**R**	S	S	S	S
Staph. aureus 0544-3	**R**	S	S	**R**	S	S	**R**	**R**	**R**	S	S	S
Staph. aureus 0544-1	**R**	S	S	**R**	**R**	S	**R**	**R**	S	**R**	**R**	S
Staph. aureus 0549-3	**R**	S	S	S	S	S	S	**R**	S	**R**	S	S
Staph. aureus 0593-3	S	S	S	S	**R**	S	**R**	**R**	S	S	S	S
Staph. aureus 0610-2	**R**	S	S	**R**	S	S	**R**	**R**	S	S	S	S
Staph. aureus 0610-3	**R**	S	S	**R**	S	S	**R**	**R**	S	S	R	S
Staph. aureus 0612-1	**R**	S	**R**	**R**	S	S	**R**	**R**	S	S	S	S
Staph. aureus 0612-3	**R**	S	S	S	S	S	**R**	**R**	S	S	R	S
Staph. aureus 0615-3	**R**	S	S	S	S	S	**R**	**R**	S	S	S	S
Staph. aureus 0735-2	**R**	S	**R**	**R**	S	S	**R**	**R**	S	S	S	S
Staph. aureus 0747-4	**R**	S	**R**	**R**	**R**	S	**R**	**R**	S	S	S	S
% Resistance	**92.86**	**0**	**21.43**	**50**	**21.43**	**14.29**	**92.86**	**100**	**14.29**	**14.29**	**21.43**	**0**

R- Resistant

S- Susceptible

Antibiotics- Ampicillin (AMP); Cephalothin (KF); Clindamycine (DA); Erythromycin (E); Gentamicin (CN); Methicillin (M); Oxacillin (OX); Penicillin G (P); Teicoplanin (TEC); Tetracycline (TE); Trimethoprim-sulfamethoxazole (SXT); vancomycin (V).

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5.5 DISCUSSION

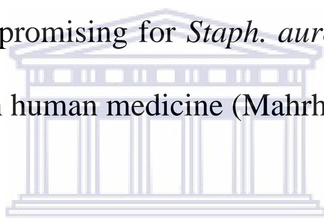
Meat serves as an important vector for the transfer of antimicrobial resistances from food animal to humans, as these animals may become colonized with resistant microorganisms, which eventually can reach humans through the food chain (Shea 2004). The exposure of feeding stuffs and drinkin water dosed with antimicrobial agents to food-producing animals, which allows for the treatment of the whole animal herd or flock, has led to an increase in the frequency of antibiotic-resistant microorganisms (Bowers and Daeschel 1999).

The occurrence of cephalothin resistance among the *Staph. aureus* from raw ostrich meat was low even though it's a commonly used antibiotic in poultry feed and is widely used in cattle (Maripandi and Al-Salamah 2010; Anderson *et al*. 2003). None of the *Staph. aureus* strains were resistant to vancomycin, which was unexpected especially since avoparcin is used exclusively as a feed additive for growth promotion and has been linked to the appearance of vancomycin-resistant enterococci and the potential for transmission of this resistance to *Staph. aureus* (Pesavento *et al*. 2007; van de Kerk-van Hoof and Heck 1999; Wegener *et al.* 1999). Vancomycin is the only drug available in hospitals to control methicillin-resistant *Staph. aureus* as it interferes by binding to the D-Ala-D-Ala terminus of peptidoglycan in bacteria, subsequently disrupting the synthesis of the cell wall by preventing the cross-linking of peptidoglycan (Mckeegan *et al.* 2002; Manie *et al.* 1999).

Higher incidence of resistance to ampicillin (92.86%), penicillin (92.86%) and teicoplanin (100%) by ostrich meat isolates may be attributed to nontherapeutic doses

being used over prolonged periods on farms that select for resistant strains or by coselection imposed by other currently approved antimicrobials, through genetic linkage between their resistance genes (Harada and Asai 2010). The antimicrobial resistance pattern of pathogenic food isolates reflects the animal treatment with antimicrobial substances (Mahrhofer *et al.* 2004).

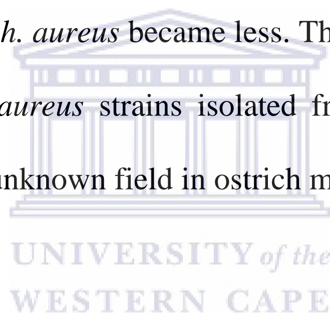
Tetracycline is active against both Gram-negative and Gram-positive bacteria and is the most common antibiotic used in animal feed (Manie *et al*. 1999). It interferes by binding to the subunit of the bacterial ribosome subsequently inhibiting protein synthesis (Tenover 2006). The incidence of tetracycline is low considering its usages in animal feed. This result is promising for *Staph. aureus* illnesses since tetracycline is the drug of second choice in human medicine (Mahrhofer *et al*. 2004).



Methicillin, a semi-synthetic penicillin, was introduced in the 1960's. Within a few years of use, outbreaks involving MRSA strains occurred (Bowers and Daeschel 1999). In this study, 21 % of the *Staph. aureus* isolates were found to show resistance to methicillin; one methicillin resistant isolate in particular showed significant multiresistance to ampicillin, erythromycin, gentamicin, penicillin, teicoplanin and trimethoprim-sulfamethoxazole. Isolates resistant to two or more antibiotics are assumed to have originated from high-risk sources of contamination like commercial farms, where antibiotics are frequently used (Gouws and Brözel 2000). Human illness caused by these multiresistant *Staph. aureus* strains might not be treatable by any commonly used antibiotics. Results in this study have demonstrated a high incidence (100%) of multiresistant *Staph. aureus* in ostrich meat, with risks of infections and possibility of transmission of resistances to other bacteria. These multiresistant *Staph.*

aureus strains may thrive when animals being fed feedstuffs supplemented with antibiotics over a prolonged period, suppresses competing bacteria from normal flora. This poses a risk, since the resulting reservoir of infectious agent can persist and transfer to other animals and eventually in to the food supply.

Selective pressure exerted by widespread antimicrobial use is the driving force in the development of antibiotic resistance. Inappropriate use of antibiotics is suspected to be a major contributory factor in the relatively high level of resistance to antimicrobial agents observed in this study. Resistant strains were isolated from every stage in succession of the ostrich processing operation and there was no stage at which resistant strains of *Staph. aureus* became less. This work provides the antibiotic resistance pattern of *Staph. aureus* strains isolated from two ostrich abattoirs and represents a study on a quite unknown field in ostrich meat production.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5.6 CONCLUSION

Staphylococcus aureus isolated from ostrich meat were multiresistant and mostly resistant to ampicillin, penicillin and teicoplanin. None of the *Staph. aureus* isolates were resistant to vancomycin. Antimicrobial resistance has become a growing area of concern in both human and veterinary medicine. The present findings demonstrates that antimicrobial resistant *Staph. aureus* isolated from ostrich meat may pose a serious problem and should be addressed in terms of their potentially negative effect on the safety of the food supply and on consumer health. Lack of information concerning antimicrobial usage in ostriches warrants continued research. If extensive usage of a spectrum of antibiotics has created an increase in the number of antibiotic-resistant foodborne pathogens then, reduction or elimination of usage of antibiotics in animal husbandry practices should result in decreased numbers of antimicrobial resistant microorganisms. However, temporarily eliminating the usage of antibiotics is not a solution since resistant microorganisms won't disappear from the gene pool. It has become imperative to understand in detail the mechanisms of and routes to resistance in pathogenic bacteria in order to make modifications in clinical behaviour in ways that decreases the growth of future resistant bacteria. The efficiency of antibiotics presently available for the control of bacterial infections may be preserved and selective pressure minimized by discretion of prescription and administration of antimicrobial drugs, supported by accurate diagnosis and subsequently cautious selection of the respective agent(s).

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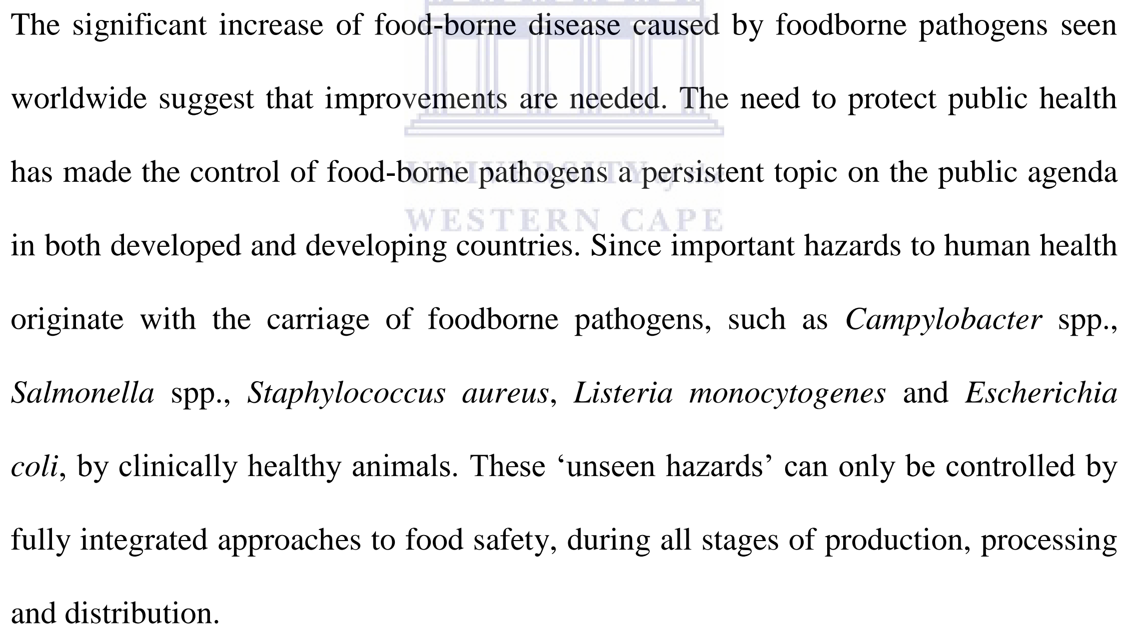
5.7 ACKNOWLEDGEMENT

This work was funded by the National Research Foundation (NRF). Sincere gratitude is extended to the members of the Klein Karoo Laboratory for their support.

CHAPTER 6

6.1 CONCLUSION

Ostrich farming in South Africa is shifting to meat, as a primary source of income it is therefore essential for the collection of information regarding the microbial quality characteristics of ostrich meat. As the ostrich has only recently started to be used as a meat animal, the prevalence of microorganisms on ostrich meat has not yet been investigated intensively.



The significant increase of food-borne disease caused by foodborne pathogens seen worldwide suggest that improvements are needed. The need to protect public health has made the control of food-borne pathogens a persistent topic on the public agenda in both developed and developing countries. Since important hazards to human health originate with the carriage of foodborne pathogens, such as *Campylobacter* spp., *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*, by clinically healthy animals. These 'unseen hazards' can only be controlled by fully integrated approaches to food safety, during all stages of production, processing and distribution.

The most important factors to direct actions in improving the microbial food safety situation are to determine the degree of the problem, the nature of the problem and the potential for change. It is therefore necessary for the ostrich industry to assess the risks of foodborne pathogens in ostrich meat, and to introduce appropriate risk

elimination, or if not possible risk reduction strategies. Before these strategies can be introduced, it is important to identify the factors and practices contributing to the spread of foodborne pathogens from the farm to the slaughtering of the animal, so that interventions can be targeted appropriately.

The microbiological condition of ostrich meat is a reflection of the microbial load associated with the live bird and the care taken to control the spread of microorganisms during the slaughter process. Current slaughtering processes allow for the contamination of carcasses and for cross-contamination between infected and uninfected carcasses, and thus acts as an amplifier for the incidence of foodborne pathogens. The lack of scientific information on the microbiological status of ostrich meat worldwide makes this the first in-depth scientific study done on the microbiological quality and safety of fresh ostrich meat by means of both conventional and molecular techniques. Data obtained from this study provides some baseline information on the quality of ostrich meat produced in South African export approved abattoirs and could be used in future studies on ostrich meat.

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In this study the total viable counts observed were generally found to be lower than counts of other studies. Log_{10} mean counts increased between pre-chill and pre-packaging stages. This is expected with the succession stages of the ostrich slaughtering process, since the more the meat is processed the greater the potential for contamination. The counts of vacuum-packed ostrich meat showed a significantly decrease from counts observed in pre-packaging samples. This highlights the importance of vacuum packaging of ostrich meat to decrease bacterial loads and prologue shelf life. *Pseudomonas* spp. was detected on ostrich meat samples and was

found to have increased in succession with the stages of the slaughter processing chain. This result is an indicator that contamination and temperature abuse in the processing chain significantly affects the quality of ostrich meat. The prevalence of samples positive for coliforms and *E. coli* was observed to be much higher than that of any other microorganism tested. This result was an indication of faecal contamination and therefore implies the significant role that cross-contamination between clean and contaminated flesh, and/ or faecal spillage when intestines and cloacae are removed plays on the final quality of ostrich meat. *Staphylococcus aureus* was found to be present in some of the ostrich meat samples and was observed to have increased in succession with the stages of the slaughter processing chain. The presence of *Staph. aureus* in the samples generally indicates contamination from human handling and/or inadequate cleaning and disinfections of processing equipment. The low prevalence of samples positive for *Salmonella* spp. and the absence of *L. monocytogenes* and *Campylobacter* spp. in this study indicate that current in-plant process practices appear not to increase nor reduce the level of ostrich meat contamination with these foodborne pathogens. However some new interventions should be developed in order to ensure the overall safety of ostrich meat, especially for the faecal contamination of the meat.

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The antibiotic resistant pattern of *Staph. aureus* positive isolated was determined. This study provides evidence that transmission of resistant bacteria or resistant genes via direct contact with food animals does in fact occur. Findings demonstrates that antimicrobial resistant *Staph. aureus* isolated from ostrich meat pose a serious problem and should be addressed in terms of their potentially negative effect on the safety of the food supply and on consumer health. Antimicrobials used in the

treatment of humans should be avoided in animal husbandry practices to reduce the resistance of foodborne pathogens ensuring the health of the public.

In general focusing on good hygiene throughout production and preparation stages and the efficient application of physical (cold-chain) principles to decrease or prevent growth of pathogenic microorganisms may contribute significantly to food safety. The aim of foodborne pathogen control is to reduce the incidence of human disease. This can be achieved by elimination of the pathogen at the most appropriate stage in the food chain. Where this is not feasible incremental risk reduction at all stages of the food chain is the approach to adopt. Implementation of Good Manufacturing Practices, which are based on proper Critical Control Point analyses, will, however, at best maintain the prevalence of contaminated ostrich meat.

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More data of the microbial quality and safety of ostrich meat are needed for comparison. Samples of ostrich meat at various processing points in the abattoir need to be evaluated for the prevalence of existing foodborne pathogens and quantification of these microorganisms would present useful information on system contamination and the extent of downstream processing steps. The use of antibiotics in ostrich needs to be monitored and alternative methods other than the use of antibiotics found. Ostrich meat produced in abattoirs across South Africa need to be studied in order to get a proper overview of the microbial quality and safety of ostrich meat.

CHAPTER 7

7.1 REFERENCES

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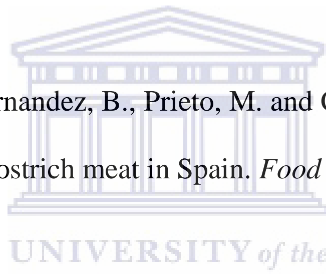
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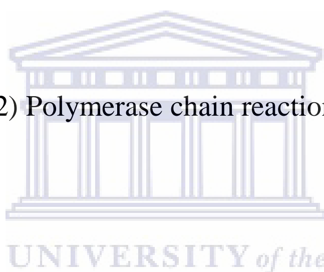

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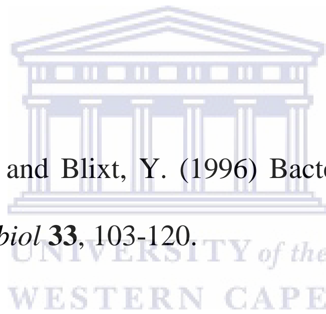
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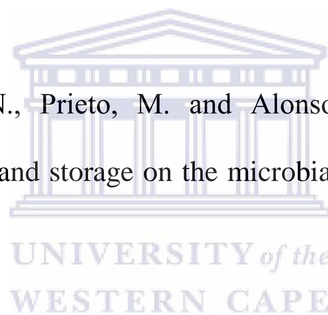
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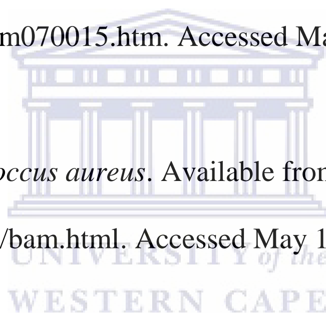
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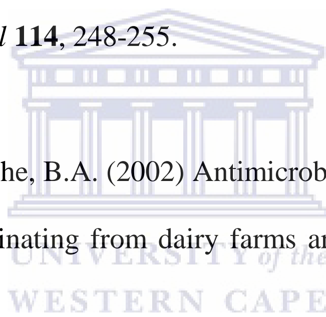
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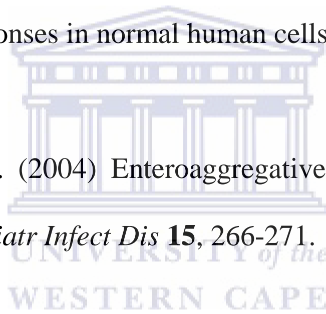

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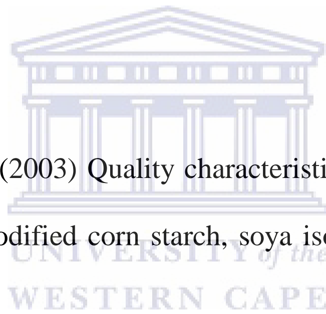
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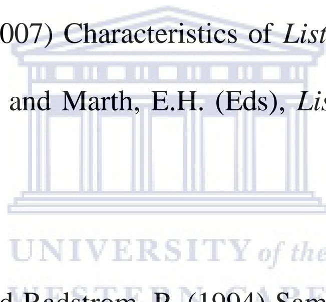
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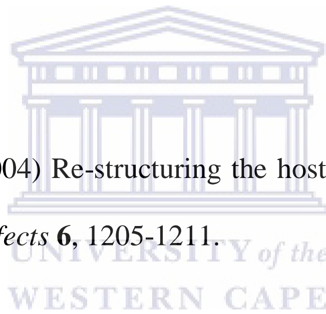
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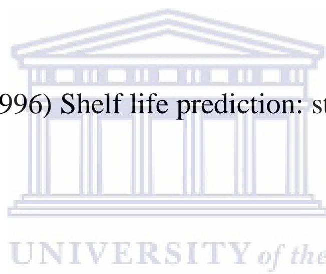
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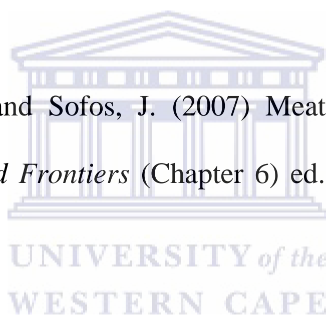

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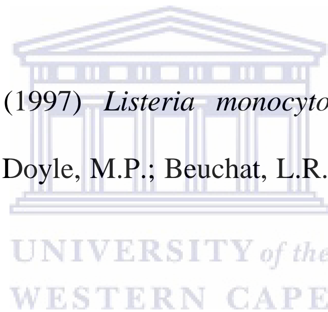
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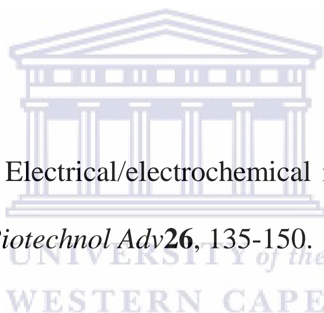
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