

Molecular characterization of *Campylobacter* isolates from free range and commercial chicken in South Africa

by

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DECLARATION

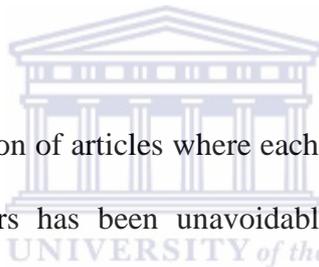
I, the undersigned, declare that '**Molecular characterization of *Campylobacter* isolates from free range and commercial chicken in South Africa**' is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

Miss Laeeqa Basardien

2612961

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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Molecular characterization of *Campylobacter* isolates from free range and commercial chicken in South Africa

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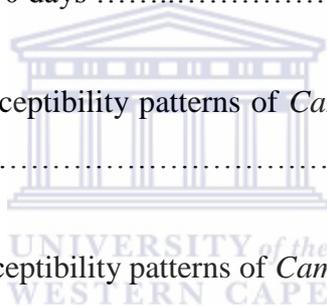
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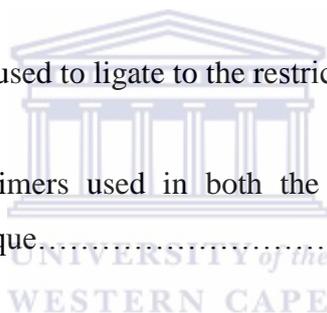
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LIST OF ABBREVIATIONS

- AFLP Amplified Fragment Length Polymorphism
- AGPs Antibiotic Growth Promoters
- ATCC American Type Culture Collection
- bp Base pairs
- BSAC British Society for Antimicrobial Chemotherapy
- CBA Columbia blood agar
- CFU.g⁻¹ Colony forming units per gram
- CFU.ml⁻¹ Colony forming units per millilitre
- CIP Collection de l'Institut Pasteur
- CLSI Clinical and Laboratory Standards Institute
- DNA Deoxyribonucleic acid
- dNTPs Deoxynucleotide triphosphates
- EDTA Ethylenediaminetetraacetic acid
- EFSA European Food Safety Authority
- *et al.* and others
- 6-FAM Carboxyfluorescein fluorescent dye
- G+C Guanine plus Cytosine
- g Gram
- GBS Guillain-Barré Syndrome
- h Hour
- HIV Human Immunodeficiency Virus
- HUS Haemolytic Uremic Syndrome
- I Intermediate resistance
- IBS Irritable Bowel Syndrome

- i.e. That is
- IgG Immunoglobulin G
- ISO International Organization for Standardization
- L-ALA L-aminopeptidase
- ml Milliliter
- min Minute
- mM Millimolar
- MAP Modified atmosphere packaging
- mCCDA modified charcoal cefoperazone deoxycholate agar
- MFS Miller Fisher Syndrome
- MHA Mueller Hinton Agar
- MLST Multilocus Sequence Type
- NHLS National Health Laboratory Services
- pH Hydrogen potential
- PCR Polymerase Chain Reaction
- PFGE Pulsed-Field Gel Electrophoresis
- R Resistance
- RCWMCH Red Cross War Memorial Children's Hospital
- ReA Reactive arthritis
- rRNA ribosomal ribonucleic acid
- ROX Carboxy-X-rhodamine dye
- RTE Ready-to-eat
- spp. Species
- S Susceptible
- TBA Tryptose blood agar
- TSA Tryptone soya agar



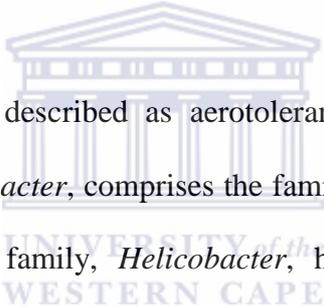
- TSB Tryptone soya broth
- μ Micro
- μl microlitre
- μm micrometer
- UPGMA unweighted Pair Group Method with Arithmetic Mean
- $^{\circ}\text{C}$ Degrees Celsius
- \leq Less than or equal to
- \geq More than or equal to



Molecular characterisation of *Campylobacter* isolates from free range and commercial chicken in South Africa

Chapter 1 – Introduction

Campylobacter species are fastidious Gram negative, microaerophilic organisms that belong to the class Epsilonproteobacteria. They are mostly spirally curved or S-shaped bacteria that transient to a coccoid form when stressed. All species besides *Campylobacter gracilis* are motile and can be identified by its characteristic corkscrew-like motion which provides the cell with an extremely rapid and spinning rotation. This movement enables the bacteria to move through and colonize the viscous mucous layer of the intestinal tract of humans and animals. Campylobacters cannot ferment carbohydrates and obtain their energy from amino acids or tricarboxylic acid cycle intermediates.

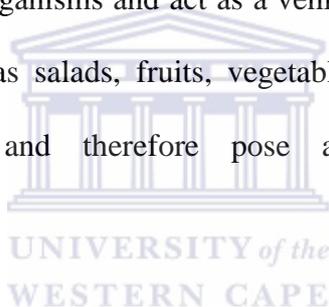


The genus *Arcobacter*, initially described as aerotolerant campylobacters, together with organisms of the genus *Campylobacter*, comprises the family *Campylobacteraceae* while the closest related bacteria to this family, *Helicobacter*, have been placed in the family *Helicobacteraceae*. The true number of species belonging to the genus *Campylobacter* remains debatable but a survey of the literature indicates that there are 22 species and eight subspecies to date.

Campylobacter species are ubiquitous in the environment and can be found in various water bodies, treated and untreated drinking water as well as sewage water and groundwater. Groundwater is often used to irrigate crops and as a result, campylobacters have been isolated from fresh produce such as leafy vegetables, cucumber, carrots, strawberries and mushrooms. They are commensals in non-mammalian species such as birds and shellfish as well as warm-

blooded animals. However, *C. fetus* is notoriously known as the causative agent of spontaneous abortion in swine, sheep and cattle.

Chicken, however, is considered as the main reservoir of *Campylobacter* and because the resulting illness, campylobacteriosis is transmitted from animals to humans, it is considered to be a zoonotic illness. Chicken are consumed on a large scale globally and especially in developing countries as it is a cheaper alternative to other meats while still maintaining the provision of protein, essential vitamins and minerals. It has a distinct taste and does not require a lot of time for its preparation. Campylobacteriosis cases occur sporadically while outbreaks are often reported in developed countries. The majority of sporadic cases are determined to be of raw chicken origin since chicken are often mishandled in the domestic kitchen during the preparation of food. Food preparation surfaces and cutlery such as knives become contaminated with the organisms and act as a vehicle for the cross contamination of ready-to-eat (RTE) foods such as salads, fruits, vegetables and cold meats. These foods require no further cooking and therefore pose a serious risk in contracting campylobacteriosis.



It is therefore of utmost importance to determine the prevalence level of *Campylobacter* species in South African free range and commercial chicken. This was done by using two isolation protocols; the Cape Town Protocol and the ISO10272-1:2006 method. The two methods were compared to determine the efficiency in the isolation of *Campylobacter* species from raw chicken. The isolates obtained were confirmed to belong to the genus *Campylobacter* by use of the Polymerase Chain Reaction (PCR) and the characterization of species was done by the use of biochemical tests specific to campylobacters. The results obtained will provide an indication of whether the South African population are at risk of acquiring campylobacteriosis from the consumption of raw or undercooked chicken and other contaminated food products. It is also important to determine the period of survival of

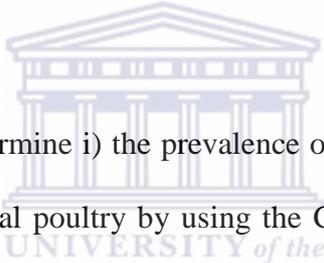
Campylobacter species strains in chicken in order to educate the public about the survival mechanisms of the organism and how the chicken and all working areas and cutlery should be handled during the preparation of food. Molecular DNA fingerprinting methods such as the Amplified Fragment Length Polymorphism (AFLP) technique are often used to determine the epidemiology of *Campylobacter* species in an outbreak.

AFLP is a PCR-based technique where the target organisms' genomic DNA is fragmented with restriction endonucleases, ligated to adaptors and exponentially amplified under stringent PCR conditions. The products are then separated by denaturing gradient gel electrophoresis using an automated DNA sequencer and the data is collected using specialized software. Dendrograms are constructed and closely related organisms are clustered together while distantly related organisms are clustered separately. The technique was applied to a selection of 22 *Campylobacter* isolates from chicken in order to determine the relatedness between the bacteria. It was also used to further confirm the subtypes of the *Campylobacter* strains obtained in the study. This is important so that the source of *Campylobacter* infections in the poultry industry are identified and control measures to reduce or completely prevent *Campylobacter* colonization of flocks can be developed.

Campylobacteriosis has a relatively low infectious dose as it is reported that 100 – 500 cells are capable of causing the illness. Clinical features of the disease include abdominal cramps, fever and diarrhoea which often become bloody after one or two days. Abdominal pain may become so intense that it mimics acute appendicitis. Young children, the elderly and immunocompromised individuals are most at risk of acquiring campylobacteriosis and in severe cases, serious secondary or post infection complications such as septicaemia, hepatitis, pancreatitis, meningitis, endocarditis as well as the neuromuscular, paralytic disease, Guillain-Barré Syndrome may arise. The illness is usually self-limiting but in the severe cases described, treatment with antibiotics is essential.

Antibiotics are used in the therapeutic treatment of human and animal infections and are also used sub-therapeutically as Antibiotic Growth Promoters (AGPs) to enhance the growth and performance of food animals. However, this sub-therapeutic use of AGPs contributes to the bacteria acquiring resistance to the antibiotics used. In recent years, it has been well documented that *Campylobacter* species have increasingly become resistant to fluoroquinolones such as ciprofloxacin and macrolides, such as erythromycin; the two antibiotics primarily used to treat patients with campylobacteriosis.

AGPs are still used in the South African commercial farming practises. The antibiotic resistance patterns of the *Campylobacter* isolates from South African chicken was determined. This is of importance so that the correct antibiotic treatment can be administered when patients with severe cases of campylobacteriosis or secondary and post infection complications are presented.



The aim of this study was to determine i) the prevalence of *Campylobacter* species in South African free range and commercial poultry by using the Cape Town and ISO10272-1:2006 protocols, ii) the period that the *Campylobacter* isolates obtained are able to survive in refrigerated and frozen chicken, iii) the antibiotic resistance patterns of the isolates to five antibiotics commonly used in the treatment of campylobacteriosis cases and iv) the genetic relatedness of the isolates using the AFLP technique.

Chapter 2 – Literature Review

2.1 INTRODUCTION

Campylobacter species belongs to the class Epsilonproteobacteria (ϵ -proteobacteria) which has one order, *Campylobacterales* and two families, *Campylobacteraceae* and *Helicobacteraceae* (Wassenaar and Newell 2007; Alonso *et al.* 2011). These microorganisms (Figure 2.1) are non-sporulating Gram-negative slender rods (Smibert 1984; Penner 1988; Vandamme 2000) that are mostly spirally curved or S-shaped (Penner 1988; Vandamme 2000) and are typically 0.2 μm to 0.8 μm in width and 0.5 μm to 5.0 μm in length (Horrocks *et al.* 2009; Alonso *et al.* 2011). Some species may be straight rods (Penner 1988; Vandamme 2000) while older or stressed cultures are able to transient to a coccoid form with a typical diameter of 1 μm (Humphrey *et al.* 2007; Alonso *et al.* 2011). They are motile by means of a polar flagellum at one or both ends and have a characteristic corkscrew-like motion (Penner 1988; Gorkiewicz *et al.* 2002; Alonso *et al.* 2011) which provides the cell with an extremely rapid and spinning rotation (Alonso *et al.* 2011) and therefore being able to move through very viscous media and colonize and pass through the mucous layer of the intestinal tract of humans and animals (Black *et al.* 1988; Alonso *et al.* 2011; Silva *et al.* 2011). Some species such as *C. gracilis* are non-motile while *C. showae* cells have multiple flagella. They are microaerophilic microorganisms that are not able to ferment carbohydrates (nonsaccharolytic) (Penner 1988; Vandamme 2000; Alonso *et al.* 2011; Silva *et al.* 2011) or degrade complex substances but obtain energy from amino acids or tricarboxylic acid cycle intermediates (Alonso *et al.* 2011; Silva *et al.* 2011).

Campylobacter organisms, specifically *C. fetus*, were originally isolated from aborted sheep fetuses in 1909 and identified as the causative agent of abortion in sheep and cattle by McFadyean and Stockman (1913) (Penner 1988; Wassenaar and Newell 2007; Ruiz-Palacios 2007; Blaser *et al.* 2008). Later, in 1919, it was cultured from aborted bovine foetal fluids by

Theobald Smith and classified as *Vibrio fetus* because of the similar morphology it shared with *V. cholera* (Smith and Taylor 1919; Allos and Lastovica 2011; Silva *et al.* 2011).

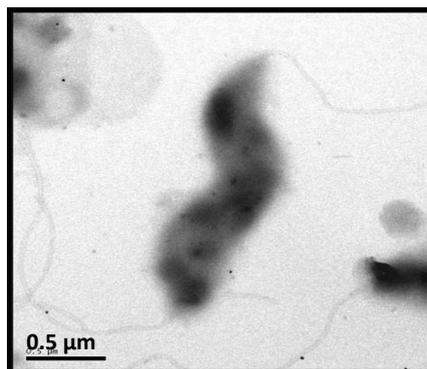


Fig. 2.1 Immunogold electron micrograph of *Campylobacter* spp (Bar = 0.5 μm; adapted from Qian *et al.* 2008)

Knowledge obtained from additional studies that were done on the microorganism suggested that *V. fetus* was different from other members of the genus *Vibrio*, as it was unable to ferment sugars and the genomic percentage of guanosine plus cytosine (G + C) content of *V. fetus* (32% to 35% G+C) was not typical of other members of its genus that had a genomic percentage G + C content of 47%. It was suggested by Sebald and Véron in 1963 that *V. fetus* is not a member of the genus *Vibrio* and the novel genus *Campylobacter*, the Greek word for curved rod, was proposed (Sebald and Véron 1963; Penner 1988; Wassenaar and Newell 2007; Blaser *et al.* 2008; Allos and Lastovica 2011; Silva *et al.* 2011).

The taxonomic structure of the genus *Campylobacter* remains a controversial matter as the true number of species belonging to the genus remains debatable with many authors citing that the genus comprises of 16 species, (On 2001; Foster *et al.* 2004; Alonso *et al.* 2011). Lastovica (2006) stated that the genus is comprised of 17 species while Fernández *et al.* (2008) stated that there are 20 species belonging to the genus. According to Debruyne *et al.* (2010), there are 21 recognised species, of which five were recently described. Furthermore eight subspecies of *Campylobacter* have been described (Debruyne *et al.* 2010). There are at

least 14 species of *Campylobacter* that have been associated with human illness (Lastovica 2006). Although this controversial matter of the taxonomic structure of the genus requires further investigation, a review of the literature shows that there are 22 recognised species of *Campylobacter* to date (Table 2.1).

Table 2.1 The 22 recognised species and eight subspecies of *Campylobacter* as compiled from a review of the literature

The species of the genus <i>Campylobacter</i>		The subspecies of the <i>Campylobacter</i> genus
<i>C. avium</i> *	<i>C. jejuni</i>	<i>C. fetus</i> subsp. <i>fetus</i> †
<i>C. canadensis</i>	<i>C. lanienae</i>	<i>C. fetus</i> subsp. <i>venerealis</i>
<i>C. coli</i>	<i>C. lari</i>	<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>
<i>C. concisus</i>	<i>C. mucosalis</i>	<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>
<i>C. cuniculorum</i> *	<i>C. peloridis</i> *	<i>C. jejuni</i> subsp. <i>doylei</i>
<i>C. curvus</i>	<i>C. rectus</i>	<i>C. jejuni</i> subsp. <i>jejuni</i> biotype 1
<i>C. fetus</i>	<i>C. showae</i>	<i>C. jejuni</i> subsp. <i>jejuni</i> biotype 2
<i>C. gracilis</i>	<i>C. sputorum</i>	<i>C. lari</i> subsp. <i>concheus</i> ‡
<i>C. hominis</i>	<i>C. subartanticus</i> *	<i>C. lari</i> subsp. <i>lari</i> ‡
<i>C. hyointestinalis</i>	<i>C. upsaliensis</i>	<i>C. sputorum</i> bv. <i>sputorum</i> §
<i>C. insulaenigrae</i>	<i>C. volucris</i> *	<i>C. sputorum</i> bv. <i>paraureolyticus</i> §

* Recently described species † Type species
‡ Recently described subspecies § Biovar – not a subspecies!

A phylogenetic tree (Figure 2.2) was constructed on the basis of sequence similarity of the 16S rRNA gene, as well as similar genotypic and phenotypic features and shows that *Campylobacter* and *Arcobacter*, first described as aerotolerant *Campylobacter* (Neill *et al.*

1978; Neill *et al.* 1979; Houf 2011), are close phylogenetic neighbours and belong to the family *Campylobacteraceae* (Vandamme 1991; Ho *et al.* 2006). The generically misclassified species *Bacteroides ureolyticus* and strains that were originally classified as free-living campylobacters, *Sulfurospirillum* species, also belong to this family. A new family, *Helicobacteraceae*, has been created to accommodate species of the genera *Helicobacter* and *Wolinella* which are the closest genetically related bacteria to those of the family *Campylobacteraceae* (Vandamme 2000; Wassenaar and Newell 2007).

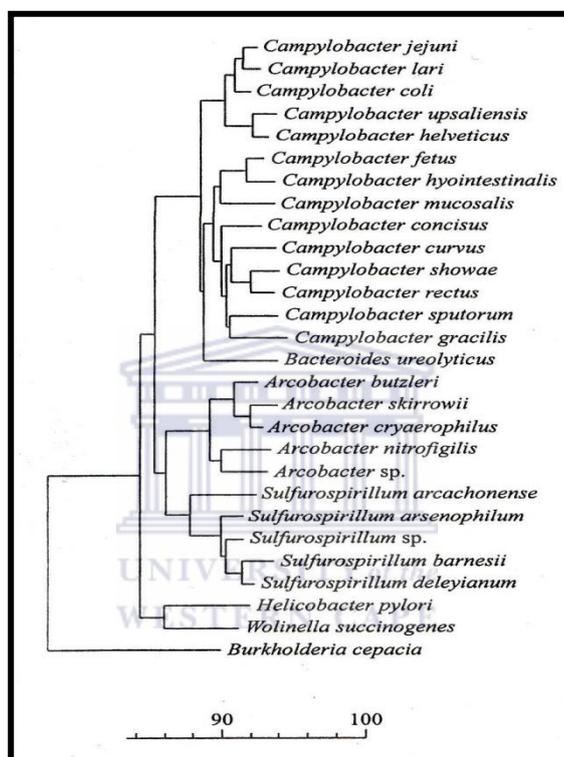


Fig. 2.2 The phylogenetic tree of the family *Campylobacteraceae* and its closest phylogenetic neighbours, members of the genera *Helicobacter* and *Wolinella*, based on percentage sequence similarity of the 16S rRNA gene. *Burkholderia cepacia* was used as an outgroup organism (Vandamme 2000)

2. 2 THE RESERVOIRS AND INFECTION ROUTES OF *CAMPYLOBACTER* SPECIES

Campylobacter is widely distributed in the environment and is considered to be amongst the normal intestinal biota of a wide range of domestic and wild animals (Alonso *et al.* 2011).

2.2.1 Environmental reservoirs harbouring *Campylobacter*

Water is thought to be one of the main transmission routes of campylobacteriosis as it is associated with all reservoirs of *Campylobacter* (Jones 2001). It has been isolated from untreated drinking water, treated drinking water (Jones 2001; Moore *et al.* 2001), recreational water such as swimming pools and a jacuzzi (Moore *et al.* 2001); water bodies such as lakes, rivers, lagoons, canals, ponds, estuaries, coastal waters, groundwater as well as sewages and farm run-offs (Stampi *et al.* 1999; Daczowska-Kozon and Brzostek-Nowakowska 2001; Jones 2001; Moore *et al.* 2001; Moreno *et al.* 2003). *Campylobacter* was also detected in very low numbers in surface and sewage waters in South Africa (Diergaardt *et al.* 2004).

Since *Campylobacter* species are commonly found in water sources, there is a substantial risk of acquiring a *Campylobacter* infection from fresh produce if contaminated groundwater is used to water crops. It is reported that *Campylobacter* is frequently associated with foodborne illnesses due to consumption of contaminated fresh produce (Olaimat and Holley 2012). Kärenlampi and Hänninen (2004) have shown that clinical and chicken *C. jejuni* strains are able to survive on lettuce, cantaloupe, cucumber, carrot and strawberries. The risk of *Campylobacter* associated with raw fruit and vegetables such as sprouts, leafy vegetables, fruit crops, cabbage, mushrooms, mixed salads, vegetables and root crops have also been reported (Chai *et al.* 2007; Verhoeff-Bakkenes *et al.* 2011).

Campylobacteriosis in humans are considered to be mainly a food-borne disease as foods of animal origin are the main source of infection and therefore the disease is considered to be zoonotic (Kemper *et al.* 2006; Hänel *et al.* 2009). *Campylobacter* spp. are commensals in non-mammalian species such as birds and shellfish as well as warm-blooded animals such as swine, sheep and cattle (Allos and Lastovica 2011) but is also associated with spontaneous abortions in the latter (Daczowska-Kozon and Brzostek-Nowakowska 2001; Zhao *et al.* 2010; Sippy *et al.* 2012).

2.2.2 Agricultural, domestic and wild animals as reservoirs of *Campylobacter*

2.2.2.1 Chicken as a reservoir of *Campylobacter*

Campylobacters are mainly associated with fresh and frozen chicken as it often colonizes live chickens in large numbers without the bird showing any clinical signs of illness (Jacobs-Reitsma 2000; Doyle and Erickson 2006; Allos and Lastovica 2011; Silva *et al.* 2011; Habib *et al.* 2012; Sippy *et al.* 2012). Chicken is consumed on a large-scale worldwide and especially so in developing countries such as South Africa (Figures 2.3 and 2.4). Naturally contaminated chicken includes the whole carcasses as well as breast, breast fillet, thigh, drumstick and wing portions (Sallam 2007; Suzuki and Yamamoto 2009; Hue *et al.* 2011).

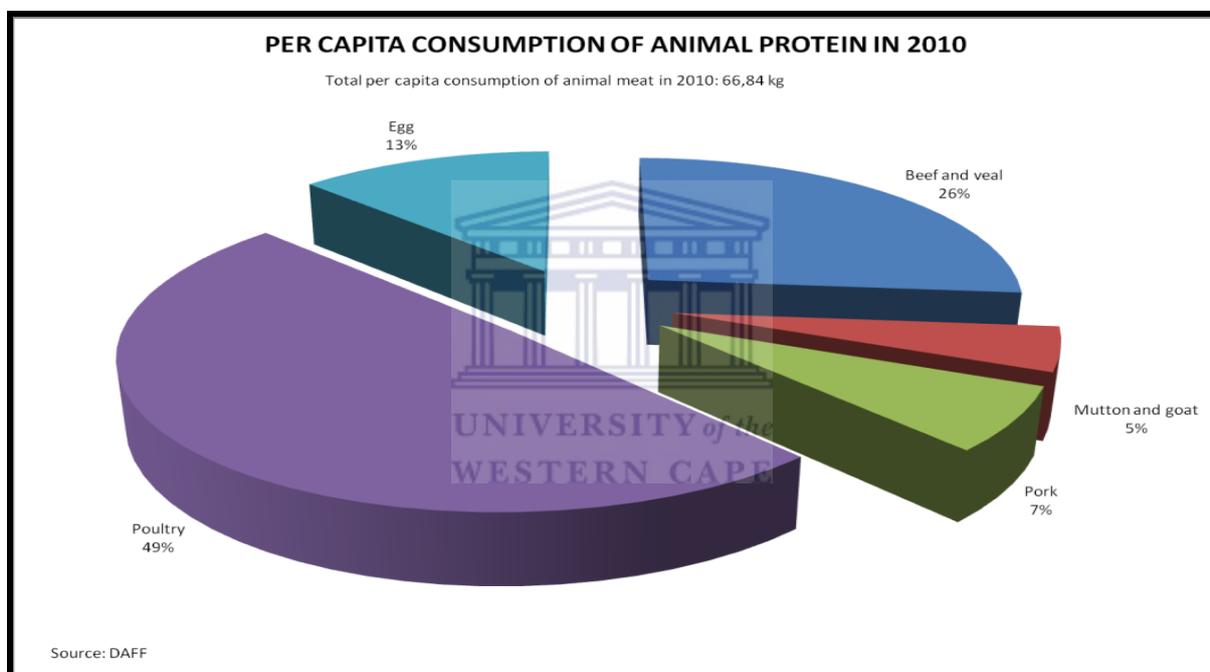


Fig. 2.3 A graph showing the consumption of different animal meat and meat products in South Africa in 2010 (adapted from Anon 2011)

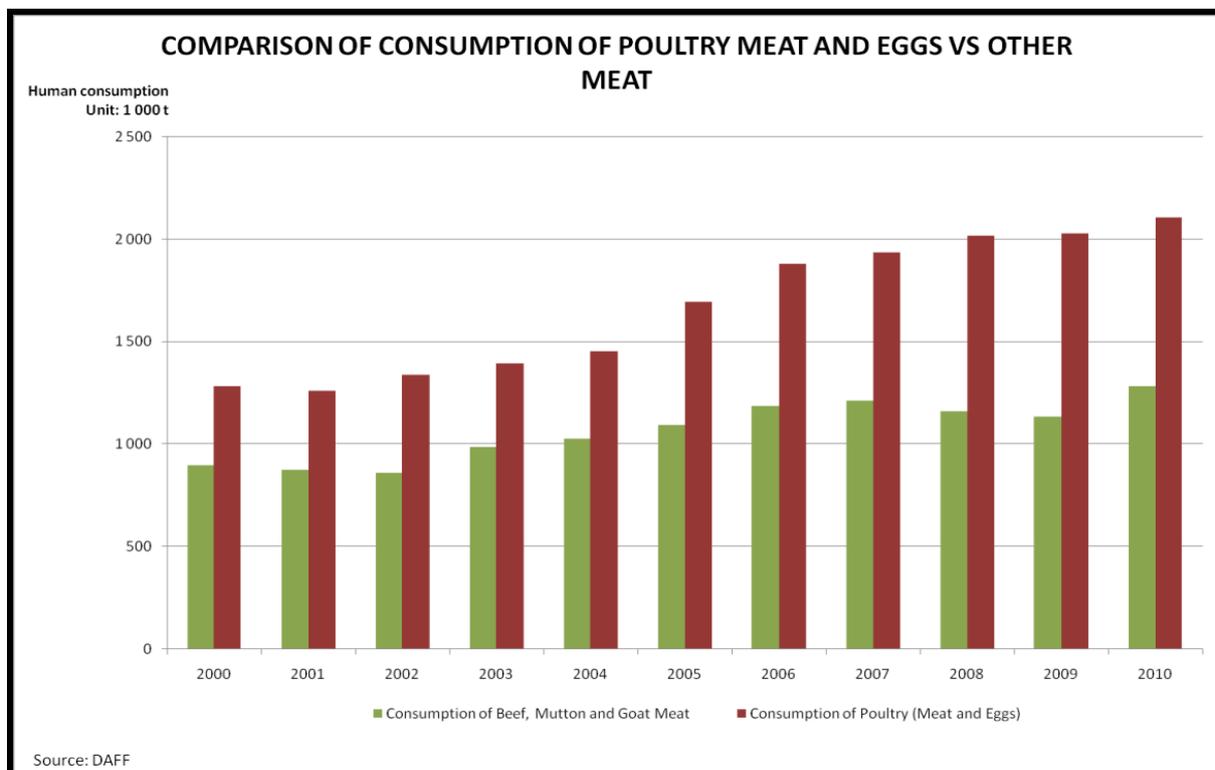
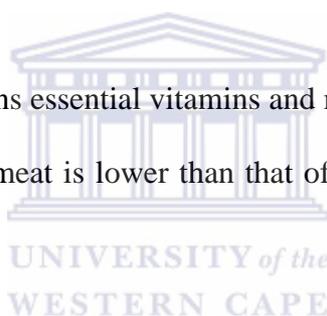


Fig. 2.4 Graph depicting the consumption of poultry in comparison to other meats in South Africa (adapted from Anon 2011)

Chicken is high in protein, contains essential vitamins and minerals (Sallam 2007; Silva *et al.* 2011) and the prices of chicken meat is lower than that of lamb, beef and pork (Figure 2.5) (Silva *et al.* 2011; Anon 2012).



Internal organs such as the large intestines, caeca and cloaca contain the highest *Campylobacter* load with levels as high as 10^5 to 10^9 CFU.g⁻¹ reported (Corry and Atabay 2001; Zweifel *et al.* 2008). During the defeathering and evisceration stages of the slaughter process, the internal organs are ripped out of the chicken carcass and it is at these stages of the slaughtering process that the chicken meat becomes contaminated with *Campylobacter* (Ono and Yamamoto 1999; Allen *et al.* 2007; Reich *et al.* 2008; Hansson *et al.* 2010). It is not uncommon to find households in South Africa, especially in rural and poor communities, rearing chicken in their back yards (subsistence farming) as it contributes significantly to their income (Mtileni *et al.* 2009). Chicken is high in protein, contains essential vitamins and

minerals (Sallam 2007; Silva *et al.* 2011) and the prices of chicken meat is lower than that of lamb, beef and pork (Figure 2.5) (Silva *et al.* 2011; Anon 2012). Chicken by-products are also widely consumed because of its low price, special taste and the short time needed for preparation (Sallam 2007; Silva *et al.* 2011). The by-products often found to be naturally contaminated with *Campylobacter* in prevalence studies in Japan include gizzards, liver and the heart (Sallam 2007; Suzuki and Yamamoto 2009).

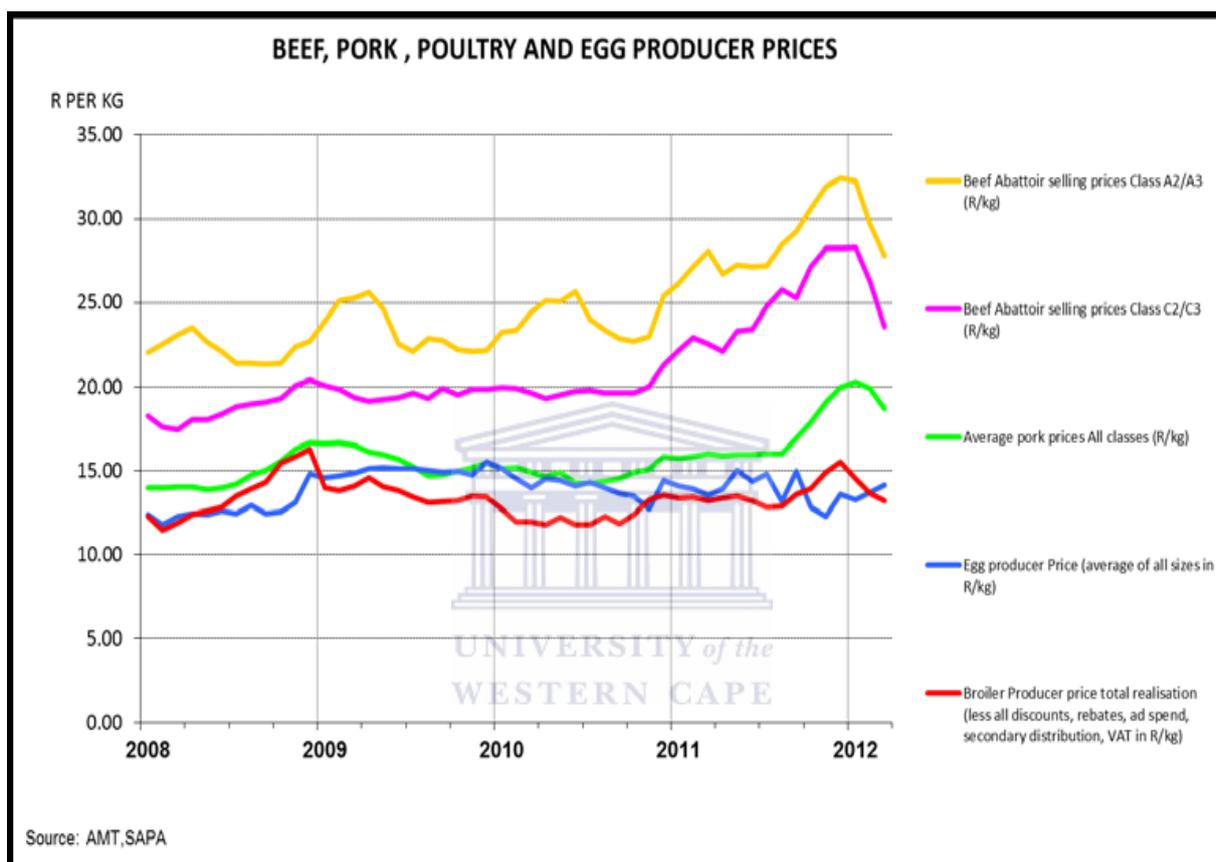


Fig. 2.5 The prices of broiler chicken per kg in comparison with beef and pork in South Africa (adapted from Anon 2012)

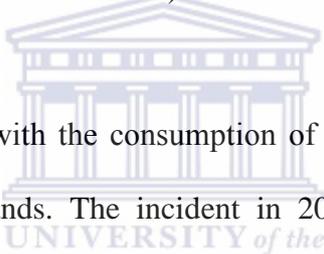
Therefore, the risk of acquiring campylobacteriosis from contaminated chicken greatly increases especially when handling raw chicken, eating undercooked chicken meat or the cross contamination of raw to cooked foods due to the variation in food preparation and preferences worldwide (Alonso *et al.* 2011).

2.2.2.2 The prevalence of *Campylobacter* in other agricultural animals

Poultry has long been considered as the main reservoir of *Campylobacter* species in the transmission of human *Campylobacter* – enteritis. However, cattle may also represent an important infectious source of a variety of *Campylobacter* species (Stanley and Jones 2003; Bae *et al.* 2007; Enokimoto *et al.* 2007). Cattle are usually asymptomatic carriers of campylobacters but it is also known that *Campylobacter* species are associated with septic abortion in these animals (Sippy *et al.* 2012). As is the case with poultry, campylobacters readily contaminate meat and meat products during the evisceration stage of the slaughtering process. Japanese consider raw beef liver and meat (sashimi) a delicacy and therefore this eating practise greatly increases the risk of acquiring campylobacteriosis (Enokimoto *et al.* 2007).

C. jejuni is the most common species associated with cattle (Horrocks *et al.* 2009) but the recovery of *C. coli*, *C. fetus* and *C. hyointestinalis* have also been reported (Enokimoto *et al.* 2007). *Campylobacter* species have previously been isolated from the intestinal contents (Saito *et al.* 2005), bile, liver (Saito *et al.* 2005; Enokimoto *et al.* 2007) as well as the carcasses of cattle and its meat products at the retail level (Gill and Harris 1982; Kotula and Stern 1984). An experiment by Enokimoto *et al.* (2007) has shown that campylobacters can survive in bile for a long period as the growth of *C. coli*, *C. fetus* and *C. jejuni* initially increased exponentially before reaching a plateau where no apparent increase in growth occurred for 28 days in spiked bile. No decline phase was observed. The same study also determined the transfer route of bacterial cells to the gallbladder by intravenously injecting the above-mentioned *Campylobacter* species in mice. The study showed that *Campylobacter* species are capable of being transferred from the bloodstream to the major organs including the bile, blood, liver, spleen, kidney and caeca as identical bacterial cells were recovered after three days post inoculation (Enokimoto *et al.* 2007).

An outbreak of campylobacteriosis due to consumption of contaminated milk occurred in August 1992 when local doctors in a town in Northamptonshire (United Kingdom - UK) reported the increase in the cases of patients with diarrhoea. After investigation by the Environmental Health Officers, it was found that the pasteurizing equipment of a local dairy had failed to reach the correct temperature required for pasteurization of the milk as all samples of milk and milk products had failed the phosphatase test (Fahey *et al.* 1995), which detects the presence of the thermally stable enzyme, phosphatase which is present in raw milk. The enzyme is deactivated when pasteurisation temperatures are reached (Burgwald 1939). The stool samples supplied by 41/53 patients were positive for *C. jejuni* and serology tests were positive for 16/41 patients. Twenty-four patients, who did not have their faeces cultured, had positive serology tests (Fahey *et al.* 1995). Another outbreak in the UK took place in 1979 where over 2500 school children became infected with *Campylobacter* after the ingestion of pasteurized milk (Jones *et al.* 1981).



Two more outbreaks associated with the consumption of raw cow's milk were reported in 2005 and 2007 in The Netherlands. The incident in 2005 occurred when 22/34 school children developed diarrhoea after a visit to a dairy farm. The consumption of raw milk during the visit could be directly linked to 86% of the cases. The outbreak in 2007 occurred when bulk tank raw milk was served and used to prepare dishes for a firm's lunch outing to a dairy farm. People (84%) who consumed the raw milk became sick and *C. jejuni* was cultured from stool specimens. The recovered *Campylobacter* species could directly be linked to the bulk tank raw milk (Heuvelink *et al.* 2009).

Campylobacter species are often isolated from the faeces of healthy and ill cows but are less efficiently isolated from milk (Fahey *et al.* 1995; Jacobs-Reitsma 2000) and this may lead to the incorrect tracing to the source of contamination. Human campylobacteriosis cases had

also previously been associated with bird-pecked milk (Moore and Rooney 2010), soft cheeses and yoghurts (Hussain *et al.* 2007; El-Sharoud 2009; Ogden *et al.* 2009).

The prevalence of *Campylobacter* in sheep is not as extensively documented as other agricultural animals (Horrocks *et al.* 2009). However, it is known that *C. fetus* subsp. *fetus* is the leading cause of spontaneous abortion in sheep (Erganis *et al.* 2002) with stillbirth and the birth of weak lambs caused by both *C. fetus* subsp. *fetus* and *C. jejuni* later in pregnancy. Campylobacteriosis is highly contagious and may cause up to 70% of ewes to abort their young (Raji *et al.* 2000). *Campylobacter* species have also been associated with the liver (Fenwick *et al.* 2000; Kramer *et al.* 2000), gallbladder (Raji *et al.* 2000; Aık and etinkaya 2006), intestinal contents (Raji *et al.* 2000; Zweifel *et al.* 2004; Aık and etinkaya 2006) and faeces (Aık and etinkaya 2006).

In comparison to poultry, cattle and sheep, the predominant *Campylobacter* species associated with swine is *C. coli* (Jensen *et al.* 2006; Horrocks *et al.* 2009; Bratz *et al.* 2012). The prevalence of *C. coli* in pigs range between 50% to 100% with excretion levels ranging between 10^2 to 10^7 CFU.g⁻¹ of faeces (Jensen *et al.* 2006; Maridor *et al.* 2008; Horrocks *et al.* 2009; Bratz *et al.* 2012). A study conducted in Limpopo, South Africa, showed that the prevalence levels of *Campylobacter* in pigs on three farms were 30.2% and that the majority of isolates, 87.5%, were *C. coli* (Uaboi-Egbenni *et al.* 2011). Gorkiewicz *et al.* (2002) reported the transmission of *C. hyointestinalis* from a pig to a 88 year old woman who was hospitalised for suffering from persistent diarrhoea, abdominal pain and intermittent vomiting for more than one month and who lived and worked on a farm that had pigs, chickens, cats and dogs. The patient's stool samples were positive for two strains of *C. hyointestinalis* and two strains of *C. jejuni*. *C. hyointestinalis* and *C. coli* were also recovered from the porcine stool samples (Gorkiewicz *et al.* 2002).

2.2.2.3 The prevalence of *Campylobacter* in domestic animals

Contact with animals, especially pets and animals in petting zoos or on farms may pose a serious health risk to immunocompromised individuals such as the elderly, pregnant women, organ recipients, cancer patients and children through zoonotic transmission of disease. Infants to pre-school aged children are especially at risk of acquiring zoonotic illnesses from their environment due to their inquisitive nature with the petting of animals and licking by pets considered the main transmission routes of zoonotic illnesses due to lack of hygiene practises after contact (Sockett and Rodgers 2001; Hemsworth and Pizer 2006).

Campylobacter has been shown to be associated with domestic pets and contact with the pet(s) is considered a major risk factor for acquiring *Campylobacter*-associated illness (Salfield and Pugh 1987; Sockett and Rodgers 2001; Hemsworth and Pizer 2006). A recent study done in North America by Chaban *et al.* (2010) has shown that pet dogs harbour a wide diversity of *Campylobacter* species: 14 species including species considered as emerging campylobacters were detected and quantified by quantitative PCR. The study also showed that 58% of healthy dogs shed detectable levels ($10^3 - 10^8$ CFU.g⁻¹ of faeces) of *Campylobacter* species while 97% of diarrheic dogs shed higher levels within the detectable range of *Campylobacter* species. Many of the dogs were infected with multiple species of *Campylobacter* (Chaban *et al.* 2010).

Studies of *Campylobacter* in dogs in the UK has shown prevalence of 38% in dogs visiting veterinary practises (Parsons *et al.* 2010) and 46% to 73% in kennelled dogs (Parsons *et al.* 2011). Carbonero *et al.* (2012) showed that 33% of healthy and diarrheic dogs in Spain harboured *Campylobacter*. These studies showed that 58.8% of ill and healthy dogs (Carbonero *et al.* 2012), 95% of dogs visiting veterinary practises (Parsons *et al.* 2010) and 62% of kennelled dogs (Parsons *et al.* 2011) harboured *C. upsaliensis* while *C. jejuni* was the second-most frequently isolated species (Parsons *et al.* 2010; Parsons *et al.* 2011; Carbonero

et al. 2012). These animals pose a risk of zoonosis to individuals who come into contact with them.

The prevalence of *Campylobacter* detected in pet cats has been less frequently reported than dogs. Acke *et al.* (2006 and 2009) showed that there is a prevalence of 75% of healthy Irish cats in shelters that harbour *Campylobacter* species (Acke *et al.* 2006) while *Campylobacter* species were isolated from 42.9% of Irish household healthy or ill cats (Acke *et al.* 2009). *C. helveticus*, *C. jejuni*, *C. upsaliensis* as well as species of the closely related genus *Helicobacter* have been detected in the stools of healthy and diarrheic cats. Co-infection of multiple species of *Campylobacter* and the related genera has been found in cats (Rossi *et al.* 2008; Koene *et al.* 2009).

2.2.2.4 The prevalence of *Campylobacter* in wild animals

Wild animals and birds have been associated with the transmission of *Campylobacter* to agricultural animals (Horrocks *et al.* 2009; Sippy *et al.* 2012). This route of transmission is implicated in the contamination of mussels, clams and oysters when the droppings of seagulls that cluster near shellfish beds wash over the shellfish when the tide is low (Abeyta *et al.* 1993; Endtz *et al.* 1997; Jacobs-Reitsma 2000). *Campylobacters* have been recovered from wild birds and small mammals (Sippy *et al.* 2012) such as rodents (squirrels) (Dipineto *et al.* 2009), reptiles (turtles) (Harvey and Greenwood 1985; Tu *et al.* 2004; Tu *et al.* 2005) as well as zoo animals (Misawa *et al.* 2000). Kemper *et al.* (2006) also reported an incidence where *C. hyointestinalis* was isolated from a semi-domesticated reindeer in a study done in Northern Finland and Norway. *Campylobacters* have also been associated with foxes, monkeys and seals (Lastovica 2006).

2.2.3 Survival of *Campylobacter* in chicken meat, red meats and the environment.

Campylobacter are microaerophilic organisms and because of this fastidious growth requirement, it is often thought that *Campylobacter* species would not be able to survive conditions outside the host and the non-favourable, harsh conditions of the environment. Approximately 500 million cases of campylobacteriosis due to the consumption of contaminated food occur annually (Horrocks *et al.* 2009). This shows that *Campylobacter* can indeed survive environmental conditions outside the host (Humphrey *et al.* 2007; Rajkovic *et al.* 2010; Sampers *et al.* 2010).

As previously mentioned, *Campylobacter* are able to survive in a variety of water bodies including treated, untreated and inadequately treated drinking water. Talibart *et al.* (2000) has shown that *C. jejuni* and *C. coli* are able to survive between 14 and 21 days in an aqueous microcosm (sterile water at pH 6). The difference in survival periods are strain dependent. Two of the test strains were able to maintain its viability for 35 days in a shaken aqueous microcosm at 4°C while being viable for more than 60 days without shaking. *Campylobacter* enter a viable but non culturable (VBNC) stage when in a stressed or non-favourable growth environment. This study went on to show that the strains could be resuscitated after inoculating nine day old fertilised eggs with 1ml of the aqueous microcosm. This shows that VBNC strains have the capability of regaining viability and subsequently have the potential to colonise and infect humans and animals. It may also be indicative of a certain factor present in the embryonic egg that is essential to the resuscitation of VBNC *Campylobacter* colonies (Talibart *et al.* 2000). Guillou *et al.* (2008) has also demonstrated the ability of *C. jejuni* to survive in bottled water although the risk of acquiring a *Campylobacter* infection is low as cells enter the VBNC state.

The incidence rate of *Campylobacter* found in poultry flocks varies across geographical regions (Suzuki and Yamamoto 2009; Sheppard *et al.* 2010). Reported incidence rates are

typically between 35% (Rosenquist *et al.* 2006) to 48% (Georgsson *et al.* 2006; Sampers *et al.* 2010). Most countries have a prevalence of 50% or more (Suzuki and Yamamoto 2009) but prevalences of up to 85% of retail chicken naturally contaminated with *Campylobacter* are often reported (Sampers *et al.* 2008; El-Shibiny *et al.* 2009a; Horrocks *et al.* 2009; Ligowska *et al.* 2011). *Campylobacters* cannot grow in temperatures below 30°C but these incidence rates are evidence that they have the ability to survive in environments where its favourable growth conditions are not met (Ligowska *et al.* 2011).

The survival of *Campylobacter* in any environment is strain-dependant (Habib *et al.* 2010). Survival studies of naturally and artificially contaminated chicken meat has demonstrated that *Campylobacter* species are more sensitive to the freezing (storage at -20°C) of chicken as a significant reduction of *Campylobacter* numbers occur on initial freezing of the sample. Subsequent freeze-thaw cycles have shown no significant reduction in *Campylobacter* numbers and therefore this stress of freezing does not completely eliminate *Campylobacter* from a sample (Georgsson *et al.* 2006; Habib *et al.* 2010; Sampers *et al.* 2010) as it could still be detected 84 days (Sampers *et al.* 2010) to 220 days (Georgsson *et al.* 2006) after inoculation. In comparison, *Campylobacter* numbers does not decrease significantly at 4°C for 14 days when subjected to freezing. The cooking of naturally and artificially contaminated food to an internal temperature above the maximum growth temperature (52°C to 58°C) of *Campylobacter* reduces the number of the bacteria to below detectable levels (Sampers *et al.* 2010).

A study by Melero *et al.* (2012) demonstrated that the combination of freezing with a high oxygen - modified atmosphere packaging (MAP) completely eliminates *C. jejuni* from fresh chicken meat burgers stored at 4°C. Comparable results were obtained by Dykes and Moorhead (2001) who demonstrated that not only are *C. jejuni* able to survive but also, there

was no significant reduction in numbers in standard vacuum and 100% carbon dioxide packaging of inoculated beef cuts at -1.5°C for 41 days (Dykes and Moorhead 2001).

The many reservoirs of *Campylobacter* spp. suggest the many transmission routes (Figure 2.6) that susceptible animals and humans can acquire *Campylobacter* infections.

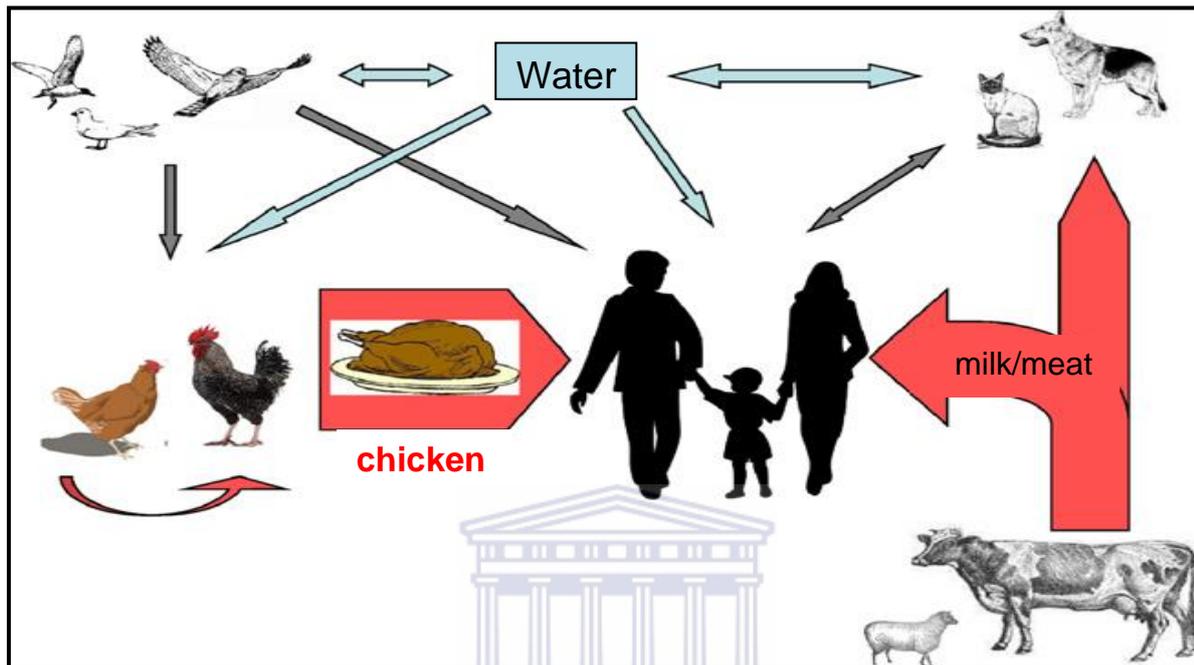


Fig. 2.6 The most important infection routes of human campylobacteriosis (adapted from Dasti *et al.* 2010)

2.3. CAMPYLOBACTERIOSIS

For nearly a century, *Campylobacter* species has been recognised as a pathogen in food-production animals. It was the causative agent of septic abortion in cattle and sheep and also the cause of diarrhoea in cattle (Zhao *et al.* 2010; Sippy *et al.* 2012). However, they have only been recognised as a human pathogen for the last three decades (Friedman *et al.* 2000; Blaser *et al.* 2008; Horrocks *et al.* 2009) and is now considered the most common bacterial cause of acute gastroenteritis worldwide (Gibreel and Taylor 2006; Colles *et al.* 2008; Horrocks *et al.* 2009; Habib *et al.* 2012).

Campylobacter infections in humans is mainly considered to be a zoonotic disease, derived from contaminated foods of animal origin and usually occurs sporadically while large community outbreaks are relatively rare (Jacobs-Reitsma 2000; Colles *et al.* 2008; Hänel *et al.* 2009; Hue *et al.* 2011; Silva *et al.* 2011; Calciati *et al.* 2012; Habib *et al.* 2012).

Campylobacteriosis or *Campylobacter* enteritis is an acute diarrhoeal disease of humans with clinical features the same as that of salmonellosis or shigellosis and is therefore difficult to differentiate from other infections of bacterial origin. A reported case can therefore only be confirmed when campylobacters are cultured from stool (Skirrow and Blaser 2000; Allos and Lastovica 2011; Alonso *et al.* 2011), blood and other site(s) of infection (Allos and Lastovica 2011). Campylobacteriosis, caused by the infection of an individual with any of a variety of *Campylobacter* species, is one of the leading causes of food-borne bacterial illnesses in developed and developing countries (Suzuki and Yamamoto 2009). Globally, it is the cause of 400 to 500 million cases of acute diarrhoea each year and an estimated 2.5 million cases of campylobacteriosis is reported in the United States and more than 340 000 cases are reported in the UK annually (Diergaardt *et al.* 2004; Humphrey *et al.* 2007; Ruiz-Palacios 2007; Colles *et al.* 2008; Horrocks *et al.* 2009). The reports show that there is a 30% increase in annual rates and it is estimated that the actual number of cases per year is closer to 450 000, taking into account the cases that are unreported. It is also estimated that 10% of the reported cases are hospitalised in the UK annually (Strachan and Forbes 2010). In 2005, annual statistics show that there were more than 51.6 reported campylobacteriosis cases per 100 000 inhabitants in 24 European Union countries (Nauta *et al.* 2009a) and 212 064 confirmed cases were reported by the European Food Safety Authority (EFSA) in 2010 (Wieczorek *et al.* 2012).

The economic burden of campylobacteriosis is largely due to the clinical costs and lost working hours. In the US, the accumulated losses are estimated to be between \$1.3 to 6.2

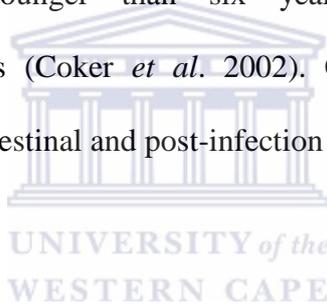
billion (Humphrey *et al.* 2007; Samie *et al.* 2007). This amount does not take into account the amount of cases that are not reported. The true incidence of campylobacteriosis cases is estimated to be 10 times more than the number of cases that are reported (Samie *et al.* 2007). The disease burden in The Netherlands is approximately €21 million, £65 million in the UK (the true estimate is probably closer to £500 million), NZ \$4.48 million in New Zealand (Humphrey *et al.* 2007) and €10.9 million in Belgium (Messens *et al.* 2009) per annum.

There are no official records of the incidences and frequency of *Campylobacter* infections in South Africa (Samie *et al.* 2007; Allos and Lastovica 2011); however, a significant number of cases are recorded each year from diarrhoeic stool and blood culture samples from paediatric patients from Red Cross Children's and Groote Schuur Hospitals in Cape Town, South Africa (Diergaardt *et al.* 2004; Alam *et al.* 2006; Lastovica 2006; Lastovica and Allos 2008). As in most developing countries, *Campylobacter* species is mostly frequently isolated in infants younger than two years of age in South Africa. It is also frequently isolated from children older than two years, asymptomatic children and adults and compares with campylobacteriosis cases in developed nations (Allos and Lastovica 2011). In a separate South African study using molecular diagnostic methods, *C. jejuni* was associated with 84.8% diarrheic cases especially amongst HIV positive individuals (Samie *et al.* 2007). The majority of campylobacteriosis cases are caused by *C. jejuni* or *C. coli*, (Skirrow and Blaser 2000; Lastovica and Skirrow 2000; Bae *et al.* 2007; Allos and Lastovica 2011) but other emerging species of *Campylobacter* are recognised to be pathogenic organisms (Lastovica 2006; Lastovica and Allos 2008; Lastovica 2009).

2.3.1 Characteristics of the disease

Campylobacteriosis has a relatively low infectious dose as only 100 - 500 organisms are capable of causing disease (Robinson 1981; Diergaardt *et al.* 2004; Calderón-Gómez *et al.* 2009; Maal-Bared *et al.* 2012). The incubation period has a mean of 3 days and ranges from

18 hours to 8 days. The illness may last as little as a few days to more than one week (Skirrow and Blaser 2000; Allos and Lastovica 2011; Alonso *et al.* 2011). The clinical features of campylobacteriosis include abdominal cramps, fever and diarrhoea (Nachamkin *et al.* 2000; Humphrey *et al.* 2007; Alonso *et al.* 2011; Calciati *et al.* 2012) which often become bloody after 1 to 2 days of diarrhoea. Nausea is also a frequent symptom but a small percentage of vomiting has been reported (Skirrow and Blaser 2000; Humphrey *et al.* 2007; Calciati *et al.* 2012). Abdominal pain may be continuous and become so intense that it may mimic acute appendicitis; this is the most frequent reason for admission of campylobacteriosis cases to hospital (Skirrow and Blaser 2000; Humphrey *et al.* 2007; Alonso *et al.* 2011). These symptoms may vary from mild to severe and the incidence of asymptomatic and milder cases of symptoms experienced is common (Skirrow and Blaser 2000; Alonso *et al.* 2011). All age groups are affected by the disease but those most at risk are very young children (younger than six years of age), the elderly and immunocompromised individuals (Coker *et al.* 2002). Complications of *Campylobacter* enteritis may give rise to extra-intestinal and post-infection complications.



2.3.2 Extra-intestinal infections

Bacteremia is the presence of bacteria in the blood (Pigrau *et al.* 1997; Prescott *et al.* 2005) and is rarely reported in campylobacteriosis. This may be that it occurs as a transient event in the early stages of infection and is not detected as blood cultures are rarely performed in the early stage of the infection and the methods for detection are not as sensitive for *Campylobacter* species, as for other bacteria. It is mostly reported in people that are 65 years and older, immunocompromised patients or almost always associated with patients with an underlying disease such as liver cirrhosis, neoplasia and immunosuppression due to chemotherapy (Pigrau *et al.* 1997; Skirrow and Blaser 2000). In a brief study of paediatric patients, 20 *Campylobacter* strains were isolated from 19 blood and one cerebrospinal fluid sample(s). Four of the isolates were *C. coli* while the rest was *C. jejuni* (Lastovica and Penner

1983). Serious cases of septicaemia have been reported as a result of *C. jejuni* infections in patients with a severely compromised immune system (Skirrow and Blaser 2000). Although rarely reported, animals such as ostriches may suffer from fatal *Campylobacter* septicaemia (A.J. Lastovica, personal communication).

Cases of hepatitis and pancreatitis have been associated with campylobacteriosis. These cases have been reported mainly in adults and have successfully been resolved after antimicrobial treatment directed at the infecting species (Skirrow and Blaser 2000). It has long been known that campylobacters, particularly *C. fetus*, are the causative agent of abortion in sheep and cattle. In addition, *C. jejuni* and *C. coli* are responsible for a significant number of ovine abortion cases. Until 2000, there have also been 30 reported cases of human septic abortion and stillbirth due to *Campylobacter* infection. Half of these cases were caused by *C. fetus*, one by *C. hyointestinalis* and the rest was caused either by *C. jejuni* or *C. coli*. The manner in which *Campylobacter* are able to infect human placenta is unknown but is thought to reach the placenta by hematogenous spread from the intestinal tract. The pathology of human abortions caused by *Campylobacter* infections are similar to that of sheep where acute placentitis causes death as a result of placental insufficiency although the infection reaches the fetus via the amniotic fluid (Skirrow and Blaser 2000). Other extra-intestinal complications include haemolytic-uremic syndrome (HUS), peritonitis, myocarditis, nephritis, prostatitis, cholecystitis, meningitis, endocarditis, abscesses and urinary tract infections (Skirrow and Blaser 2000; Allos and Lastovica 2011; Alonso *et al.* 2011).

2.3.3 Post-infective sequelae

Complications may arise after the individual has had a *Campylobacter* infection and include Guillain-Barré Syndrome (GBS), Miller Fisher Syndrome (MFS), Reactive Arthritis (ReA) and Crohn's Disease (Goddard *et al.* 1997; Nachamkin *et al.* 1998; Endtz *et al.* 2000; Hannu *et al.* 2004; Zhang *et al.* 2009).

GBS is an autoimmune disorder of the peripheral nervous system and the most common cause of acute flaccid neuromuscular paralysis since the eradication of polio globally. It results in the weakness of the limbs and respiratory muscles and areflexia (loss of reflexes). The weakness is usually developed symmetrically and evolves over a period of several days or more. Intensive care is needed for a full recovery that takes place over weeks or months (Nachamkin *et al.* 1998). It is frequently preceded by a number of bacterial and viral infections but *Campylobacter* – enteritis is now recognized as the most identified infection preceding GBS (Goddard *et al.* 1997; Endtz *et al.* 2000; Nachamkin 2002). The recovery of some patients may be uneventful and complete while some are left with severe neurologic deficits.

The first case of GBS following a *Campylobacter* infection was reported in 1982. The 45 year-old man developed irreversible neurological damage just two weeks following the gastrointestinal illness. Thereafter, more reports had described the development of GBS following a *C. jejuni* infection. Males are more likely to develop GBS than females with a ratio of 3:1 (Nachamkin *et al.* 1998). The incidence of the development of GBS following a *Campylobacter* infection is one in 1000 (Endtz *et al.* 2000; Nachamkin 2002; Humphrey *et al.* 2007). The risk increases to approximately to one in 200 for patients infected with *C. jejuni* serotype HS:19 (Nachamkin 2002; Humphrey *et al.* 2007).

Prior to 1997, there have been a few reports of GBS in children. However, a study done over a period of 15 months at the Red Cross War Memorial Children's Hospital (RCWMCH) in Cape Town, South Africa between June 1994 and August 1995 showed that *Campylobacter* spp. were isolated from stool samples in nine of 14 paediatric patients admitted with GBS (Goddard *et al.* 1997). Six of the isolates were identified as *C. jejuni* subsp. *jejuni* biotype 2, serotype 0:41. These six patients had a more severe disease than the others from whom *C. concisus*, *C. upsaliensis* and *C. jejuni* subsp. *jejuni* biotype 1 were isolated. *C. jejuni* subsp.

jejuni biotype 2, serotype 0:41 is an extremely rare sero-biotype of *Campylobacter* in the RCWMCH region (Goddard *et al.* 1997; Wassenaar *et al.* 2000). There are several different factors that contribute to the risk of developing GBS following a *C. jejuni* infection and include the age of the host and serotype-specific risks (Goddard *et al.* 1997; McCarthy and Giesecke 2001).

Miller Fisher Syndrome is a non-paralytic, rare variant of GBS (Endtz *et al.* 2000) and is characterised by the acute onset of unsteadiness of gait (ataxia), areflexia and an inability to move the eyes, usually associated with non-reactive pupils (ophthalmoplegia) (Nachamkin *et al.* 1998).

Reactive arthritis (ReA) is a non-purulent joint inflammation condition that is triggered by gut - or urogenital tract infections. The mechanism of pathogenesis of this *Campylobacter* – mediated rheumatologic disease is not fully understood. Different serotypes of *C. jejuni* are amongst the different pathogens that are able to trigger this reaction (Hannu *et al.* 2002; Hannu *et al.* 2004). In a population-based study, the mean appearance of pain and swelling of joints after the onset of bowel symptoms characteristic of campylobacteriosis was 14 days and lasts from several weeks to several months and in rare cases lasts up to one year (Skirrow and Blaser 2000). Studies done on *Campylobacter* outbreaks show that the occurrence rate of ReA following an outbreak is between 0.7% and 2.6% (Hannu *et al.* 2004). Reiter's Syndrome, a manifestation collectively of the inflammatory arthritis of large joints, conjunctivitis and urethritis in men or cervicitis in women, has also been observed in some patients with reactive arthritis associated with *Campylobacter* infections (Skirrow and Blaser 2000).

Irritable Bowel Syndrome(s) (IBS), which includes Crohn's Disease and ulcerative colitis, is characterised by abdominal pain and sometimes an altered bowel habit, has increasingly been

linked to campylobacteriosis. Little is known about the role of *Campylobacter* pathogenesis in IBS (Zhang *et al.* 2009; Alonso *et al.* 2011).

Campylobacteriosis is a normally self-limiting disease (Bester and Essack 2008; Alonso *et al.* 2011) but treatment with antibiotics is often necessary in young children, pregnant women and immunocompromised patients who have a greater possibility of experiencing an increased severity or duration of infection. It is an absolute necessity to treat patients that has developed secondary or post-infection complications with antibiotics (Zirnstein *et al.* 1999; Ishihara *et al.* 2004; Silva *et al.* 2011).

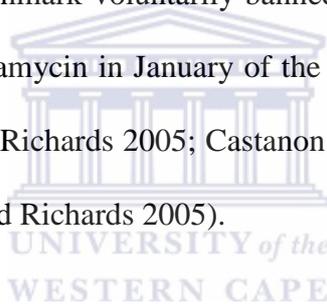
2.4 ANTIBIOTIC THERAPY AND ACQUIRED ANTIBIOTIC RESISTANCE IN *CAMPYLOBACTER* SPECIES

Natural antibiotics originate from microorganisms and are chemical compounds that inhibit the growth of other microbes in its surrounding environment. The many classes of antibiotics are classified by its structural type as well as its mechanism of action in inhibiting or killing the target cell(s) (Davies 2006) such as preventing the synthesis of bacterial proteins by antibiotics belonging to the class of aminoglycoside antibiotics (Graeme and Pollack 1996; Butaye *et al.* 2003).

Antibiotics were first discovered by Sir Alexander Fleming and used exclusively by the military during World War II. As novel antibiotics were discovered and newer techniques employed for its production, it became widely available for use by the general public (Alanis 2005; Hiramatsu *et al.* 2012). Coincidentally, it was discovered that antibiotics were also beneficial to the health of animals, especially food animals, not only therapeutically but also as an agent for enhancing the growth and performance of these animals when added to their feed at sub-therapeutic concentrations (van den Bogaard and Stobberingh 2000; Castanon

2007; Martinez 2009; Silva *et al.* 2011). As was the case in human therapy during World War II, there was a clear correlation with the acquired resistance of bacteria to the antibiotics used in the animal feed (Alanis 2005; Dibner and Richards 2005).

An increase in the trend of antibiotic resistance continued during the years that antibiotics were used as growth promoters. This increasing trend was a cause of concern to Swann in 1969 which led him to suggest the banning of the use of sub-therapeutic antibiotics to the British Parliament (Dibner and Richards 2005). The 1980's saw the vast emergence of antibiotic resistant pathogens globally and this led to the recommendation to ban all growth promoters. Sweden was the first country to discontinue the use of antibiotic growth promoters (AGPs) in 1986 and in 1997, avoparcin was banned in Denmark with the Commission of the European Union banning it in all European Union (EU) member states in 1997. Food-animal/agricultural farmers in Denmark voluntarily banned the use of all AGPs in February 1998 after the banning of virginiamycin in January of the same year (Aarestrup *et al.* 1998; Casewell *et al.* 2003; Dibner and Richards 2005; Castanon 2007). Coccidiostats are still used in the poultry industry (Dibner and Richards 2005).



The use of antimicrobials was banned in poultry production by the European Council in 1998 when the realisation became apparent that the efficacy of the antimicrobials in human therapy may weaken (European Economic Community 1998). Since the introduction of antibiotic therapy, vast numbers of antibiotic-resistant bacteria has developed throughout the world. This acquired resistance is primarily a direct result of many years of underuse, misuse and overuse of antibiotics by humans (Fàbrega *et al.* 2008; Davies and Davies 2010).

AGPs are used intensively in animal health in South Africa. It is used therapeutically to treat diseases as well as sub-therapeutically for the prevention of diseases which increases animal production. The South African Government acknowledges that this practise may lead to the

development of antibiotic resistant bacteria, which in turn, could compromise human therapy (Xingwana 2008).

Patients with campylobacteriosis that require antibiotic therapy are primarily treated with macrolides and fluoroquinolones (Avrain *et al.* 2003; Ishihara *et al.* 2004; Moore *et al.* 2006; Bester and Essack 2008) while tetracyclines, although rarely used, have been suggested as an alternative choice of treatment. Serious cases of *Campylobacter* infections, such as bacteremia or other systemic infections may necessitate the administration of intravenous aminoglycoside (gentamicin) therapy (Aerestrup and Engberg 2001; Wiczorek *et al.* 2012).

Campylobacteriosis is considered a zoonotic illness as it is mainly acquired from the handling and consumption of raw and inadequately cooked contaminated chicken. Therefore, antibiotic resistant *Campylobacter* strains are likely to be consumed if contaminated chicken are ingested (Bester and Essack 2010). The South African government are however concerned about the range and extent of antibiotic use in animal production as there are an estimated 7096 of antibiotics used as AGPs according to the Union of Concerned Scientists. However, this practise cannot be eradicated because of the type of farming methods practised where many animals are housed in confined spaces (Xingwana 2008).

2.5 DIAGNOSIS OF CAMPYLOBACTERIOSIS

2.5.1 Growth conditions

Campylobacters are fastidious, slow-growing microorganisms that require specific microaerophilic incubation conditions for its survival (Stoyanchev 2004; Humphrey *et al.* 2007). Most strains of *C. jejuni* subsp. *jejuni* and *C. coli* grow relatively fast when compared with *C. upsaliensis*, *C. rectus*, *C. hyointestinalis* and *C. concisus* (Lastovica 2006). Hydrogen-enriched microaerophilic conditions essential for optimal growth of campylobacters (Lastovica and le Roux 2003a; Diergaardt *et al.* 2004; Lynch *et al.* 2010) can

be obtained with commercially available anaerobic gas generating kits such as the Oxoid BR038 Gas Generating Kit (Lastovica and le Roux 2003a; Diergaardt *et al.* 2004). A hydrogen enriched microaerobic atmosphere containing 5% O₂, 10% CO₂ and 85% N₂ is required by most *Campylobacter* species for optimal growth (Kaakoush *et al.* 2007; Potturi-Venkata *et al.* 2007; Alonso *et al.* 2011). A primary incubation temperature of 42°C allows for the growth of usually just *C. jejuni* and *C. coli*. This temperature is used as it is the internal temperature of chicken. However, an incubation temperature of 37°C is more appropriate for the isolation and maintenance of *Campylobacter* spp. that are able to infect humans as other *Campylobacter* species grow poorly or not at all at 42°C (Lastovica 2006).

2.5.2 Culture dependent detection methods

Campylobacter spp. can be detected from food, environmental and faecal samples by direct plating with the use of selective media or by the enrichment of the culture from the sample when there may be a few target cells present in the sample (Williams *et al.* 2009). Food products may only have small numbers of campylobacters due to the microorganisms' sensitivity to processing procedures such as freezing, cooling, heating and salting (Jacobs-Reitsma 2000). The enrichment procedure allows for the better recovery of *Campylobacter* spp. as factors such as pH, oxidative stress and temperature inhibits the recovery from complex sample types (Williams *et al.* 2009). Although the International Organization for Standardisation (ISO) protocol ISO 10272-1:2006 states that Bolton broth should be used for enrichment (Anon 2006), Preston and Exeter broth are also widely used for enrichment of cultures. Bolton (Humphrey *et al.* 2007; Williams *et al.* 2009) and Exeter broths were shown to yield a better recovery in food, water and environmental samples than Preston broth (Baylis *et al.* 2000; Williams *et al.* 2009).

The conventional methods that are used for the detection of any microorganism from samples include the use of media that it supports and in most cases, is specific for the growth

of the particular microorganism. *Campylobacter*, when compared to most other pathogenic microorganisms, does not however have a standard protocol for its detection in samples (Humphrey *et al.* 2007; Potturi-Venkata *et al.* 2007). There is a wide range of selective media that can be used for the isolation of *Campylobacter* and include *Campylobacter* agar base, *Campylobacter*, Campy-Line (CL), modified Campy-Cefex (mCC) and modified charcoal cefoperazone deoxycholate agar (mCCDA). A study done in 2007 by Potturi-Venkata *et al.* to evaluate these different selective media types showed that *Campylobacter* agar base, *Campylobacter*, mCC and mCCDA showed similar isolation rates whereas CL showed a lower efficacy of isolation of *Campylobacter* spp. from broilers reared in battery cages. *Campylobacter* spp. was also isolated more consistently in samples obtained from faeces of commercial live broilers with the use of mCC, while mCC and mCCDA showed similar isolation rates from cecal samples. CL agar showed a lower rate of identification for both fecal and cecal samples (Potturi-Venkata *et al.* 2007). CCDA is commonly used worldwide as well as other selective media containing antimicrobials. The selectivity in the latter is given by the specific antimicrobial used as different *Campylobacter* strains are resistant to specific antimicrobials (Potturi-Venkata *et al.* 2007). The usage of antimicrobials in media leads to the unsuccessful isolation of emerging campylobacters from stool and other clinical samples as these organisms might be susceptible to the specific type of antimicrobial being used in the medium (Lastovica 2006; Humphrey *et al.* 2007).

The Cape Town Protocol allows for the efficient isolation of *Campylobacter* spp. from stool samples where the stool samples are filtered through a 0.6 µm pore size membrane filter onto an antibiotic-free tryptose blood agar (TBA) plate that contains 10% unlysed horse or sheep blood (Lastovica and le Roux 2003a; Alam *et al.* 2006; Lastovica 2006). An impressive three-fold increase in the number of *Campylobacter*-stools was observed after the implementation of the Cape Town Protocol when compared to the numbers obtained by the use of antibiotic-containing selective media at the Red Cross Children's Hospital. The Cape

Town protocol can be modified when blood or other clinical samples such as gastric biopsy sample material are to be analysed. The use of the filter paper should be eliminated in such cases (Alam *et al.* 2006; Lastovica 2006). The Cape Town Protocol is superior to the variety of other selective media for efficient and optimal isolation of campylobacters from stool samples (Lastovica and le Roux 2003a, b; Jacob *et al.* 2011).

2.5.3 Molecular detection methods

2.5.3.1 The Polymerase Chain Reaction (PCR) Technique

Conventional techniques used for the detection and identification of *Campylobacter* species are laborious, time-consuming and problems of contamination are often encountered. Therefore, it is desired that a simple and rapid species detection and identification method be used particularly for the detection of *Campylobacter* in foods as the cells are often stressed by unfavourable growth conditions and may not be detected by conventional growth methods (Candrian 1995; Humphrey *et al.* 2007; Williams *et al.* 2009; Alonso *et al.* 2011). The PCR technique provides the answer to this need. The Polymerase Chain Reaction (PCR) technique is the most widely used of all molecular techniques as it is highly sensitive, specific and rapid for the detection of food-borne pathogens (Samosornsuk *et al.* 2007). It is a technique whereby a specific DNA fragment is amplified dramatically with the aid of primers that are designed to specifically bind to the template DNA and synthesize more of the target DNA in a reaction catalysed by a thermostable DNA polymerase in the presence of free deoxyribonucleoside triphosphates (dNTPs) (Harris and Griffiths 1992).

Limitations to the PCR technique is that the amplified DNA may be derived from living and dead bacteria in a food sample (Humphrey *et al.* 2007; Alonso *et al.* 2011) as well as the target DNA may be repressed from amplification by the polymerase due to inhibitors that are inherently present in the extracted DNA sample. Blood and certain tissues have inhibitors that are inherent to a sample that cannot be separated from the extracted DNA sample by DNA

purification methods adopted by laboratories (Candrian 1995; Al-Soud and Rådström 2001). Heme, leukocyte DNA, immunoglobulin G (IgG) and added anticoagulants such as EDTA and heparin have been identified as inhibitors that are inherent to blood (Al-Soud and Rådström 2001). Another limitation of the technique is that a standardized protocol cannot be established due to the technique not being reproducible between laboratories. The technique may work well in the laboratory that developed the protocol but may not allow for the comparison of results in other laboratories following the same protocol (Humphrey *et al.* 2007).

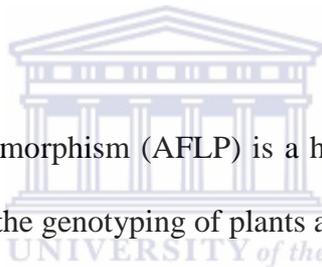
2.5.3.2 DNA fingerprinting

The genomic ‘fingerprint’ of a *Campylobacter* strain has the ability to distinguish different strains from one another and to trace the source and routes of cases and/or outbreaks (Newell *et al.* 2000; Humphrey *et al.* 2007). Genetic subtyping methods have enhanced sensitivity and discrimination and are commonly applied in epidemiological studies (Newell *et al.* 2000).

Multilocus sequence typing (MLST) is a typing method that amplifies and sequences defined regions of seven moderately conserved *Campylobacter* housekeeping genes. Regions of loci with distinct sequences are assigned unique allele numbers while unique sequence types (ST) are assigned to each unique allelic profile (Humphrey *et al.* 2007; Miller *et al.* 2012). Data generated by MLST studies can also provide insight to lateral gene transfer and evolution as well as genotypic information for novel species that are diverse phenotypically (Miller *et al.* 2012).

Pulse-Field Gel Electrophoresis (PFGE) is generally accepted as one of the most discriminatory genotypic methods for the subtyping of *Campylobacter* species. The principal behind the method is based on macrorestriction profiling where bacterial cells are embedded in chromosomal-grade agarose (referred to as plugs) and lysed *in situ* to prevent DNA

shearing and the DNA is subsequently fragmented by rare-cutting enzymes. The DNA fragments are then separated by a special electrophoresis method (Newell *et al.* 2000; Ribot *et al.* 2001). The PFGE method was also used to determine the genome sizes of *C. jejuni*, *C. coli*, *C. lari* and *C. fetus* and to construct a genetic map of a *C. jejuni* strain. The discriminatory power of the PFGE method is excellent, however, it does present with some disadvantages such as (i) the time-consuming and tedious preparation of the DNA-containing agarose plugs, (ii) The DNase of some *Campylobacter* strains must be inactivated to ensure that the DNA does not degrade before electrophoresis. The inactivation usually requires the pretreatment of bacterial cells with the toxic chemical formaldehyde, (iii) The apparatus used for the electrophoresis is specialized and expensive, (iv) The enzymes commonly used does not digest the DNA of some strains, (v) The genetic instability of the bacterial cells can lead to minor and major changes in profiles and therefore the interpretation of the results become difficult (Newell *et al.* 2000).



Amplified Fragment Length Polymorphism (AFLP) is a high resolution genotyping method that was originally developed for the genotyping of plants and to a lesser extent, animals (Vos *et al.* 1995; Meudt and Clarke 2007). It has also been adapted for the genotyping of bacteria (Duim *et al.* 1999; Kokotovic and On 1999; Duim *et al.* 2000). The powerful advantage of the AFLP technique is that it requires no prior knowledge of the target genome's sequence (Vos *et al.* 1995; Savelkoul *et al.* 1999; Duim *et al.* 2000; Messens *et al.* 2009). The technique involves the digestion of chromosomal DNA with two restriction enzymes and the subsequent ligation of restriction-site specific oligonucleotide adaptors. This creates a template for the PCR amplification by adaptor-specific primers containing one or more selective nucleotides, ensuring that only a subset of fragments will be amplified under stringent PCR conditions (Vos *et al.* 1995; Duim *et al.* 1999; On and Harrington 2000; Messens *et al.* 2009). The generated fragments are detected by a fluorescently labelled primer

and analyzed on gel or capillary based automated DNA sequencers (Duim *et al.* 2001; Messens *et al.* 2009).

Newell *et al.* (2000) agree that AFLP and PFGE appear to provide the same level of discrimination. However, a comparative DNA fingerprinting study by Lindstedt *et al.* (2000) demonstrated that the AFLP technique has a better discriminatory power to PFGE as it was able to differentiate between strains with identical PFGE profiles (Lindstedt *et al.* 2000). Another disadvantage is that PFGE involves the use of several restriction enzymes for optimal discrimination while AFLP uses a single procedure. The AFLP technique is rapid and easily standardized and has become feasible due to the increasing availability of automated DNA sequencers. However, this equipment is expensive but the digitization of AFLP results allows accurate interpretation, ease of data storage and ready data exchange between laboratories (Newell *et al.* 2000).

2.6 CONTROL OF *CAMPYLOBACTER* FROM THE FARM TO THE FORK

Campylobacters are ubiquitous in the natural environment (Newell and Wagenaar 2000; Allos and Lastovica 2011) and it is therefore expected that animals in direct contact with the environment are more likely to be colonised with the organisms (Humphrey *et al.* 2007). It is for this reason that free-range chickens are more likely to be infected with *Campylobacter* from their environment than their housed counterparts (Humphrey *et al.* 2007; Colles *et al.* 2008; Hermans *et al.* 2011). The majority of poultry flocks are colonized within two to four weeks after hatching (Hermans *et al.* 2011). The rest of the flock becomes colonized within days of the first chick being infected (van Gerwe *et al.* 2009) and carry high numbers of campylobacters in their intestinal tract (Hermans *et al.* 2011). They remain colonized until slaughter where the carcasses are readily contaminated especially during the evisceration stage of the slaughtering process (Reich *et al.* 2008). Risk assessments of *Campylobacter* in poultry meat has been developed to assist in the implementation of control strategies to

minimise the presence of *Campylobacter* throughout the poultry meat production chain (Nauta *et al.* 2009b) and it is agreed upon that the focus should be to reduce *Campylobacter* levels on broiler carcasses after the evisceration stage of production rather than to reduce the prevalence of *Campylobacter* in the environment (Rosenquist *et al.* 2006).

Many control practises developed has been aimed at reducing the prevalence of *Campylobacter* before it is introduced or transmitted in the flocks or to reduce the intestinal counts which leads to the reduction of contamination levels of the carcasses after processing (Hermans *et al.* 2011).

2.6.1 Biosecurity

This is the prevention of colonisation of birds with campylobacters from outside sources. This is very important since the spread of campylobacter colonization through a flock may be rapid once it is introduced (Newell and Wagenaar 2000; Humphrey *et al.* 2007; Hermans *et al.* 2011). Hygiene is an important factor when entering flocks. Visits to flocks should be limited to essential personnel and dipping boots in disinfectant and changing it frequently has been shown to reduce infection rates by 50% (Humphrey *et al.* 2007). The entry of rodents and insects into the flocks should also be controlled. In two separate studies, this control has been shown to reduce the colonisation of flocks by 40% and 58% (van de Giessen *et al.* 1998).

2.6.2 Drinking water treatment

The treatment of drinking water with organic acids has a strong bactericidal effect on *Campylobacter* species. The addition of these acids may reduce the numbers of infected chickens and may possibly reduce or prevent the transmission of *Campylobacter* through the flocks (Hermans *et al.* 2011). The addition of 0.44% lactic acid (Byrd *et al.* 2001) and monocaprin (Thormar *et al.* 2006) to drinking water reduces the colonization rates in chicken.

The chlorination of drinking water also aids in reducing the risk of *Campylobacter* colonization (Hermans *et al.* 2011).

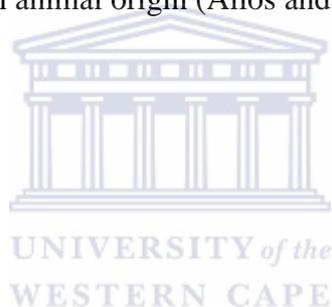
2.6.3 Bacteriophage treatment

Bacteriophages, simply known as phages are viruses that specifically infect bacteria (Humphrey *et al.* 2007), which have been shown to be effective in attacking campylobacters and immediately reducing the numbers of already-contaminated chicken caeca by an approximate three logs (Wagenaar *et al.* 2005). Another study has shown an immediate two log CFU.g⁻¹ reduction in caecal *Campylobacter* levels (El-Shibiny *et al.* 2009b) but campylobacters have the ability to establish itself to its original counts after a sudden drop. This indicates that phage therapy could be administered just before slaughtering to reduce caecal bacterial load. A study by Carvalho *et al.* (2010) has shown that the administration of phages in the feed is more effective than oral administration and that *C. jejuni* was not able to recover and re-establish itself to its original count. El-Shibiny *et al.* (2009b) has also shown that a small percentage of *Campylobacter* exposed to virulent phages develop phage-resistance. Carvalho *et al.* (2010) was also able to isolate phage-resistant *Campylobacter* at a frequency of 13% from chicks previously exposed to phage therapy. Natural phage-resistance was also observed at a lower frequency. Further research on this topic is required as the efficacy of phages to control *C. jejuni* cannot be ensured.

2.6.4 Competitive exclusion – Treatment with prebiotics and probiotics

This method of control aims to prevent *Campylobacter* colonization by prophylactic administration of a cocktail of non-pathogenic gut bacteria. This approach has not yet been successful in the control of *Campylobacter* as the exact exclusion mechanism is not known (Humphrey *et al.* 2007; Hermans *et al.* 2011).

After years of research, there are still no effective, reliable and practical control measures in place to reduce or to completely prevent *Campylobacter* colonisation (Humphrey *et al.* 2007; Hermans *et al.* 2011). It is of outmost importance to identify the sources and routes of infection before control can be applied (Humphrey *et al.* 2007). The development of a reliable and effective strategy to eradicate *Campylobacter* from the environment will probably be overcome by the combination of several strategies tackling the hurdles faced particularly in the poultry industry (Hermans *et al.* 2011). It is therefore imperative that the transmission of infection from animals, animal products and the environment to humans be prevented. Awareness should be raised to adhere to basic hygiene guidelines such as the adequate washing of hands before and after handling pets or petting animals at animal zoos, animal meats and products especially products made with raw chicken for food preparation. It is also important to raise awareness about the necessity for the proper cooking and storage of foods, especially those foods from animal origin (Allos and Lastovica 2011).



2.7 REFERENCES

Aarestrup, F.M., Bager, F., Jensen, N.E., Madsen, M., Meyling, A. and Wegener, H.C. (1998) Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *APMIS* **106**, 606-622.

Aarestrup, F.M. and Engberg, J. (2001) Antimicrobial resistance of thermophilic *Campylobacter*. *Vet Res* **32**, 311-321.

Abeyta, C., Deeter, F.G., Kaysner, C.A., Stott, R.F. and Wekell, M.M. (1993) *Campylobacter jejuni* in a Washington State shellfish growing bed associated with illness. *J Food Prot* **56**, 232-235.

Açık, M.N. and Çetinkaya, B. (2006) Heterogeneity of *Campylobacter jejuni* and *Campylobacter coli* strains from healthy sheep. *Vet Microbiol* **115**, 370-375.

Acke, E., Whyte, P., Jones, B.R., McGill, K., Collins, J.D. and Fanning, S. (2006) Prevalence of thermophilic *Campylobacter* species in cats and dogs in two animal shelters in Ireland. *Vet Rec* **158**, 51-54.

Acke, E., McGill, K., Golden, O., Jones, B.R., Fanning, S. and Whyte, P. (2009) Prevalence of thermophilic *Campylobacter* species in household cats and dogs in Ireland. *Vet Rec* **164**, 44-47.

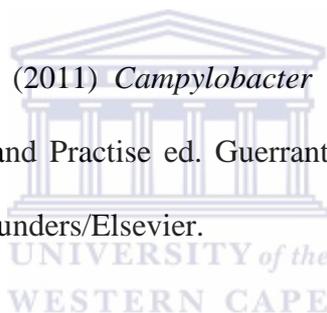
Al-Soud, W. A. and Rådström, P. (2001) Purification and Characterization of PCR-Inhibitory Components in Blood Cells. *J Clin Microbiol* **39**, 485-493.

Alam, K., Lastovica, A. J., le Roux, E., Hossain, M. A., Islam, M. N., Sen, S. K., Sur, G. C., Nair, G. B. and Sack, D. A. (2006) Clinical Characteristics and Serotype Distribution of *Campylobacter jejuni* and *Campylobacter coli* Isolated from Diarrhoeic Patients in Dhaka, Bangladesh, and Cape Town, South Africa. *Bangladesh J Microbiol* **23**, 121-124.

Alanis, A.J. (2005) Resistance to Antibiotics: Are We in the Post-Antibiotic Era? *Arch Med Res* **36**, 697-705.

Allen, V.M., Bull, S.A., Corry, J.E.L., Domingue, G., Jørgensen, F., Frost, J.A., Whyte, R., Gonzalez, A., Elviss, N. and Humphrey, T.J. (2007) *Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonisation. *Int J Food Microbiol* **113**, 54-61.

Allos, B.M. and Lastovica, A.J. (2011) *Campylobacter* Infections. In *Tropical Infectious Diseases: Principles, Pathogens and Practise* ed. Guerrant, R.L., Walker, D.H. and Weller, P.F. pp.145-149. Philadelphia: Saunders/Elsevier.



Alonso, R., Girbau, C. and Astorga, A.F. (2011) *Campylobacter*. In *Molecular Detection of Human Bacterial Pathogens* ed. Liu, D. pp.1125-1139. Boca Raton, Florida: CRC Press, Taylor & Francis Group.

Anonymous (2006) The Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp.: Part 1 – Detection method. ISO 10272-1:2006, International Organisation for Standardisation. SANS, Pretoria, South Africa.

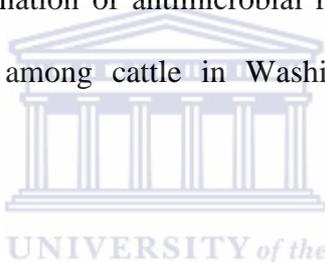
Anonymous (2011) *The South African Poultry Industry Profile for 2010*. Southern African Poultry Association. Honeydew, South Africa: Southern African Poultry Association.

Anonymous (2012) *SAPA Broiler Price Report for the First Quarter of 2012*. Southern African Poultry Association (1Q2012). Honeydew, South Africa: Southern African Poultry Association.

Astorga, A.F. and Alonso, R. (2010) *Campylobacter*. In *Molecular Detection of Foodborne Pathogens* ed. Liu, D. pp.345-360. Boca Raton, Florida: CRC Press, Taylor & Francis Group.

Avrain, L., Humbert, F., L'Hospitalier, R., Sanders, P., Vernozy-Rozand, C. and Kempf, I. (2003) Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. *Vet Microbiol* **96**, 267-276.

Bae, W., Hancock, D.D., Call, D.R., Park, Y.H., Berge, A.C.B., Finger, R.M., Sisco, W.M. and Besser, T.E. (2007) Dissemination of antimicrobial resistant strains of *Campylobacter coli* and *Campylobacter jejuni* among cattle in Washington State and California. *Vet Microbiol* **122**, 306-315.



Baylis, C.L., MacPhee, S., Martin, K.W., Humphrey, T.J. and Betts, R.P. (2000) Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. *J Appl Microbiol* **89**, 884-891.

Bester, L.A. and Essack, S.Y. (2008) Prevalence of antibiotic resistance in *Campylobacter* isolates from commercial poultry suppliers in KwaZulu-Natal, South Africa. *J Antimicrob Chemother* **62**, 1298-1300.

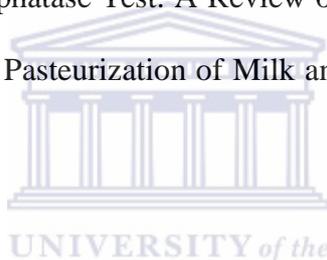
Bester, L.A. and Essack, S.Y. (2010) Antibiotic resistance via the food chain: Fact or Fiction? *S Afr J Sci* **106**, 1-5.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P. and Blaser, M.J. (1988) Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* **157**, 472-479.

Blaser, M. J., Newell, D. G., Thompson, S. A. and Zechner, E. L. (2008) Pathogenesis of *Campylobacter fetus*. In *Campylobacter* ed. Nachamkin, I., Szymanski, C. M. and Blaser, M. J. pp. 401-428. Washington, D.C.: ASM Press.

Bratz, K., Bückler, R., Gölz, G., Zakrzewski, S.S., Janczyk, P., Nöckler, K. and Alter, T. (2012) Experimental infection of weaned piglets with *Campylobacter coli* – Excretion and translocation in a pig colonisation trial. *Veterinary Microbiology*. Article in press. <http://dx.doi.org/10.1016/j.vet-mic.2012.08.016>.

Burgwald, L.H. (1939) The Phosphatase Test. A Review of the Literature on its Application for Detecting Irregularities in the Pasteurization of Milk and Dairy Products. *J Dairy Sci* **22**, 853-873.



Butaye, P., Devriese, L.A. and Haesebrouck, F. (2003) Antimicrobial Growth Promoters Used in Animal Feed: Effects of Less Well Known Antibiotics on Gram-Positive Bacteria. *Clin Microbiol Rev* **16**, 175-188.

Byrd, J.A., Hargis, B.M., Caldwell, D.J., Bailey, R.H., Herron, K.L., McReynolds, J.L., Brewer, R.L., Anderson, R.C., Bischoff, K.M., Callaway, T.R. and Kubena, L.F. (2001) Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on *Salmonella* and *Campylobacter* contamination of broilers. *Poult Sci* **80**, 278-283.

Calciati, E., Lafuente, S., De Simó, M., Balfagon, P., Bartolomé, R. and Caylá, J. (2012) A *Campylobacter* outbreak in a Barcelona school. *Enferm Infecc Microbiol Clin*. **30**, 243-245.

Calderón-Gómez, L.I., Hartley, L.E., McCormack, A., Ringoir, D.D. and Korolik, V. 2009. Potential use of characterised hyper-colonising strain(s) of *Campylobacter jejuni* to reduce circulation of environmental strains in commercial poultry. *Vet Med* **134**, 353-361.

Candrian, U. (1995) Polymerase chain reaction in food microbiology. *J Microbiol Meth* **23**, 89-103.

Carbonero, A., Torralbo, A., Borge, C., García-Bocanegra, I., Arenas, A. and Perea, A. (2012) *Campylobacter* spp., *C. jejuni* and *C. upsaliensis* infection-associated factors in healthy and ill dogs from clinics in Cordoba, Spain. Screening tests for antimicrobial susceptibility. *Comp Immunol Microbiol and Infect Dis*. Article in press. <http://dx.doi.org/10.1016/j.cimid.2012.05.002>.

Carvalho, C.M., Gannon, B.W., Halfhide, D.E., Santos, S.B., Hayes, C.M., Roe, J.M. and Azredo, J. (2010) The *in vivo* efficacy of two administration routes of a phage cocktail to reduce numbers of *Campylobacter coli* and *Campylobacter jejuni* in chickens. *BMC Microbiol* **10**, 232.

Casewell, M., Friis, C., Marco, E., McMullin, P. and Phillips, I. (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother* **52**, 159-161.

Castanon, J.I.R. (2007) History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poult Sci* **86**, 2466-2471.

Chaban, B., Ngeleka, M. and Hill, J.E. (2010) Detection and quantification of 14 *Campylobacter* species pet dogs reveals an increase in species richness in feces of diarrheic animals. *BMC Microbiol* **10**, 1-7.

Chai, L.C., Robin, T., Ragavan, U.M., Gunsalam, J.W., Abu Bakar, F., Ghazali, F.M., Radu, S.F. and Kumar, M.P. (2007) Thermophilic *Campylobacter* spp. in salad vegetables in Malaysia. *Int J Food Microbiol* **117**, 106-111.

Coker, A.O., Isokpehi, R.D., Thomas, B.N., Amisu, K.O. and Obi, C.L. (2002) Human *Campylobacteriosis* in Developing Countries. *Emerg Infect Dis* **8**, 237-243.

Colles, F.M., Jones, T.A., McCarthy, N.D., Sheppard, S.K., Cody, A.J., Dingle, K.E., Dawkins, M.S. and Maiden, M.C.J. (2008) *Campylobacter* infection of broiler chickens in a free range environment. *Environ Microbiol* **10**, 2042-2050.

Corry, J.E.L. and Atabay, H.I. (2001) Poultry as a source of *Campylobacter* and related organisms. *J Appl Microbiol* **90**, 96-114.

Daczkowska-Kozon, E. and Brzostek-Nowakowska, J. (2001) *Campylobacter* spp. in waters of three main Western Pomerania water bodies. *Int J Hyg Environ Health* **203**, 435-443.

Dasti, J.I., Tareen, A.M., Lugert, R., Zautner, A.E. and Groß, U. (2010) *Campylobacter jejuni*: A brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int J Med Microbiol* **300**, 205-211.

Davies, J. (2006) Are antibiotics naturally antibiotics? *J Ind Microbiol Biotechnol* **33**, 496-499.

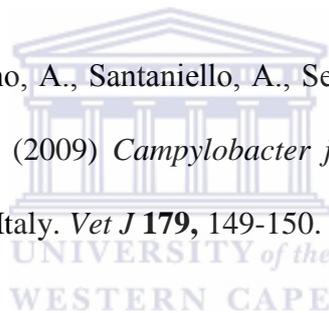
Davies, J. and Davies, D. (2010) Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev* **74**, 417-433.

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S.L.W. and Vandamme, P. (2010) *Campylobacter volucris* sp. nov., isolated from black-headed gulls (*Larus ridibundus*). *Int J Syst Evol Microbiol* **60**, 1870-1875.

Dibner, J.J. and Richards, J.D. (2005) Antibiotic Growth Promoters in Agriculture: History and Mode of Action. *Poult Sci* **84**, 634-643.

Diergaardt, S. M., Venter, S. N., Spreeth, A., Theron, J. and Brözel, V. S. (2004) The occurrence of campylobacters in water sources in South Africa. *Water Res* **38**, 2589-2595.

Dipineto, L., Gargiulo, A., Cuomo, A., Santaniello, A., Sensale, M., Borrelli, L., D'Angelo, L., Menna, L.F. and Fioretti, A. (2009) *Campylobacter jejuni* in the red squirrel (*Sciurus vulgaris*) population of Southern Italy. *Vet J* **179**, 149-150.



Doyle, M.P. and Erickson, M.C. (2006) Reducing the carriage of foodborne pathogens in livestock and poultry. *Poult Sci* **85**, 960-973.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J. (1999) High-Resolution Genotyping of *Campylobacter* Strains Isolated from Poultry and Humans with Amplified Fragment Length Polymorphism Fingerprinting. *Appl Environ Microbiol* **65**, 2369-2375.

Duim, B., Ang, C.W., van Belkum, A., Rigter, A., van Leeuwen, N.W.J., Endtz, H.P. and Wagenaar, J.A. (2000) Amplified Fragment Length Polymorphism Analysis of *Campylobacter jejuni* Strains Isolated from Chickens and from Patients with Gastroenteritis or Guillain-Barré or Miller Fisher Syndrome. *Appl Environ Microbiol* **66**, 3917-3923.

Duim, B., Vandamme, P.A.R., Rigter, A., Laevens, S., Dijkstra, J.R. and Wagenaar, J.A. (2001) Differentiation of *Campylobacter* species by AFLP fingerprinting. *Microbiol* **147**, 2729-2737.

Dykes, G.A. and Moorhead, S.M. (2001) Survival of *Campylobacter jejuni* on vacuum or carbon dioxide packaged primal beef cuts stored at -1.5°C. *Food Control* **12**, 553-557.

El-Sharoud, W.M. (2009) Prevalence and survival of *Campylobacter* in Egyptian dairy products. *Food Res Int* **42**, 622-626.

El-Shibiny, A., Connerton, P. and Connerton, I. (2009a) Survival at refrigeration and freezing temperatures of *Campylobacter coli* and *Campylobacter jejuni* on chicken skin applied as axenic and mixed inoculums. *Int J Food Microbiol* **131**, 197-202.

El-Shibiny, A., Scott, A., Timms, A., Metawea, Y., Connerton, P. and Connerton, I. (2009b) Application of a group II *Campylobacter* bacteriophage to reduce strains of *Campylobacter jejuni* and *Campylobacter coli* colonizing broiler chickens. *J Food Prot* **72**, 733-740.

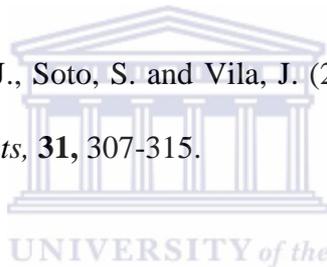
Endtz, H.P., Vliegthart, J.S., Vandamme, P., Weverink, H.W., van den Braak, N.P., Verbrugh, H.A. and van Belkum, A. (1997) Genotypic diversity of *Campylobacter lari* isolated from mussels and oysters in The Netherlands. *Int J Food Microbiol* **34**, 79-88.

Endtz, H.P., Ang, C.W., van den Braak, N., Duim, B., Rigter, A., Price, L.J., Woodward, D.L., Rodgers, F.G., Johnson, W.M., Wagenaar, J.A., Jacobs, B.C., Verbrugh, H.A. and van Belkum, A. (2000) Molecular Characterization of *Campylobacter jejuni* from Patients with Guillain-Barré and Miller Fisher Syndromes. *J Clin Microbiol* **38**, 2297-2301.

Enokimoto, M., Kubo, M., Bozono, Y., Mieno, Y. and Misawa, N. (2007) Enumeration and identification of *Campylobacter* species in the liver and bile of slaughtered cattle. *Int J Food Microbiol* **118**, 259-263.

Erganis, O., Kaya, O., Hadimli, H.H. and Guler, L. (2002) Rapid diagnosis of ovine *Brucella*, *Campylobacter* and *Salmonella* infections from fetal stomach contents by coagglutination test. *Small Rum Res* **45**, 123-127.

Fàbrega, A., Sánchez-Céspedes, J., Soto, S. and Vila, J. (2008). Quinolone resistance in the food chain. *Int J Antimicrob Agents*, **31**, 307-315.



Fahey, T., Morgan, D., Gunneburg, C., Adak, G.K., Majid, F. and Kaczmarek, E. (1995) An Outbreak of *Campylobacter jejuni* Enteritis Associated with Failed Milk Pasteurisation. *J Infect* **31**, 137-143.

Fenwick, S.G., West, D.M., Hunter, J.E.B., Sargison, N.D., Ahmed, F., Lumsden, J.S. and Collett, M.G. (2000) *Campylobacter fetus fetus* abortions in vaccinated ewes. *N Z Vet J* **48**, 155-157.

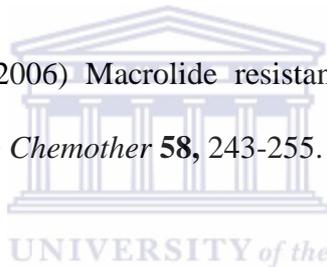
Fernández, H., Vera, F., Villanueva, M.P. and Garcia, A. (2008) Occurrence of *Campylobacter* Species in Healthy Well-Nourished and Malnourished Children. *Braz J Microbiol* **39**, 56-58.

Foster, G., Holmes, B., Steigerwalt, A.G., Lawson, P.A., Thorne, P., Byrer, D.E., Ross, H.M., Xerry, J., Thompson, P.M. and Collins, M.D. (2004) *Campylobacter insulaenigrae* sp. nov., isolated from marine mammals. *Int J Syst Evol Microbiol* **54**, 2369-2373.

Friedman, C. R., Neimann, J., Wegener, H. C. and Tauxe, R. V. (2000) Epidemiology of *Campylobacter jejuni* Infections in the United States and Other Industrialized Nations. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 121-138. Washington, D.C.: ASM Press.

Georgsson, F., Þorkelsson, A.E., Geirsdóttir, M., Reiersen, J. and Stern, N.J. (2006) The influence of freezing and duration of storage on *Campylobacter* and indicator bacteria in broiler carcasses. *Food Microbiol* **23**, 677-683.

Gibreel, A. and Taylor, D.E. (2006) Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J Antimicrob Chemother* **58**, 243-255.



Gill, C.O. and Harris, L.M. (1982) Contamination of red-meat carcasses by *Campylobacter fetus* subsp. *jejuni*. *Appl Environ Microbiol* **43**, 977-980.

Goddard, E. A., Lastovica, A. J. and Argent, A. C. (1997) *Campylobacter* 0:41 isolation in Guillain-Barré Syndrome. *Arch Dis Child* **76**, 526-528.

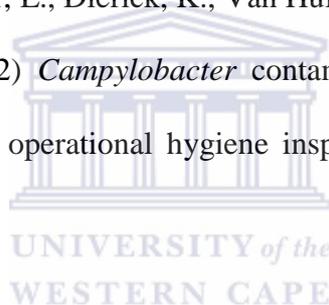
Gorkiewicz, G., Feierl, G., Zechner, R. and Zechner, E.L. (2002) Transmission of *Campylobacter hyointestinalis* from a pig to a human. *J Clin Microbiol* **40**, 2601-2605.

Graeme, K. A. and Pollack, C.V. (1996) Antibiotic use in the emergency department II. The Aminoglycosides, Macrolides, Tetracyclines, Sulfa Drugs and Urinary Antiseptics. *J Emerg Med* **14**, 361-371.

Guillou, S., Leguerinel, I., Garrec, N., Renard, M.A., Cappelier, J.M. and Federighi, M. (2008) Survival of *Campylobacter jejuni* in mineral bottled water according to difference in mineral content: Application of the Weibull model. *Water Res* **42**, 2213-2219.

Habib, I., Uyttendaele, M. and De Zutter, L. (2010) Survival of poultry-derived *Campylobacter jejuni* of multilocus sequence type clonal complexes 21 and 45 under freeze, chill, oxidative, acid and heat stresses. *Food Microbiol* **27**, 829-834.

Habib, I., Berkvens, D., De Zutter, L., Dierick, K., Van Huffel, X., Speybroeck, N., Geeraerd, A.H. and Uyttendaele, M. (2012) *Campylobacter* contamination in broiler carcasses and correlation with slaughterhouses operational hygiene inspection. *Food Microbiol* **29**, 105-112.



Hänel, I., Borrmann, E., Müller, J., Müller, W., Pauly, B., Liebler-Tenorio, E.M. and Schulze, F. (2009) Genotypic and phenotypic changes of *Campylobacter jejuni* strains after passage of the chicken gut. *Vet Microbiol* **136**, 121-129.

Hannu, T., Mattila, L., Rautelin, H., Pelkonen, P., Lahdenne, P., Siitonen, A. and Leirisalo-Repo, M. (2002) *Campylobacter*-triggered reactive arthritis: a population-based study. *Rheumatol* **41**, 312-318.

Hannu, T., Kauppi, M., Tuomala, M., Laaksonen, I. and Klemets, P. (2004) Reactive Arthritis Following an Outbreak of *Campylobacter jejuni* Infection. *J Rheumatol* **31**, 528-530.

Hansson, I., Pudas, N., Harbom, B. and Engvall, E.O. (2010) Within-flock variations of *Campylobacter* loads in caeca and on carcasses from broilers. *Int J Food Microbiol* **141**, 51-55.

Harris, L. J. and Griffiths, M. W. (1992) The detection of foodborne pathogens by the polymerase chain reaction (PCR). *Food Res Int* **25**, 457-469.

Harvey, S. and Greenwood, J.R. (1985) Isolation of *Campylobacter fetus* from a Pet Turtle. *J Clin Microbiol* **21**, 260-261.

Hemsworth, S. and Pizer, B. (2006) Pet ownership in immunocompromised children – A review of the literature and survey of existing guidelines. *Eur J Oncol Nurs* **10**, 117-127.

Hermans, D., Van Deun, K., Messens, W., Martel, A., Van Immerseel, F., Haesebrouck, F., Rasschaert, G., Heyndrickx, M. and Pasmans, F. (2011) *Campylobacter* control in poultry by current intervention measures ineffective: Urgent need for intensified fundamental research. *Vet Microbiol* **152**, 219-228.

Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J.J.H.C., Bos, M.H., Heilmann, F.G.C., Hofhuis, A., Hoekstra, T., de Boer, E. (2009) Two outbreaks of campylobacteriosis associated with the consumption of raw cow's milk. *Int J Food Microbiol* **134**, 70-74.

Hiramatsu, K., Igarashi, M., Morimoto, Y., Baba, T., Umekita, M. and Akamatsu, Y. (2012) Curing bacteria of antibiotic resistance: reverse antibiotics, a novel class of antibiotics in nature. *Int J Antimicrob Agents* **39**, 478-485.

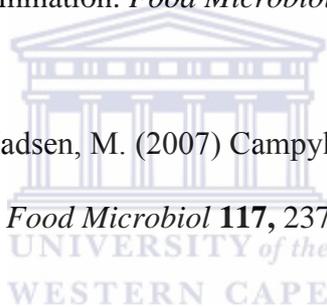
Ho, H.T.K., Lipman, L.J.A. and Gaastra, W. (2006) *Arcobacter*, what is known and unknown about a potential foodborne zoonotic agent! *Vet Microbiol* **115**, 1-13.

Horrocks, S.M., Anderson, R.C., Nisbet, D.J. and Ricke, S.C. (2009) Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* **15**, 18-25.

Houf, K. (2011) *Arcobacter*. In Molecular Detection of Human Bacterial Pathogens ed. Liu, D. pp.1111-1123. Boca Raton, Florida: CRC Press, Taylor & Francis Group.

Hue, O., Allain, V., Laisney, M., Le Bouquin, S., Lalande, F., Petetin, I., Rouxel, S., Quesne, S., Gloaguen, P., Picherot, M., Santolini, J., Bougeard, S., Salvat, G. and Chemaly, M. (2011) *Campylobacter* contamination of broiler caeca and carcasses at the slaughterhouse and correlation with *Salmonella* contamination. *Food Microbiol* **28**, 862-868.

Humphrey, T., O'Brien, S. and Madsen, M. (2007) Campylobacters as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* **117**, 237-257.



Hussain, I., Mahmood, M.S., Akhtar, M. and Khan, A. (2007) Prevalence of *Campylobacter* species in meat, milk and other food commodities in Pakistan. *Food Microbiol* **24**, 219-222.

Ishihara, K., Kira, T., Ogikubo, K., Morioka, A., Kojima, A., Kijima-Tanaka, M., Takahashi, T. and Tamura, Y. (2004) Antimicrobial susceptibilities of *Campylobacter* isolated from food-producing animals on farms (1999–2001): results from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. *Int J Antimicrob Agents* **24**, 63-69.

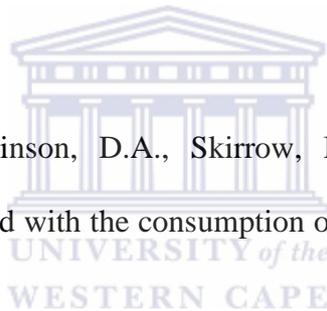
Jacob, P., Mdegela, R.H. and Nonga, H.E. (2011) Comparison of Cape Town and Skirrow's *Campylobacter* isolation protocols in humans and broilers in Morogoro, Tanzania. *Trop Anim Health Prod* **43**, 1007-1013.

Jacobs-Reitsma, W. (2000) *Campylobacter* in the Food Supply. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 467-481. Washington, D.C.: ASM Press.

Jensen, A.N., Dalsgaard, A., Baggesen, D.L. and Nielsen, E.M. (2006) The occurrence and characterization of *Campylobacter jejuni* and *C. coli* in organic pigs and their outdoor environment. *Vet Microbiol* **116**, 96-105.

Jones, K. (2001) *Campylobacters* in water, sewage and the environment. *J Appl Microbiol* **90**, 68-79.

Jones, P.H., Willis, A.T., Robinson, D.A., Skirrow, M.B. and Josephs, D.S. (1981) *Campylobacter* enteritis associated with the consumption of free school milk. *J Hyg* **87**, 155-162.



Kaakoush, N. O., Miller, W. G., De Reuse, H. and Mendz, G. L. (2007) Oxygen requirement and tolerance of *Campylobacter jejuni*. *Res Microbiol* **158**, 644-650.

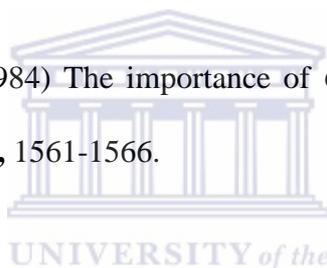
Kärenlampi, R. and Hänninen, M.L. (2004) Survival of *Campylobacter jejuni* on various fresh produce. *Int J Food Microbiol* **97**, 187-195.

Kemper, N., Aschfalk, A. and Höller, C. (2006) *Campylobacter* spp., *Enterococcus* spp., *Escherichia coli*, *Salmonella* spp., *Yersinia* spp., and *Cryptosporidium* oocysts in semi-domesticated reindeer (*Rangifer tarandus tarandus*) in Northern Finland and Norway. *BMC Acta Vet Scand* **48**, 1-7.

Koene, M.G.J., Houwers, D.J., Dijkstra, J.R., Duim, B. and Wagenaar, J.A. (2009) Strain variation within *Campylobacter* species in faecal samples from dogs and cats. *Vet Microbiol* **133**, 199-205.

Kokotovic, B. and On, S.L.W. (1999) High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. *FEMS Microbiol Lett* **173**, 77-84.

Kotula, A.W. and Stern, N.J. (1984) The importance of *Campylobacter jejuni* to the meat industry: a review. *J Anim Sci* **58**, 1561-1566.



Kramer, J.M., Frost, J.A., Bolton, F.J. and Wareing, D.R.A. (2000) *Campylobacter* contamination of raw meat and poultry at retail sale: Identification of multiple types and comparison with isolates from human infection. *J Food Prot* **63**, 1654-1659.

Lastovica, A. J. (2006) Emerging *Campylobacter* spp.: the Tip of the Iceberg, *Clin Microbiol Newslett* **28**, 49-56.

Lastovica, A. J. (2009) Clinical relevance of *Campylobacter concisus* isolated from pediatric patients. *J Clin Microbiol* **47**, 2360.

Lastovica, A.J. and Penner, J.L. (1983) Serotypes of *Campylobacter jejuni* and *Campylobacter coli* in Bacteremic, Hospitalized Children. *J Infect Dis* **147**, 592.

Lastovica, A.J. and Skirrow, M.B. (2000) Clinical Significance of *Campylobacter* and Related Species Other than *Campylobacter jejuni* and *C. coli*. In *Campylobacter* ed Nachamkin, I. and Blaser, M.J. pp. 89-120. Washington, D.C.: ASM Press.

Lastovica, A. J. and le Roux, E. (2003a) Optimal detection of *Campylobacter* spp in stools. *J Clin Pathol* **56**, 480.

Lastovica, A. J. and le Roux, E. (2003b) Prevalence and optimal detection of *Campylobacter upsaliensis* in stools. *Clin Infect Dis* **36**, 1624-1625.

Lastovica, A. J. and Allos, B. M. (2008) Clinical Significance of *Campylobacter* and Related Species other than *Campylobacter jejuni* and *Campylobacter coli*. In *Campylobacter* ed. Nachamkin, I., Szymanski, C. M. and Blaser, M. pp. 123-149. Washington, D.C.: ASM Press.

Ligowska, M., Cohn, M.T., Stabler, R.A., Wren, B.W. and Brønsted, L. (2011) Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int J Food Microbiol* **145**, 111-115.

Lindstedt, B.A., Heir, E., Vardund, T., Melby, K.K. and Kapperud, G. (2000) Comparative Fingerprinting Analysis of *Campylobacter jejuni* subsp. *jejuni* Strains by Amplified-Fragment Length Polymorphism Genotyping. *J Clin Microbiol* **38**, 3379-3387.

Lynch, O.A., Cagney, C., McDowell, D.A. and Duffy, G. (2010) A method for the growth and recovery of 17 species of *Campylobacter* and its subsequent application to inoculated beef. *J Microbiol Meth* **83**, 1-7.

Maal-Bared, R., Bartlett, K.H., Bowie, W.R. and Hall, E.R. (2012) *Campylobacter* spp. distribution in biofilms on different surfaces in an agricultural watershed (Elk Creek, British Columbia): Using biofilms to monitor for *Campylobacter*. *Int J Hyg Environ Health* **215**, 270-278.

Maridor, M.L., Denis, M., Lalande, F., Beaurepaire, B., Cariolet, R., Fravallo, P., Federighi, M., Seegers, H. and Belloc, C. (2008) Experimental infection of specific pathogen –free pigs with *Campylobacter*: Excretion in faeces and transmission to non-inoculated pigs. *Vet Microbiol* **131**, 309-317.

Martinez, J.L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* **157**, 2893-2902.



McCarthy, N. and Giesecke, J. (2001) Incidence of Guillain-Barré Syndrome following Infection with *Campylobacter jejuni*. *Am J Epidemiol* **153**, 610-614.

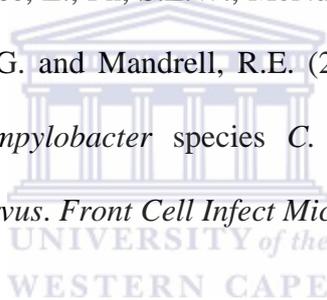
McFadyean, J. & Stockman, S. (1913). *Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion. Part III. Abortion in sheep.* London: HMSO.

Melero, B., Diez, A.M., Rajkovic, A., Jamie, I. and Rovira, J. (2012) Behaviour of non-stressed and stressed *Listeria monocytogenes* and *Campylobacter jejuni* on fresh chicken burger meat packaged under modified atmosphere and inoculated with protective culture. *Int J Food Microbiol* **158**, 107-112.

Messens, W., Herman, L., De Zutter, L. and Heyndrickx, M. (2009) Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. *Vet Microbiol* **138**, 120-131.

Meudt, H.M. and Clarke, A.C. (2007) Almost Forgotten or Latest Practise? AFLP applications, analyses and advances. *Trends Plant Sci* **12**, 106-117.

Miller, W.G., Chapman, M.H., Yee, E., Pn, S.L.W., McNulty, D.K., Lastovica, A.J., Carroll, A.M., McNamara, E.B., Duffy, G. and Mandrell, R.E. (2012) Multilocus sequence typing methods for the emerging *Campylobacter* species *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus*, and *C. curvus*. *Front Cell Infect Microbiol* **2**, 1-12.



Misawa, N., Shinohara, S., Satoh, H., Itoh, H., Shinohara, K., Shimomura, K., Kondo, F. and Itoh, K. (2000) Isolation of *Campylobacter* species from zoo animals and polymerase chain reaction-based random amplified polymorphism DNA analysis. *Vet Microbiol* **71**, 59-68.

Moore, J., Caldwell, P. and Millar, B. (2001) Molecular detection of *Campylobacter* spp. in drinking, recreational and environmental water supplies. *Int J Hyg Environ Health* **204**, 185-189.

Moore, J.E., Barton, M.D., Blair, I.S., Corcoran, D., Dooley, J.S.G., Fanning, S., Kempf, I., Lastovica, A.J., Lowery, C.J., Matsuda, M., McDowell, D.A., McMahon, A., Millar, B.C.,

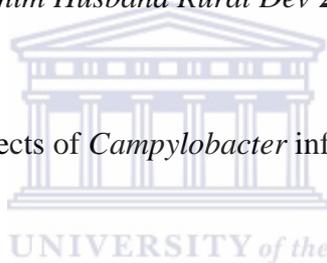
Rao, J.R., Rooney, P.J., Seal, B.S., Snelling, W.J. and Tolba, O. (2006) The epidemiology of antibiotic resistance in *Campylobacter*. *Microbe Infect* **8**, 1955-1966.

Moore, J.E. and Rooney, P.J. (2010) Human campylobacteriosis and bird-pecked milk. *Br Food J* **112**, 151-154.

Moreno, Y., Botella, S., Alonso, J.L., Ferrús, M.A., Hernández, M. and Hernández, J. (2003) Specific Detection of *Arcobacter* and *Campylobacter* Strains in Water and Sewage by PCR and Fluorescent In situ Hybridization. *Appl Environ Microbiol* **69**, 1181-1186.

Mtileni, B.J., Muchadeyi, F.C., Maiwashe, A., Phitsane, P.M., Halimani, T.E., Chimonyo, M. and Dzama, K. (2009) Characterisation of production systems for indigenous chicken genetic resources of South Africa. *Appl Anim Husband Rural Dev* **2**, 18-22.

Nachamkin, I. (2002) Chronic effects of *Campylobacter* infection. *Microbe Infect* **4**, 399-403.



Nachamkin, I., Allos, B. M. and Ho, T. (1998) *Campylobacter* species and Guillain-Barré Syndrome. *Clin Microbiol Rev* **11**, 555-567.

Nachamkin, I., Engberg, J. and Aarestrup, F. M. (2000) Diagnosis and Antimicrobial Susceptibility of *Campylobacter* Species. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 45-66. Washington, D.C.: ASM Press.

Nauta, M. J., van der Wal, F. J., Putirulan, F. F., Post, J., van de Kasstele, J. and Bolder, N. M. (2009a) Evaluation of the “testing and scheduling” strategy for control of *Campylobacter* in broiler meat in the Netherlands. *Int J Food Microbiol* **134**, 216-222.

Nauta, M., Hill, A., Rosenquist, H., Brynstad, S., Fetsch, A., van der Logt, P., Fazil, A., Christensen, B., Katsma, A., Borck, B. and Havelaar, A. (2009b) A comparison of risk assessments on *Campylobacter* in broiler meat. *Int J Food Microbiol* **129**, 107-123.

Neill, S.D., Ellis, W.A. and O'Brien, J.J. (1978) The biochemical characteristics of *Campylobacter*-like organisms from cattle and pigs. *Res Vet Sci* **25**, 368-372.

Neill, S.D., Ellis, W.A. and O'Brien, J.J. (1979) Designation of aerotolerant *Campylobacter*-like organisms from porcine and bovine abortions to the genus *Campylobacter*. *Res Vet Sci* **27**, 180-186.

Newell, D.G. and Wagenaar, J.A. (2000) Poultry Infections and their Control at the Farm Level. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 497-509. Washington, D.C.: ASM Press.

Newell, D.G., Frost, J.A., Duim, B., Wagenaar, J.A., Madden, R.H., van der Plas, P. and On, S.L.W. (2000) New Developments in the Subtyping of *Campylobacter* Species. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 27-44. Washington, D.C.: ASM Press.

Ogden, I.D., Dallas, J.F., MacRae, M., Rotariu, O., Reay, K.W., Leitch, M., Thomson, A.P., Sheppard, S.K., Maiden, M., Forbes, K.J. and Strachan, N.J.C. (2009) *Campylobacter* Excreted into the Environment by Animal Sources: Prevalence, Concentration Shed, and Host Association. *Foodborne Pathog Dis* **6**, 1161-1170.

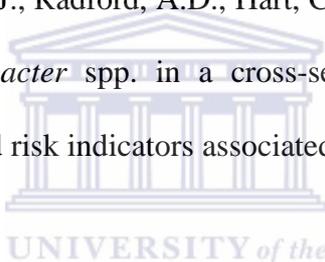
Olaimat, A.N. and Holley, R.A. (2012) Factors influencing the microbial safety of fresh produce: A review. *Food Microbiol* **32**, 1-19.

On, S.L.W. (2001) Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. *J Appl Microbiol* **90**, 1S-15S.

On, S.L.W. and Harrington, C.S. (2000) Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. *FEMS Microbiol Lett* **193**, 161-169.

Ono, K. and Yamamoto, K. (1999) Contamination of meat with *Campylobacter jejuni* in Saitama, Japan. *Int J Food Microbiol* **47**, 211-219.

Parsons, B.N., Porter, C.J., Ryvar, R., Stavisky, J., Williams, N.J., Pinchbeck, G.L., Birtles, R.J., Christley, R.M., German, A.J., Radford, A.D., Hart, C.A., Gaskell, R.M. and Dawson, S. (2010) Prevalence of *Campylobacter* spp. in a cross-sectional study of dogs attending veterinary practices in the UK and risk indicators associated with shedding. *Vet J* **184**, 66-70.



Parsons, B.N., Williams, N.J., Pinchbeck, G.L., Christley, R.M., Hart, C.A., Gaskell, R.M. and Dawson, S. (2011) Prevalence and shedding patterns of *Campylobacter* spp. in longitudinal studies of kennelled dogs. *Vet J* **190**, 249-254.

Penner, J. L. (1988) The Genus *Campylobacter*: a Decade of Progress. *Clin Microbiol Rev* **1**, 157-172.

Pigrau, C., Bartolome, R., Almirante, B., Planes, A., Gavalda, J. and Pahissa, A. (1997) Bacteremia Due to *Campylobacter* Species: Clinical Findings and Antimicrobial Susceptibility Patterns. *Clin Infect Dis* **25**, 1414-1420.

Potturi-Venkata, L. P., Backert, S., Lastovica, A. J., Vieira, S. L., Norton, R. A., Miller, R. S., Pierce, S. and Oyarzabal, O. A. (2007) Evaluation of Different Plate Media for Direct Cultivation of *Campylobacter* Species from Live Broilers. *Poult Sci* **86**, 1304-1311.

Prescott, L. M., Harley, J. P. and Klein, D. A. (2005) *Microbiology*, New York, McGraw-Hill.

Qian, H., Pang, E., Chang, J., Toh, S.K., Ng, F.K., Tan, A.L. and Kwang, J. (2008) Monoclonal antibody binding to the major outer membrane protein of *Campylobacter coli*. *J Immunol Meth* **339**, 104-113.

Raji, M.A., Adekeye, J.O., Kwaga, J.K.P. and Bale, J.O.O. (2000) Bioserogroups of *Campylobacter* species isolated from sheep in Kaduna State, Nigeria. *Small Ruminant Research* **37**, 215 – 221.

Rajkovic, A., Tomic, N., Smigic, N., Uyttendaele, M., Ragaert, P. and Devlieghere, F. (2010) Survival of *Campylobacter jejuni* on raw chicken legs packed in high-oxygen or high-carbon dioxide atmosphere after the decontamination with lactic acid/sodium lactate buffer. *Int J Food Microbiol* **140**, 201-206.

Reich, F., Atanassova, V., Haunhorst, E. and Klein, G. (2008) The effects of *Campylobacter* numbers in caeca on the contamination of broiler carcasses with *Campylobacter*. *Int J Food Microbiol* **127**, 116-120.

Ribot, E.M., Fitzgerald, C., Kubota, K., Swaminathan, B. and Barrett, T.J. (2001) Rapid Pulsed-Field Gel Electrophoresis Protocol for Subtyping of *Campylobacter jejuni*. *J Clin Microbiol* **39**, 1889-1894.

Robinson, D.A., 1981. Infective dose of *Campylobacter jejuni* in milk. *BMJ* **282**, 1584.

Rosenquist, H., Sommer, H.M., Nielsen, N.L. and Christensen, B.B. (2006) The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *Int J Food Microbiol* **108**, 226-232.

Rossi, M., Hänninen, M.L., Revez, J., Hannula, M. and Zanoni, R.G. (2008) Occurrence and species level diagnostics of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in healthy and diarrheic dogs and cats. *Vet Microbiol* **129**, 304-314.

Ruiz-Palacios, G. M. (2007) The Health Burden of *Campylobacter* Infection and the Impact of Antimicrobial Resistance: Playing Chicken. *Clin Infect Dis* **44**, 701-703.

Saito, S., Yatsuyanagi, J., Harata, S., Ito, Y., Shinagawa, K., Suzuki, N, Amano, K. and Enomoto, K. (2005) *Campylobacter jejuni* isolated from retail poultry meat, bovine feces and bile, and human diarrheal samples in Japan: comparison of serotypes and genotypes. *FEMS Immunol Med Microbiol* **45**, 311-319.

Salfield, N.J. and Pugh, E.J. (1987) *Campylobacter* enteritis in young children living in households with puppies. *BMJ* **294**, 21-22.

Sallam, K.I. (2007) Prevalence of *Campylobacter* in chicken and chicken by-products retailed in Sapporo area, Hokkaido, Japan. *Food Control* **18**, 1113-1120.

Samie, A., Obi, C.L., Barrett, L.J., Powell, S.M. and Guerrant, R.L. (2007) Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: Studies using molecular diagnostic methods. *J Infect* **54**, 558-566.

Samosornsuk, W., Asakura, M., Yoshida, E., Taguchi, T., Nishimura, K., Eampokalap, B., Phongsisay, V., Chaicumpa, W. and Yamasaki, S. (2007) Evaluation of a Cytolethal Distending Toxin (*cdt*) Gene-Based Species-Specific Multiplex PCR Assay for the Identification of *Campylobacter* Strains Isolated from Poultry in Thailand. *Microbiol Immunol* **51**, 909-917.

Sampers, I., Habib, I., Berkvens, D., Dumoulin, A., De Zutter, L. and Uyttendaele, M. (2008) Processing practises contributing to *Campylobacter* contamination in Belgian chicken meat preparations. *Int J Food Microbiol* **128**, 297-303.

Sampers, I., Habib, I., De Zutter, L., Dumoulin, A. and Uyttendaele, M. (2010) Survival of *Campylobacter* spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *Int J Food Microbiol* **137**, 147-153.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. and Lenstra, J.A. (1999) Amplified Fragment Length Polymorphism Analysis: the State of an Art. *J Clin Microbiol* **37**, 3083-3091.

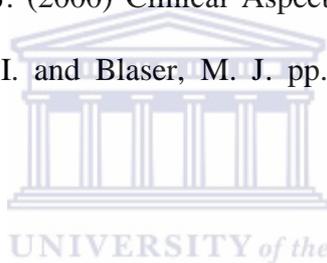
Sebald, M. and Véron, M. (1963) Teneur en bases de l'AND et classification des vibrions. *Ann Inst Pasteur* **105**, 897-910.

Sheppard, S.K., Colles, F., Richardson, J., Cody, A.J., Elson, R., Lawson, A., Brick, G., Meldrum, R., Little, C.L., Owen, R.J., Maiden, M.C.J. and McCarthy, N.D. (2010) Host Association of *Campylobacter* Genotypes Transcends Geographical Variation. *Appl Environ Microbiol* **76**, 5269-5277.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. and Teixeira, P. (2011) *Campylobacter* spp. as a foodborne pathogen: a review. *Front Microbiol* **2**, 1-12.

Sippy, R., Sandoval-Green, C.M.J., Sahin, O., Plummer, P., Fairbanks, W.S., Zhang, Q. and Blanchong, J.A. (2012) Occurrence and molecular analysis of *Campylobacter* in wildlife on livestock farms. *Vet Microbiol* **157**, 369-375.

Skirrow, M. B. and Blaser, M. J. (2000) Clinical Aspects of *Campylobacter* Infection. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 69-88. Washington, D.C.: ASM Press.



Smibert, R. M. 1984. Genus *Campylobacter*. In *Bergey's manual of systematic bacteriology Volume 1* ed. Krieg, N. R. and Holt, J.G. pp. 111-118. Baltimore: Williams and Wilkins.

Smith, T. and Taylor, M. (1919) Some morphological and biological characters of the spirilla (*Vibrio fetus*, n. sp.) associated with disease of the fetal membranes in cattle. *J Exp Med* **30**, 299-311.

Sockett, P.N. and Rodgers, F.G. (2001) Enteric and foodborne disease in children: A review of the influence of food- and environment-related risk factors. *Paediatr Child Health* **6**, 203-209.

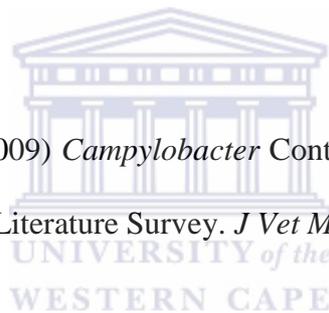
Stampi, S., De Luca, G., Varoli, O. and Zanetti, F. (1999) Occurrence, Removal and Seasonal Variation of Thermophilic *Campylobacters* and *Arcobacter* in Sewage Sludge. *Zbl Hyg Umweltmed* **202**, 19-27.

Stanley, K. and Jones, K. (2003) Cattle and sheep farms as reservoirs of *Campylobacter*. *J Appl Microbiol* **94**, 104-113.

Strachan, N.J.C. and Forbes, K.J. (2010) The growing UK epidemic of human campylobacteriosis. *Lancet* **376**, 665-666.

Stoyanchev, T. T. (2004) Detection of *Campylobacter* using standard culture and PCR of 16S rRNA gene in freshly chilled poultry and poultry products in a slaughterhouse. *Trakia J Sci* **2**, 59-64.

Suzuki, H. and Yamamoto, S. (2009) *Campylobacter* Contamination in Retail Poultry Meats and By-Products in the world: A Literature Survey. *J Vet Med Sci* **71**, 255-261.



Talibart, R., Denis, M., Castillo, A., Cappelier, J.M. and Ermel, G. (2000) Survival and recovery of viable but noncultivable forms of *Campylobacter* in aqueous microcosm. *Int J Food Microbiol* **55**, 263-267.

Thormar, H., Hilmansson, H. and Bergsson, G. (2006) Stable concentrated emulsions of the 1-monoglyceride of capric acid (monocaprin) with microbicidal activities against the food-borne bacteria *Campylobacter jejuni*, *Salmonella* spp., and *Escherichia coli*. *Appl Environ Microbiol* **72**, 522-526.

Tu, Z.C., Zeitlan, G., Gagner, J.P., Keo, T., Hanna, B.A. and Blaser, M.J. (2004) *Campylobacter fetus* of Reptile Origin as a Human Pathogen. *J Clin Microbiol* **42**, 4405-4407.

Tu, Z.C., Eisner, W., Kreiswirth, B.N. and Blaser, M.J. (2005) Genetic Divergence of *Campylobacter fetus* Strains of Mammal and Reptile Origins. *J Clin Microbiol* **43**, 3334-3340.

Uaboi-Egbenni, P.O., Bessong, P.O., Samie, A. and Obi, C.L. (2011) Prevalence, haemolysis and antibiograms of *Campylobacters* isolated from pigs from three farm settlements in Venda region, Limpopo province, South Africa. *Afr J Biotechnol* **10**, 703-711.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) Revision of *Campylobacter*, *Helicobacter* and *Wolinella* Taxonomy: Emendation of Generic Descriptions and Proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* **41**, 88-103.

Vandamme, P. (2000) Taxonomy of the family *Campylobacteraceae* In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 3-26. Washington, D.C.: ASM Press.

van de Giessen, A.W., Tilburg, J.J., Ritmeester, W.S. and van der Plas, J. (1998) Reduction of *Campylobacter* infections in broiler flocks by application of hygiene measures. *Epidemiol Infect* **121**, 57-66.

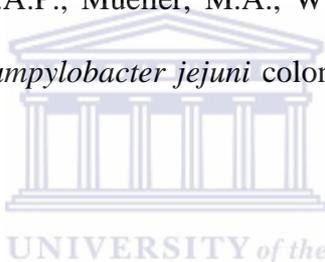
van den Bogaard, A.E. and Stobberingh, E.E. (2000) Epidemiology of resistance to antibiotics Links between animals and humans. *Int J Antimicrob Agents* **14**, 327-335.

van Gerwe, T., Miflin, J.K., Templeton, J.M., Bouma, A., Wagenaar, J.A., Jacobs-Reitsma, W.F., Stegeman, A. and Klinkenberg, D. (2009) Quantifying transmission of *Campylobacter jejuni* in commercial broiler flocks. *Appl Environ Microbiol* **75**, 625-628.

Verhoeff-Bakkenes, L., Jansen, H.A.P.M., in 't Veld, P.H. and Beumer, R.R., Zwietering, M.H. and van Leusden, F.M. (2011) Consumption of raw vegetables and fruits: A risk factor for *Campylobacter* infections. *Int J Food Microbiol* **144**, 406-412.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijyers, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407-4414.

Wagenaar, J.A., Van Bergen, M.A.P., Mueller, M.A., Wassenaar, T.M. and Carlton, R.A. (2005) Phage therapy reduces *Campylobacter jejuni* colonization in broilers. *Vet Microbiol* **109**, 275-283.



Wassenaar, T.M., Fry, B.N., Lastovica, A.J., Wagenaar, J.A., Coloe, P.J. and Duim, B. (2000) Genetic Characterization of *Campylobacter jejuni* 0:41 Isolates in Relation with Guillain-Barré Syndrome. *J Clin Microbiol* **38**, 874-876.

Wassenaar, T. M. and Newell, D. G. (2007) The Genus *Campylobacter*. In *The Prokaryotes* ed. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. and Stackebrandt, E. pp.119-138. New York: Springer-Verlag.

Wieczorek, K., Szewczyk, R. and Osek, J. (2012) Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. *Vet Med* **57**, 293-299.

Williams, L. K., Jørgensen, F., Grogono-Thomas, R. and Humphrey, T. J. (2009) Enrichment culture for the isolation of *Campylobacter* spp: Effects of incubation conditions and the inclusion of blood in selective broths. *Int J Food Microbiol* **130**, 131-134.

Xingwana, L. (2008) *Publication of South African Policy on Animal Feeds*. Department of Agriculture, Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act no.36 of 1947). No. 31005 Notice 511 of 2008. pp 181-194. Government Gazette.

Zhang, L., Man, S.M., Day, A.S., Leach, S.T., Lemberg, D.A., Dutt, S., Stormon, M., Otley, A., O'Loughlin, E.V., Magoffin, A., Ng, P.H.Y. and Mitchell, H. (2009) Detection and Isolation of *Campylobacter* Species Other than *C. jejuni* from Children with Crohn's Disease. *J Clin Microbiol* **47**, 453-455.

Zhao, H., Liu, H., Du, Y., Liu, S., Ni, H., Wang, Y., Wang, C., Si, W. Yang, J. and Ling, J. (2010) Development and evaluation of an indirect enzyme-linked immunosorbent assay for the detection of antibodies against *Campylobacter fetus* in cattle. *Res Vet Sci* **88**, 446-451.

Zirnstein, G., Li, Y., Swaminathan, B. and Angulo, F. (1999) Ciprofloxacin Resistance in *Campylobacter jejuni* Isolates: Detection of *gyrA* Resistance Mutations by Mismatch Amplification Mutation Assay PCR and DNA Sequence Analysis. *J Clin Microbiol* **37**, 3276-3280.

Zweifel, C., Zychowska, M.A. and Stephan, R. (2004) Prevalence and characteristics of Shiga toxin-producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. isolated from slaughtered sheep in Switzerland. *Int J Food Microbiol* **92**, 45-53.

Zweifel, C., Scheu, K.D., Keel, M., Renggli, F. and Stephan, R. (2008) Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. *Int J Food Microbiol* **125**, 182-187.



Chapter 3 – The isolation and biochemical characterization of *Campylobacter* species from South African free range and commercial chicken

3.1 ABSTRACT

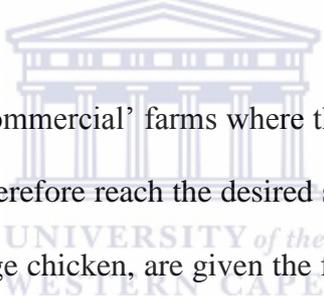
Campylobacter species are the most common bacteria associated with acute diarrhoea and is responsible for 400 to 500 million reported cases globally. It is not uncommon for 35% to 85% of chicken flocks to be infected with campylobacters and it is because of this high prevalence that chicken is considered to be the primary source of *Campylobacter* contamination in the domestic setting. Therefore, a very high risk of acquiring campylobacteriosis is associated with the mishandling and consumption of contaminated chicken. The present study had isolated a total number of 156 *Campylobacter* isolates, of which 102 isolates were *C. jejuni* and 51 were *C. coli*. The speciation of 3 *Campylobacter* isolates could not be determined. It had shown that there is a high prevalence of *Campylobacter* in South African chicken. Retail chicken (n = 84) has a lower prevalence of 27% whereas chicken sampled directly from the abattoir (n = 182), but also intended for human consumption, had an average prevalence of 73%. It also showed that free range chicken (n = 118) has a higher prevalence (average of 79%) of *Campylobacter* than commercial chicken (n = 64) (average of 56%). It is for this reason that free range chicken is not always the safer option considering that the purchasing of free range chicken is becoming more popular for health reasons. There is no standardized universal isolation protocol for *Campylobacter* species and the current isolation techniques creates a bias for the optimal growth of *C. jejuni* and *C. coli*, the two thermotolerant species most commonly associated with human illnesses. Recently, the non-selective Cape Town Protocol was designed for efficient isolation of campylobacters from clinical samples and proved to be superior to the former techniques in the isolation of the thermotolerant campylobacters as well as emerging

campylobacters. However, the protocol is not suited to the isolation of *Campylobacter* from food samples. This study successfully optimized the Cape Town protocol by incorporating the use of the selective Bolton broth for the recovery and enrichment of injured cells from raw chicken samples. The technique proved to be equal in isolation efficiency to the ISO 1272-1:2006 method but loses its ability to recover all campylobacters that may be present in the food sample. It is for this reason that a non selective enrichment broth should be sought but the technique boasts superiority over the ISO 10272-1:2006 method in that it reduces the time in obtaining the results at least by 48 h and is more cost effective.



3.2 INTRODUCTION

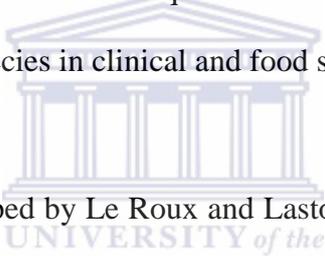
Campylobacter species are considered to be the leading bacterial causative agent of human gastroenteritis globally (Colles *et al.* 2008; Habib *et al.* 2012). It is responsible for approximately 2.5 million reported cases of gastroenteric illnesses in the US and in excess of 340 000 cases in the UK annually while statistics show that it causes 400 to 500 million acute cases of diarrhoea each year, globally (Humphrey *et al.* 2007; Colles *et al.* 2008; Horrocks *et al.* 2009). It is found at a high prevalence in raw poultry carcasses, most often between 35% (Rosenquist *et al.* 2006) and 85% (Sampers *et al.* 2008; El-Shibiny *et al.* 2009; Horrocks *et al.* 2009; Ligowska *et al.* 2011). These prevalences suggest that the mishandling of raw poultry and the consumption of undercooked poultry contribute significantly to the amount of campylobacteriosis cases reported annually (Oyarzabal *et al.* 2005; Colles *et al.* 2008).



Chickens are mainly reared on ‘commercial’ farms where the chickens are housed and fed *ad libitum*. They grow rapidly and therefore reach the desired slaughter weight between 35 to 42 days. Their counterparts, free range chicken, are given the freedom to roam outdoors (Avrain *et al.* 2003; Humphrey *et al.* 2007) and their feed is not supplemented with antibiotic growth promoters (AGPs) (Colles *et al.* 2008). They therefore take longer to grow and are slaughtered at approximately 80 days of age (Avrain *et al.* 2003). They are marketed as ‘healthier’ chickens compared to their housed counterparts and as a result, free range and organic chicken (those chickens fed a strict organic diet) have become more popular with consumers for animal welfare and human health reasons (Cui *et al.* 2005; Colles *et al.* 2008). However, campylobacters are ubiquitous in the natural environment (Allos and Lastovica 2011) and as a result, animals in direct contact with the environment are most likely to acquire the organisms. It is therefore not surprising that free range chicken are more likely to

be *Campylobacter* – positive than their housed counterparts (Humphrey *et al.* 2007; Colles *et al.* 2008).

Campylobacter species are non-spore forming, fastidious microaerophilic organisms that grow best in an atmosphere with low oxygen levels (5% O₂, 10% CO₂ and 85% N₂). It is for this reason that several selective media containing oxygen quenchers such as blood, ferrous iron and pyruvate have been developed. Antibiotics are used as the selective agents in liquid and solid media to suppress the growth of competing microorganisms (Silva *et al.* 2011). Isolation techniques of this type are labour intensive and time consuming and can take up to one week to obtain a result (Wisessombat *et al.* 2009). Isolation protocols are often optimized to recover the two most important *Campylobacter* species associated with human gastroenteritis, *C. coli* and *C. jejuni* and therefore dramatically reduces the recovery and isolation rates of all other species of *Campylobacter* (Lastovica 2006). It is for this reason that alternative, non-selective isolation techniques are sought to promote the recovery and isolation of all *Campylobacter* species in clinical and food samples.



The Cape Town Protocol, developed by Le Roux and Lastovica (1998), enables the recovery of a wider variety of *Campylobacter* species. The protocol incorporates the use of a membrane filter which exploits the natural motility of campylobacters while simultaneously acting as a selective barrier against non-motile and larger motile competing organisms. Therefore antibiotic-free basal agar is used, eliminating the need for antibiotics that suppress the growth of many species (Le Roux and Lastovica 1998; Lastovica 2006; Wisessombat *et al.* 2009; Lynch *et al.* 2010).

The aim of this study was to compare the isolation rates of *Campylobacter* species from free range and commercial retail and abattoir chicken meat in South Africa using both the Cape Town Protocol and the ISO standard method, ISO 10272-1:2006.

3.3 MATERIALS AND METHODS

3.3.1 Retail sample collection and analysis

3.3.1.1 Collection of samples across South Africa

Fresh whole chicken carcasses (n = 42) and neck skin (n = 42), of which 32 were of free range farming origin and 10 were of a commercial farming origin for both the carcasses and neck skins samples, was delivered to the laboratory by a well-known retailer in South Africa. The carcasses were supplied to the retailer from free range and commercial farms in both the KwaZulu Natal (KZN) and Western Cape (WC) provinces in South Africa. From the total supply of chicken samples, 24 carcasses and 24 neck skins were from free range farms in the WC whereas six carcasses and neck skins were from commercial chicken farms in the WC. The rest of the samples were supplied from the KZN region; eight carcasses and neck skins were of free range origin and four carcasses and neck skins were of commercial farming origin. The chicken was kept below 10°C until it had reached the laboratory. Upon receipt, the chicken carcasses were refrigerated and analysed immediately or within 24 h. Each sample was analysed using both the Cape Town Protocol and the ISO 10272-1: 2006 method.

3.3.1.2 Examination of chicken samples for Campylobacter species using the Cape Town Protocol

Tryptose Blood Agar Base (CM0233 – Oxoid, Basingstoke, UK) supplemented with 10% unlysed citrated horse blood (MRC, Delft, South Africa) (hereafter wholly referred to as TBA plates) was used for the routine isolation and sub-culturing of *Campylobacter* species for the Cape Town Protocol. The detailed preparation procedure of the growth media plates are described in Appendix A. Excess moisture was removed from the TBA plates prior to use by drying the plates for one hour in a class two biohazardous Bio-Flow laminar flow cabinet

(Labotec, South Africa). All incubations for the Cape Town Protocol were carried out at 37°C in a microaerobic atmosphere achieved by using one sachet of the BR0038 anaerobic gas generating kit (Oxoid, Basingstoke, UK) (used according to manufacturer's instructions) in an anaerobic jar (Oxoid, Basingstoke, UK) stacked with a maximum of 12 TBA plates.

In general, a sample portion:diluent ratio of 1:10 (mass/volume) was used in the preparation of the sample for analysis. Whole chicken carcasses, of which 25 g of the chicken meat with skin (hereafter referred to as chicken meat) was aseptically removed and used for analysis, was rinsed with 225 ml of sterile 1 × Phosphate Buffer Saline (PBS) (Appendix B). The mass of each neck skin sample was determined and placed in sterile Whirl-Pak (Nasco, USA) bags. The neck skin samples were rinsed with nine times its volume of 1 × PBS. Each bag containing the chicken rinse sample was pulsified (Pulsifier[®], Filtraflex Ltd, Almonté, Canada) for 30 s. Thereafter, a sterile 10 µl plastic loop (LP Italian Spa, Italy) was dipped into each mixture and then streaked across the surface of a fresh TBA plate. Simultaneously, 200 µl of each respective rinse sample was placed drop-wise on the surface of a 0.65 µm cellulose nitrate filter (Sartorius Stedim Biotech, Germany) that was left to adhere to the surface of a fresh TBA plate. The filter paper was removed from the TBA plate after leaving it to stand for 15 min at room temperature. The plates were then incubated in a microaerobic atmosphere for six days while performing checks every second day for the visible characteristic growth of *Campylobacter* species.

No presumptive positive isolates were obtained using this method and therefore no further sub-culturing for the identification of isolates were done.

3.3.1.3 Examination of chicken samples for *Campylobacter* species using an adaptation of the ISO10272-1: 2006 method

The Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp.: Part 1 – Detection method, hereinafter referred to as the ISO 10272-1:2006 method (Anon 2006), was used to analyse the samples for the presence of *Campylobacter* species as a comparison to the Cape Town Protocol. The method entails enriching one part of the sample portion with nine parts of Bolton broth (Oxoid, Basingstoke, UK) (1:10 ratio sample portion:enrichment broth). This was done for 25 g of the chicken meat and skin that was aseptically cut from the chicken carcass as well as the neck skin samples, which were placed in sterile Whirl-Pak bags. The enrichment sample was pulsed for 30 s and incubated in a microaerobic atmosphere achieved by using a CO₂ incubator with a 5% CO₂ flow at 37°C for 4 h to 6 h and then increasing the temperature to 41.5°C for 44 h ± 4 h. A 10 µl loopful of the culture was then streaked across a fresh modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid, Basingstoke, UK) plate and incubated at 41.5°C in a microaerobic atmosphere for 44 h ± 4 h. The ISO 10272-1:2006 method was adapted by not using a second solid selective medium as the non-selective TBA plate was used in the Cape Town Protocol as a comparison to the ISO 10272-1:2006 method.

Colonies exhibiting typical *Campylobacter* morphology on mCCDA were selected and sub-cultured on Columbia blood agar (CBA) (Oxoid, Basingstoke, UK) (detailed preparation procedure outlined in Appendix A) and incubated microaerobically for 44 h ± 4 h at 41.5°C. A Gram stain, motility, oxidase (Merck, South Africa), catalase, hippurate (Sigma Aldrich, Germany), indoxyl acetate (Sigma Aldrich, Germany) and L-ALA (Sigma Aldrich, Germany) tests were performed on presumptive positive colonies (where applicable, all test procedures were performed according to manufacturers protocol). The selected presumptive positive colonies were also sub-cultured on two CBA plates and were subjected to aerobic growth at

41.5°C and microaerobic growth at 25°C respectively. The antibiotic sensitivity to nalidixic acid (30 µg disc; Oxoid, Basingstoke, UK) and cephalothin (30 µg disc; Oxoid, Basingstoke, UK) were performed on each presumptive positive colony.

3.3.2 Collection and analysis of abattoir samples

Free range (a total of 30 chicken legs and 28 neck skins was obtained in three different batches each at different time intervals) and one batch of commercial samples (chicken legs, n = 32) were obtained directly from the supplier(s) after the completion of the slaughtering process. All the samples supplied were from farms in the Western Cape province. The samples were kept below 10°C while in transit. Upon reaching the laboratory, the samples were refrigerated and processed for analysis immediately or within 24 h. These samples were also analysed using both the Cape Town Protocol and the above-mentioned adapted ISO 10272-1:2006 method; however, the Cape Town protocol was adapted in a method outlined below.



3.3.2.1 Analysis of chicken samples

The chicken meat with skin (25 g) were aseptically removed from the leg bone and placed in sterile Whirl-Pak bags. The mass of the neck skin samples were determined and also placed in sterile Whirl-Pak bags. The Bolton broth (225 ml) was added to the chicken meat samples and the 1:10 sample portion:enrichment broth (mass/volume) ratio was followed for the addition of Bolton broth to the neck skin samples. All samples were pulsed for 30 s and incubated without closing the Whirl-Pak bags in the CO₂ incubator with a 5% CO₂ flow at 37°C for 4 h to 6 h and thereafter increasing the temperature to 41.5°C for 44 h ± 4 h.

3.3.2.1.1 Further analysis of chicken samples for the detection of *Campylobacter* species using an adapted Cape Town Protocol

Initially, the efficacy of two different types of filter paper was tested with the first batch of samples. The filter paper used were the Schleicher and Schuell (S&S) 0.6 μm mixed cellulose ester (MCE) filter paper made from cellulose acetate and cellulose nitrate and has a smoother, more uniform surface with high porosity (Schleicher and Schuell, Germany, now owned by Whatman, London) than the Sartorius Stedim Biotech 0.65 μm cellulose nitrate (CN) filter paper used (Sartorius Stedim Biotech, Germany), which, as the name implies, is made only of cellulose nitrate and has a narrow distribution of pores.

The two different types of filters were aseptically cut in half and one half of each type was aseptically placed and left to adhere to the surface of a fresh TBA plate for 5 min. The culture of each sample obtained from 3.3.2.1 above (200 μl) was placed drop-wise on the surface of the filter paper and left to stand at room temperature for 15 min. The filter paper was then aseptically removed and discarded. The plates were incubated in a microaerobic atmosphere generated in the anaerobic jars with the use of the anaerobic gas generating kit at 37°C. The plates were incubated up to six days, while checking for characteristic *Campylobacter* growth and replacing the gas sachet every second day.

Presumptive positive colonies were selected and each colony was sub-cultured on two fresh TBA plates to yield enough growth to perform biochemical tests for confirmation of identity. Gram stains, motility, oxidase (Merck, South Africa), catalase, hippurate (Sigma Aldrich, Germany), indoxyl acetate (Sigma Aldrich, Germany) and L-ALA (Sigma Aldrich, Germany) tests were performed on presumptive positive colonies (where applicable, all tests were performed according to manufacturer's instructions). The isolates were also sub-cultured on two TBA plates and were subjected to aerobic growth at 37°C and microaerobic growth at

25°C respectively. The antibiotic sensitivity to nalidixic acid (30 µg disc; Oxoid, Basingstoke) and cephalothin (30 µg disc; Oxoid, Basingstoke) were performed on each presumptive positive colony.

*3.3.2.1.2 Further analysis of chicken samples for the detection of *Campylobacter* species using the adapted ISO 10272-1:2006 method.*

The cultures obtained from 3.3.2.1 above were further analysed using the adapted ISO 10272-1:2006 method as outlined in 3.3.1.3 above.

The efficiency of the protocols was calculated based on the number of positive samples from the total number of samples (Figures 3.4 and 3.5).

3.3.3 Further confirmation of isolates using the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) technique was employed to further confirm the identity of the isolates subsequent to the biochemical identification and characterization of the isolates as belonging to the genus *Campylobacter*.

3.3.3.1 Genomic DNA extraction from reference strains

The DNA of reference strains of species belonging to the genus *Campylobacter* and foodborne pathogens not belonging to the genus were used to validate that the primers used in the PCR reaction solely amplified the *Campylobacter* 16S rRNA gene.

The reference cultures *C. jejuni* subsp. *jejuni* biotype 1 (216.09 BC), *C. jejuni* (GSH 5953887), *C. concisus* (197.97), *Arcobacter butzleri* (Dsp 1520) and *Helicobacter pylori* (Z29 HpCa24) were revitalized from Microbank™ beads (Pro-lab Diagnostics, USA) by aseptically transferring 2-4 beads of each respective vial into 300 µl Luria Bertani (LB) broth

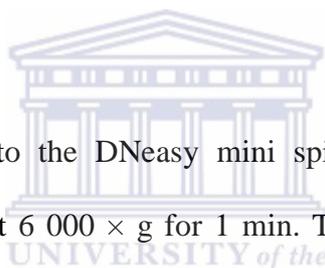
(Appendix A). It was then allowed to shake at 100 rpm in a G24 Environmental Shaker Incubator (New Brunswick Scientific Co. Inc., USA) at 37°C for 10 min to displace the culture from the porous beads. The LB broths of the respective cultures were then placed drop wise onto the surface of a TBA plate. The beads were also placed on the plate and the closed petri dish was incubated at room temperature for 30 min to allow the LB broth on the agar surface to dry. The plates were then inverted and incubated in a microaerobic atmosphere achieved by placing the plates in an anaerobic jar with 1 sachet of anaerobic gas generating kit. The plates were examined at 48 h intervals for characteristic *Campylobacter* growth for up to 6 days, replacing the gas sachet at each interval. Subculturing, to obtain pure colonies was done after the appearance of characteristic *Campylobacter* colonies and grown in a microaerobic atmosphere to obtain sufficient growth for the DNA isolation procedure.

Listeria monocytogenes (1/2b) CIP 105.448, *Staphylococcus aureus* (MRSA) ATCC® 33591™ and *Salmonella* spp. were used as negative bacterial controls in the PCR experiment. Viable strains were obtained from fellow researchers in the laboratory and sub-cultured on Tryptic Soy Agar (TSA) (Merck, South Africa). This was then incubated overnight at 37°C. A single colony of each respective bacterial strain was then aseptically inoculated into nine milliliters sterile Tryptic Soy Broth (TSB) (Merck, South Africa). Turbid growth of pure strains of the Gram positive bacteria was obtained for DNA extraction by allowing the cultures to grow with gentle shaking (75 rpm) at 37°C for 16 h.

The Qiagen DNeasy® Blood and Tissue Kit was used to extract DNA from all reference strains. The DNA of the reference cultures *C. jejuni* subsp. *jejuni* biotype 1 (216.09 BC), *C. jejuni* (GSH 5953887), *C. concisus* (197.97), *A. butzleri* (Dsp 1520), *H. pylori* (Z29 HpCa24) and *Salmonella* spp. were extracted following the manufacturers protocol for Gram negative bacteria while the DNA of *L. monocytogenes* (1/2b) CIP 105.448 and *Staph. aureus* (MRSA) ATCC® 33591™ was extracted following the manufacturers protocol for Gram positive

bacteria. All buffers and reagents besides the enzymatic lysis buffer used in the extraction of Gram positive bacteria protocol, was supplied by the manufacturer. All vortexing was done at the highest setting for 15 s.

Briefly, 20 μl of each Gram negative bacterial culture was removed from the agar plate using a 10 μl loop and washed by resuspending the respective cultures in two milliliters sterile distilled water and then centrifuged in a Spectrafuge (Labnet, USA) for 10 min at $15\,000 \times g$. The supernatant was discarded and the pellet was resuspended in 180 μl Buffer ATL. Proteinase K (20 μl of $20\text{mg}\cdot\text{ml}^{-1}$) was added and mixed thoroughly by vortexing. It was incubated at 56°C for 1 h until the bacteria were completely lysed. The mixture was then vortexed and 200 μl of Buffer AL was added. This mixture was vortexed again and 200 μl of 100% ethanol (Kimix, Cape Town, South Africa) was added and then mixed by vortexing again.



The mixture was transferred into the DNeasy mini spin column that was placed in a collection tube and centrifuged at $6\,000 \times g$ for 1 min. The flow-through in the collection tube was discarded and the DNeasy mini spin column was placed in a new collection tube. Buffer AW1 (500 μl) was added to the DNeasy mini spin column and centrifuged at $6\,000 \times g$ for 1 min. The flow-through in the collection tube was discarded and the DNeasy mini spin column was placed in a new collection tube. Buffer AW2 (500 μl) was added to the DNeasy mini spin column and centrifuged for 5 min at $15\,000 \times g$. The flow-through in the collection tube was discarded. The DNeasy mini spin column was placed in a clean 2 ml microcentrifuge tube and 200 μl Buffer AE was placed directly onto the DNeasy membrane. This was incubated at room temperature for 1 min and centrifuged for 1 min at $6\,000 \times g$ to elute the DNA from the column.

The turbid growth of Gram positive bacteria (2 ml) was centrifuged at $15\,000 \times g$ and the supernatant was discarded. The bacterial pellet was resuspended in 180 μl enzymatic lysis buffer (prepared according to the protocol listed in the Qiagen manual) and incubated at 37°C for 30 min. Proteinase K (25 μl of $20\text{mg}\cdot\text{ml}^{-1}$) and 200 μl Buffer AL was added and mixed by vortexing. This was incubated for 30 min at 56°C . Thereafter, 200 μl 100% ethanol was added to the sample and mixed thoroughly by vortexing.

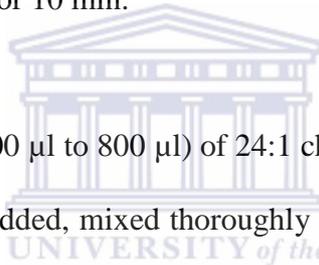
The mixture was transferred into the DNeasy mini spin column that was placed in a collection tube and centrifuged at $6\,000 \times g$ for 1 min. The flow-through in the collection tube was discarded and the DNeasy mini spin column was placed in a new collection tube. Buffer AW1 (500 μl) was added to the DNeasy mini spin column and centrifuged at $6\,000 \times g$ for 1 min. The flow-through in the collection tube was discarded and the DNeasy mini spin column was placed in a new collection tube. Buffer AW2 (500 μl) was added to the DNeasy mini spin column and centrifuged for 5 min at $15\,000 \times g$. The flow-through in the collection tube was discarded. The DNeasy mini spin column was placed in a clean 2 ml microcentrifuge tube and 200 μl Buffer AE was placed directly onto the DNeasy membrane. This was incubated at room temperature for 1 min and centrifuged for 1 min at $6\,000 \times g$ to elute the DNA from the column.

3.3.3.2 Genomic DNA extraction from Campylobacter isolates

The detailed preparation of all buffers and chemicals used in the extraction of the genomic DNA is listed in Appendix B. All mixing by use of the vortex was done at the highest setting for 15 s. All centrifugation steps were done at $15\,000 \times g$ for 5 min.

The genomic DNA of the isolates was extracted using an adaptation of the miniprep of bacterial genomic DNA protocol (Wilson 1994). The bacterial isolates were sub-cultured on a

fresh CBA (for *Campylobacter* isolates that were isolated using the adapted ISO 10272-1:2006 method) or TBA plate (for *Campylobacter* isolates that were isolated using the adapted Cape Town Protocol) and incubated for 48 h in a microaerobic atmosphere. The resulting bacterial growth (10-20 μl) was removed from the plate using a sterile 10 μl plastic loop and completely resuspended in 567 μl TE buffer by repeated pipetting. Thereafter, 30 μl of 10% sodium dodecyl sulfate (SDS) (Merck, South Africa) and 3 μl of 20 $\text{mg}\cdot\text{ml}^{-1}$ proteinase K (Roche Diagnostics, South Africa) to give a final concentration of 100 $\mu\text{g}\cdot\text{ml}^{-1}$ proteinase K in 0.5% SDS. The mixture was thoroughly mixed by vortexing and placed in a Scientific Series 9000 incubator (Lasec, South Africa) at 37°C for 1 h or overnight (~16 h). Thereafter, 100 μl of 5 M NaCl (Merck, South Africa) was added to give a final concentration of 0.7 M and mixed thoroughly by vortexing. The CTAB/NaCl solution (80 μl) (preheated in a waterbath at 65°C for 15 – 30 min) was added and thoroughly mixed by vortexing and incubated at 65°C for 10 min.



An approximate equal volume (700 μl to 800 μl) of 24:1 chloroform:isoamyl alcohol (Merck, South Africa) was subsequently added, mixed thoroughly by vortexing and then centrifuged. The aqueous viscous supernatant was transferred to a fresh microcentrifuge tube and an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Merck, South Africa) was added. The mixture was vortexed and then centrifuged. The resulting supernatant was transferred to a fresh microcentrifuge tube and 0.6 volume (of the volume transferred) isopropanol (Merck, South Africa) was added to precipitate the nucleic acids. The microcentrifuge tubes were shaken back and forth until the stringy white DNA precipitate became visible and then centrifuged. The liquid mixture in the tube was carefully poured off. The DNA was then washed with 200 μl 70% ice cold ethanol (Kimix, Cape Town, South Africa) and subsequently centrifuged. The ethanol was then carefully poured off and the DNA pellet was air-dried by placing it in a class two Bio-Flow laminar flow for 1 h or until all residual

ethanol has evaporated. The pellet was dissolved in 100 µl TE buffer in a refrigerator overnight.

The concentration of the DNA (ng.µl⁻¹) was determined using the NanoDrop[®] ND-1000 (Thermo Scientific, USA) and all DNA samples were diluted to ~50 ng.µl⁻¹ with TE buffer for use in the PCR reaction.

3.3.3.2 The PCR method

The primers (Table 3.1) used to amplify the 16S rRNA gene of the reference strains and *Campylobacter* isolates were previously published by Linton *et al.* (1996) while the internal amplification control (IAC) amplicon was designed by Inglis and Kalischuk (2003). The forward (C412F) and reverse (C1228R) primers amplified an 816 bp product (Linton *et al.* 1996) while the forward (C412F) and mutagenic reverse (C1228RIC) primers amplified a 475 bp product. The latter primers produced a product that was used as an internal control in each reaction (Inglis and Kalischuk 2003). All primers used in this study were synthesized by Integrated DNA Technologies (IDT, Whitehead Scientific, South Africa).

Table 3.1 Primer sequences for the amplification of bacteria of the genus *Campylobacter*

PCR target gene	Primer	Sequence (5' to 3')	Amplicon (bp)	Reference
16S rRNA	C412F	GGATGACACTTTTCGGAGC	816	Linton <i>et al.</i> 1996
16S rRNA	C1228R	CATTGTAGCACGTGTGTC		
IAC	C412F	GGATGACACTTTTCGGAGC	475	Inglis & Kalischuk 2003
IAC	C1228RIC	TCCCCAGGCGGTACACTTAATG		

The PCR conditions were optimized from Inglis and Kalischuk (2003). The mixture consisted of a total volume of 20 µl containing 1× PCR reaction buffer (Bioline, UK), 1.5 mM MgCl₂ (Bioline, UK), 0.075 mM dNTPs (KapaBiosystems, South Africa), 0.2 µM forward and reverse primer, 0.1 µM mutagenic reverse primer, 0.5 µl BioTaq (Bioline, UK) and 50 ng DNA. The mixture was supplemented with sterile distilled water to the final volume.

The PCR was performed in the MJ Mini Cycler (Bio-Rad, South Africa). The cycling parameters for DNA amplification were 1 cycle of initial denaturation at 95°C for 2 min; 25 cycles of denaturation at 94°C for 30 s, 30 s at the annealing temperature of 55°C and a 30 s extension at 72°C; with a final extension of 2 min at 72°C. The PCR products (5 µl) were electrophoresed in a 1.5% Tris-acetic acid-EDTA- (TAE) - agarose gel (Lonza, Switzerland) at 70 V for 90 min. The products were sized using the 100 bp ladder (Promega, USA).

The products were visualized by post staining the gel in 3 × GelRed (Biotium, USA) for 1 h and viewed under UV light.



3.4 RESULTS

3.4.1 The *Campylobacter* isolation analysis of retail free range and commercial chicken carcasses and neck skins from the Western Cape and KwaZulu Natal provinces.

Please refer to the results on the next page.

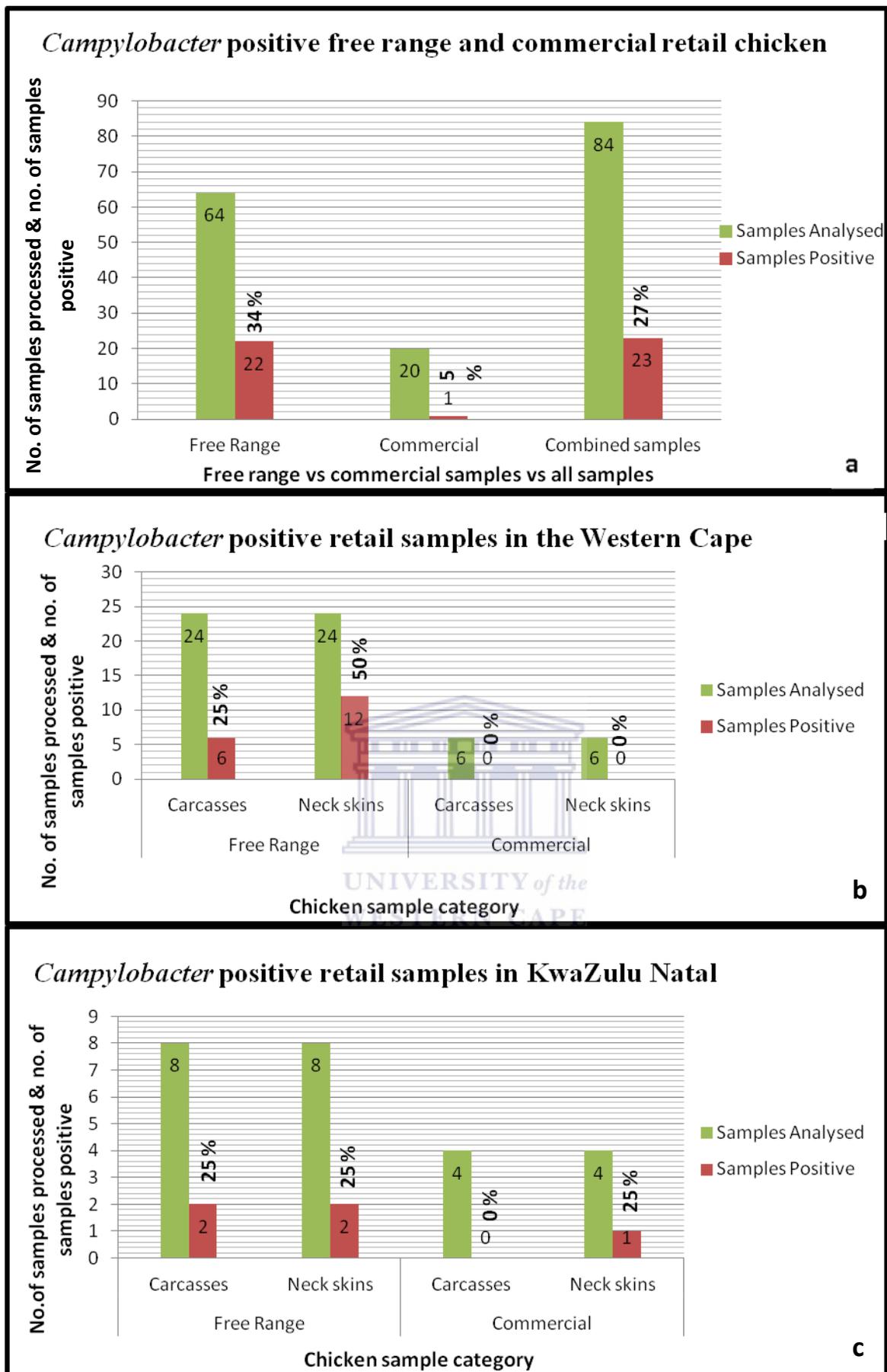


Fig. 3.1 Group analysis of retail chicken: *Campylobacter* positive chicken samples in the (a) combined free range and commercial samples, (b) Western Cape and (c) KwaZulu Natal

3.4.2 *Campylobacter* isolation analysis of free range and commercial abattoir samples using the Cape Town (CPT) Protocol and the ISO 10272-1:2006 methods

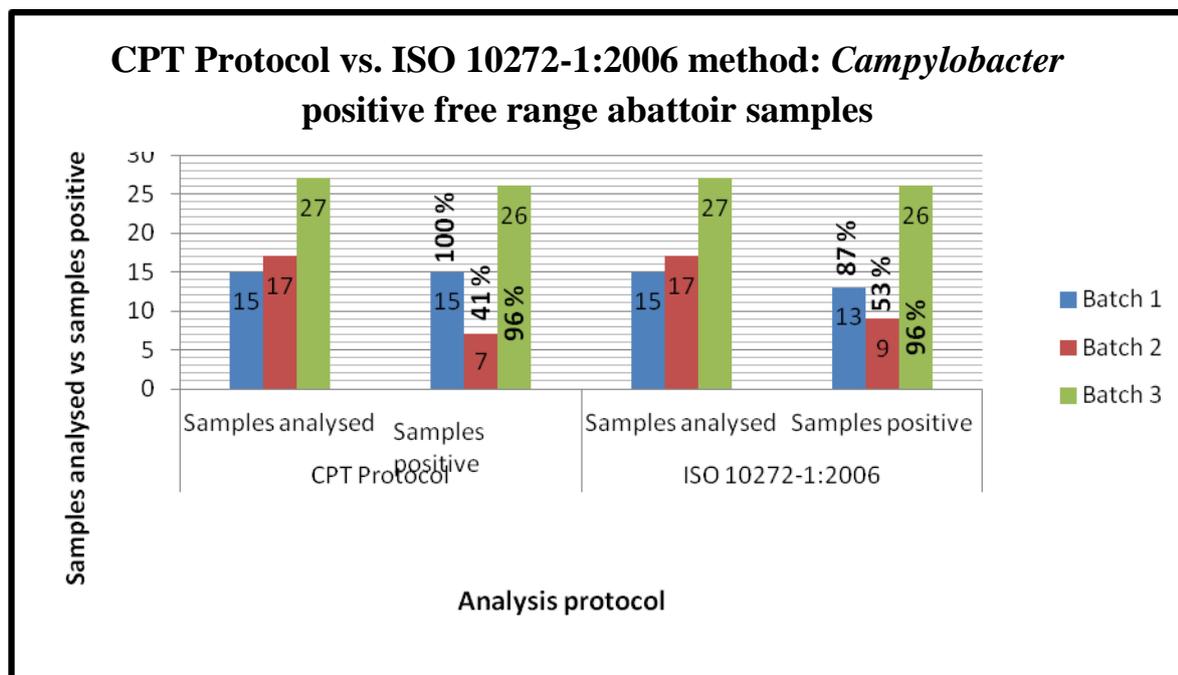


Fig. 3.2 The numbers of *Campylobacter* positive free range chicken in batches of abattoir samples using both the Cape Town Protocol and the ISO 10272-1:2006 method

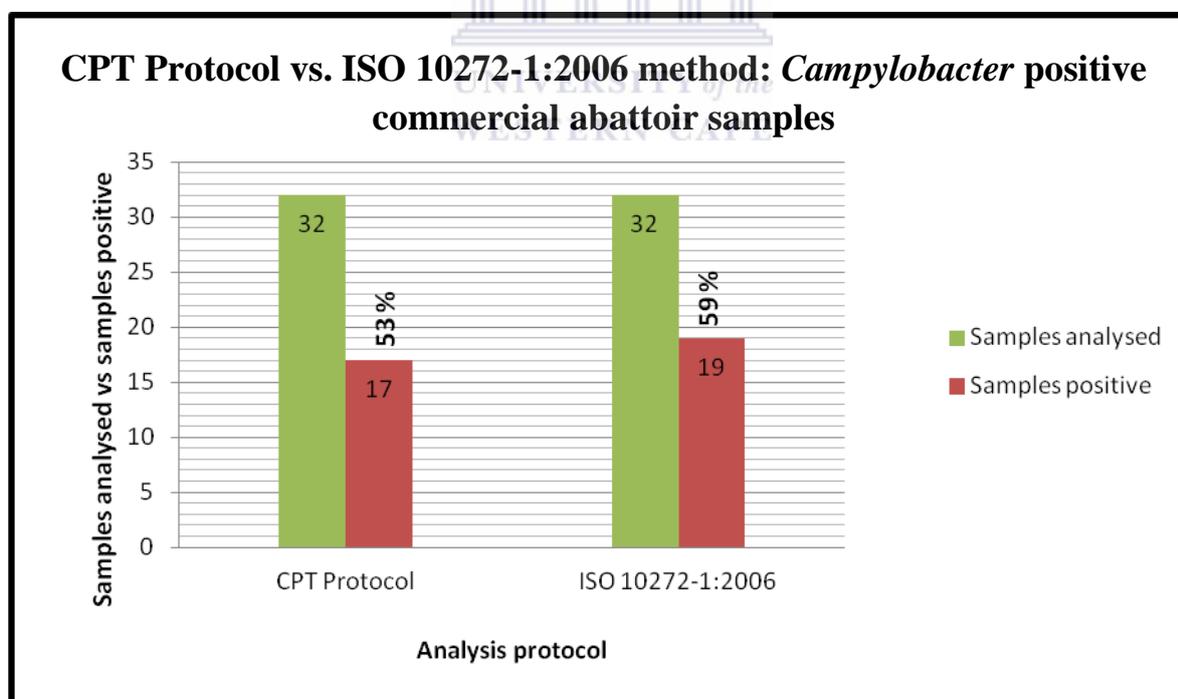


Fig. 3.3 The numbers of *Campylobacter* positive commercial abattoir samples using both the Cape Town Protocol and the ISO 10272-1:2006 method

3.4.3 The comparison efficiency in isolation of *Campylobacter* between the two Cape Town Protocols and the adapted ISO 10272-1:2006 methods.

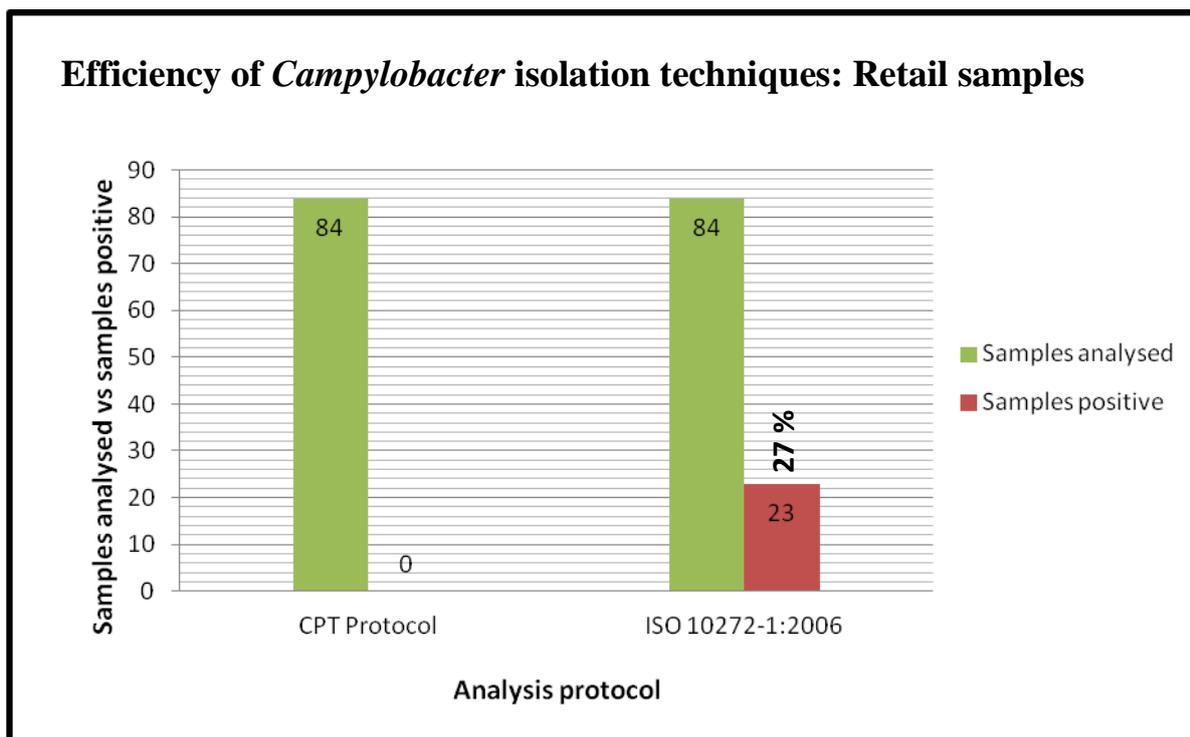


Fig. 3.4 The efficiency of the Cape Town Protocol and adapted ISO 10272-1:2006 method in isolating *Campylobacter* species

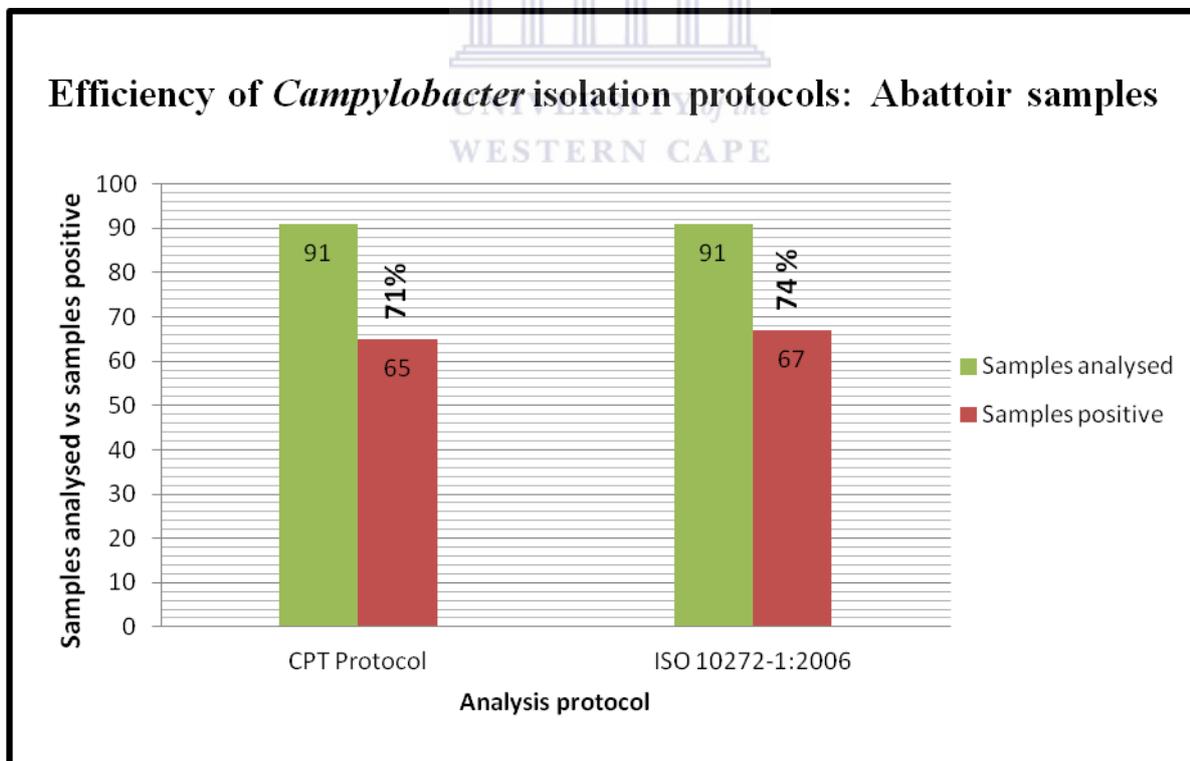


Fig. 3.5 The efficiency of the adapted Cape Town Protocol and adapted ISO 10272-1:2006 method in isolating *Campylobacter* species

3.4.4 Comparison of *Campylobacter* positive chicken in retail and abattoir samples

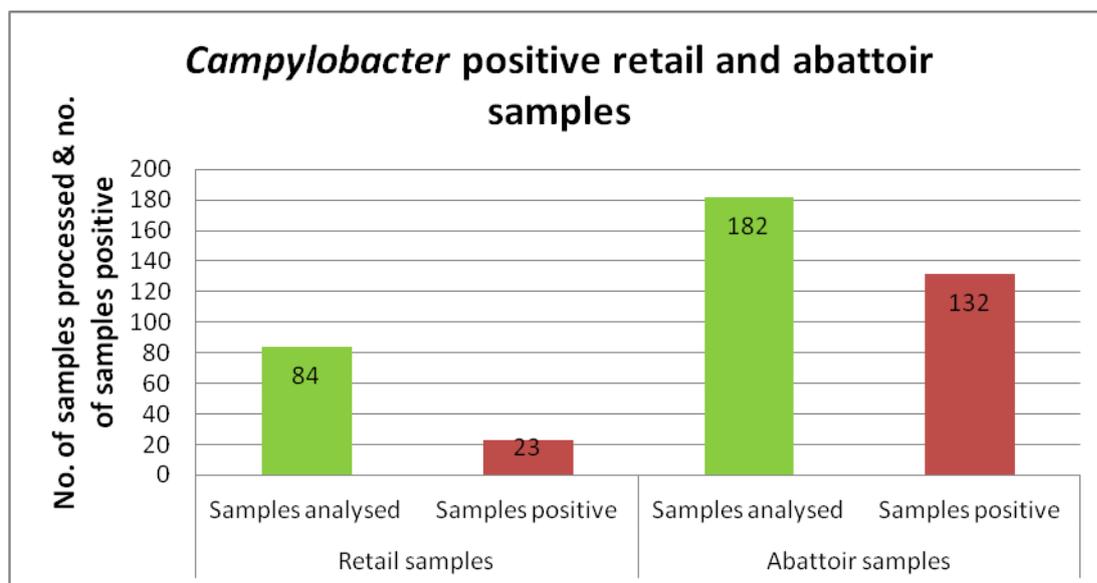


Fig 3.6 *Campylobacter* positive chicken in combined (free range and commercial) retail and abattoir samples



3.4.5 PCR confirmation of bacterial isolates as belonging to the genus *Campylobacter*

3.4.5.1 Amplification of reference strains DNA using the *Campylobacter*-specific 16S rRNA primers

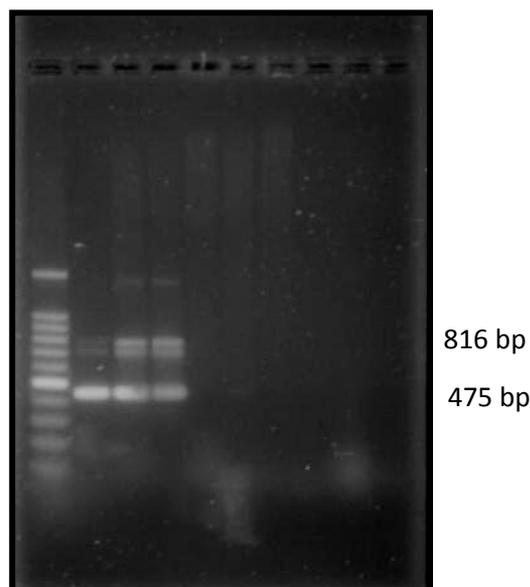


Fig 3.7 Electrophoretogram of reference strains confirming the fidelity of the primers for *Campylobacter* species only; Lanes (M) 100 bp ladder, (1) *C. jejuni* subsp. *jejuni* biotype 1 (216.09 BC), (2) *C. jejuni* (GSH 5953887), (3) *C. concisus* (197.97), (4) *A. butzleri* (Dsp 1520), (5) *H. pylori* (Z29 HpCa24), (6) *L. monocytogenes* (½ b), (7) *Salmonella* spp., (8) *Staph. aureus* (MRSA) ATCC® 33591™, (9) Negative water control

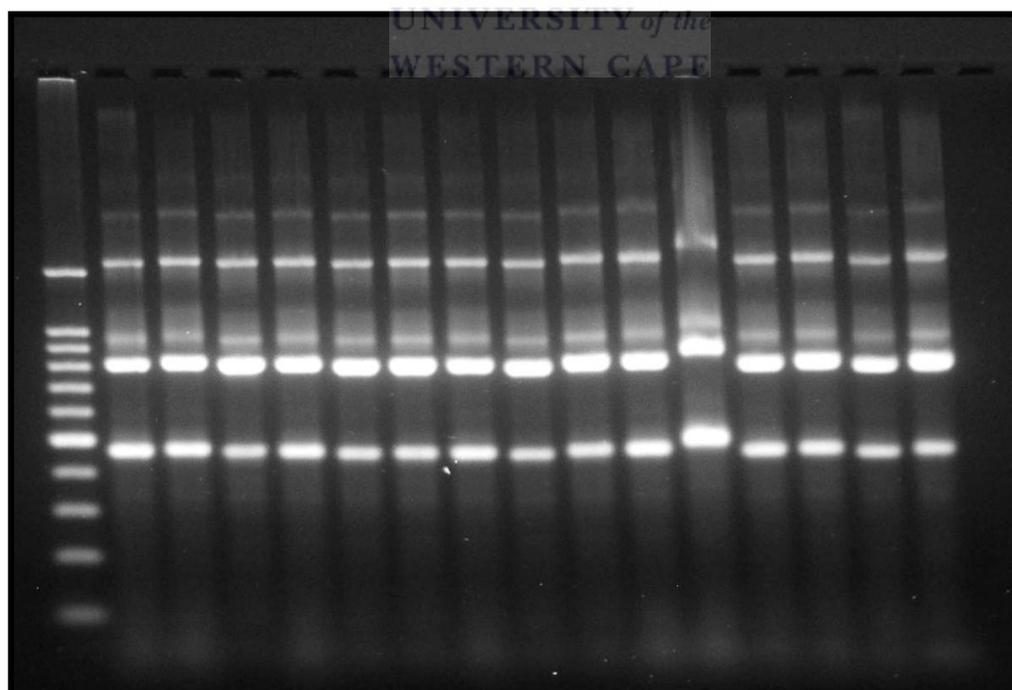


Fig. 3.8 Electrophoretogram depicting PCR products obtained before optimization of the PCR

3.4.5.2 Amplification of bacterial isolate's DNA using the *Campylobacter*-specific 16S rRNA primers to further confirm its identity as belonging to the genus *Campylobacter*

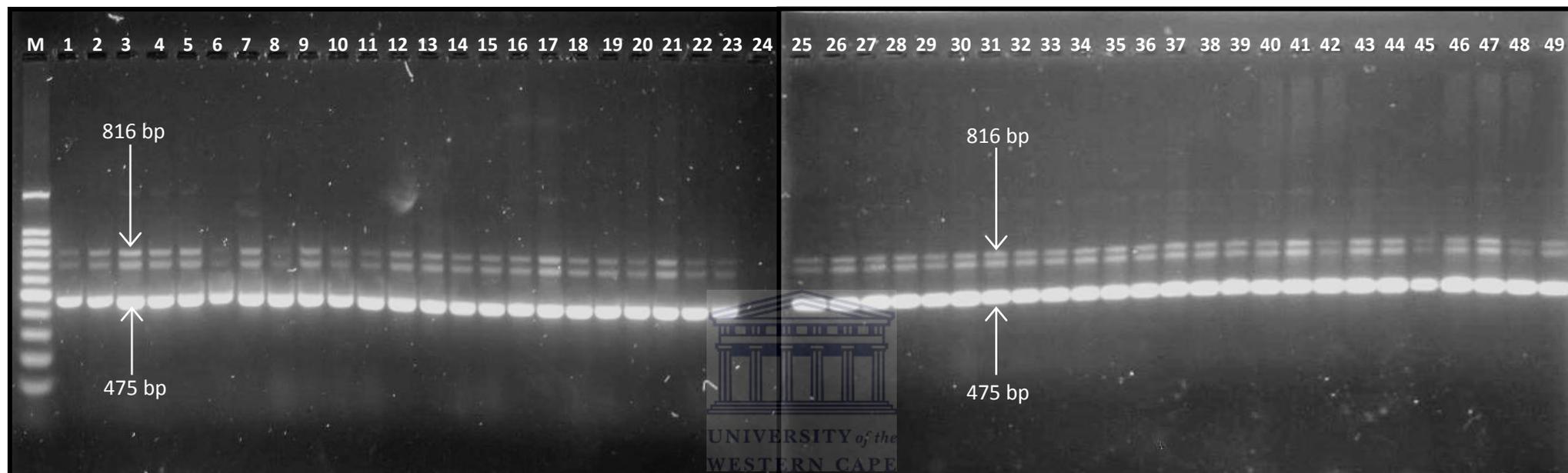


Fig. 3.9 Electrophoretogram confirming the identity of *Campylobacter* isolates from abattoir free range chicken origin. Lanes (M) 100 bp ladder, (1) Positive control *C. jejuni* subsp. *jejuni* 216.09 BC, (2) L1b, (3) L2b, (4) L3b, (5) L4b, (6) L5b, (7) L6b, (8) L7b, (9) L8b, (10) L9b, (11) L10b, (12) L15a, (13) L18a, (14) L31a, (15) L32a, (16) L33a, (17) L34a, (18) L35a, (19) L36a, (20) L37a, (21) L38a, (22) L39a, (23) L40a, (24) Negative water control, (25) NS1b, (26) NS2b, (27) NS3b, (28) NS4b, (29) NS5b, (30) NS6b, (31) NS7b, (32) NS8b, (33) NS9b, (34) NS10b, (35) NS11b, (36) NS22a, (37) NS23a, (38) NS24a, (39) NS25a, (40) NS26a, (41) NS27a, (42) NS28a, (43) NS29a, (44) NS30a, (45) NS31a, (46) NS32a, (47) NS33a, (48) NS34a, (49) NS35a

Refer to Table 3.2 for the corresponding *Campylobacter* species assignment of the respective strain codes in the above electrophoretogram.

Table 3.2 *Campylobacter* species assignments for strain codes used in Figure 3.8

L1b <i>C. coli</i>	L15a <i>C. jejuni</i>	L39a <i>C. jejuni</i>	NS9b <i>C. jejuni</i>	NS29a <i>C. jejuni</i>
L2b <i>C. coli</i>	L18a <i>C. jejuni</i>	L40a <i>C. jejuni</i>	NS10b <i>C. jejuni</i>	NS30a <i>C. jejuni</i>
L3b <i>C. coli</i>	L31a <i>C. jejuni</i>	NS1b <i>C. coli</i>	NS11b <i>C. jejuni</i>	NS31a <i>C. jejuni</i>
L4b <i>C. coli</i>	L32a <i>C. jejuni</i>	NS2b <i>C. coli</i>	NS22a <i>C. jejuni</i>	NS32a <i>C. jejuni</i>
L5b <i>C. coli</i>	L33a <i>C. jejuni</i>	NS3b <i>C. coli</i>	NS23a <i>C. jejuni</i>	NS33a <i>C. jejuni</i>
L6b <i>C. coli</i>	L34a <i>C. jejuni</i>	NS4b <i>C. coli</i>	NS24a <i>C. jejuni</i>	NS34a <i>C. jejuni</i>
L7b <i>C. coli</i>	L35a <i>C. jejuni</i>	NS5b <i>C. coli</i>	NS25a <i>C. jejuni</i>	NS35a <i>C. jejuni</i>
L8b <i>C. coli</i>	L36a <i>C. jejuni</i>	NS6b <i>C. jejuni</i>	NS26a <i>C. jejuni</i>	
L9b <i>C. coli</i>	L37a <i>C. jejuni</i>	NS7b <i>C. jejuni</i>	NS27a <i>C. jejuni</i>	
L10b <i>C. coli</i>	L38a <i>C. jejuni</i>	NS8b <i>C. jejuni</i>	NS28a <i>C. jejuni</i>	

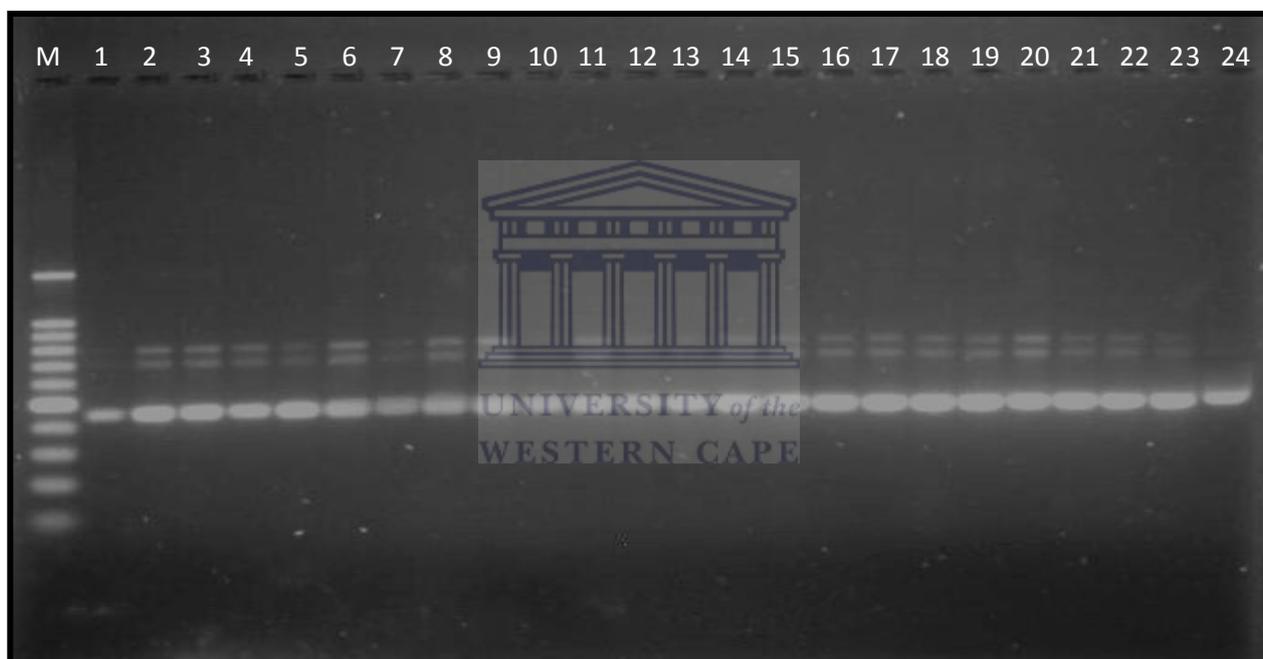


Fig. 3.10 Electrophoretogram confirming the identity of the *Campylobacter* isolates of abattoir commercial farming origin. Lanes (M) 100 bp ladder, (1) Positive control *C. jejuni* subsp. *jejuni* 216.09 BC, (2) S1a, (3) S2a, (4) S3a, (5) S4a, (6) S5a, (7) S11a, (8) CF6b, (9) CF7b, (10) CF8b, (11) CF9b, (12) CF11b, (13) CF12b, (14) T1a, (15) T3a, (16) T12a, (17) R11a woBB, (18) R2b, (19) R8b, (20) R9b, (21) T1b, (22) T3b, (23) S1b and (24) S3b

Refer to Table 3.3 for *Campylobacter* species assignment of the respective strain codes in the above electrophoretogram.

Table 3.3 *Campylobacter* species assignments for strain codes used in Figure 3.9

S1a <i>C. coli</i>	S11a <i>C. jejuni</i>	CF11b <i>C. jejuni</i>	R11woBB <i>C. jejuni</i>	T3b <i>C. jejuni</i>
S2a <i>C. jejuni</i>	CF6b <i>C. jejuni</i> subsp. <i>jejuni</i> 2	CF12b <i>C. jejuni</i>	R2b <i>C. jejuni</i>	S1b <i>C. jejuni</i>
S3a <i>C. jejuni</i>	CF7b <i>C. jejuni</i>	T1a <i>C. jejuni</i>	R8b <i>C. jejuni</i>	S3b <i>C. jejuni</i>
S4a <i>C. jejuni</i>	CF8b <i>C. jejuni</i>	T3a <i>C. jejuni</i>	R9b <i>C. jejuni</i>	
S5a <i>C. jejuni</i>	CF9b <i>C. jejuni</i>	T12a <i>C. jejuni</i>	T1b <i>C. jejuni</i>	

3.5 DISCUSSION

The total number of *Campylobacter* species isolated in this study using both the Cape Town Protocol and the ISO10272-1:2006 method was 156, of which 102 isolates were *C. jejuni* and 51 were *C. coli*. The speciation of 3 *Campylobacter* isolates could not be determined.

3.5.1 The *Campylobacter* isolation analysis of retail free range and commercial chicken carcasses and neck skins from the Western Cape and KwaZulu Natal provinces.

The Cape Town Protocol was designed for the efficient isolation of *Campylobacter* from clinical samples (Le Roux and Lastovica 1998; Lastovica and Le Roux 2003; Lastovica 2006). Jacob *et al.* (2011) reported that this protocol was also a useful technique for the isolation of *Campylobacter* from food products. The protocol relies on the natural motility ability of *Campylobacter* organisms to pass through a 0.6 µm membrane filter (Le Roux and Lastovica 1998; Silva *et al.* 2011) and therefore eliminates the need for chemical selective agents such as antibiotics as those used in the ISO 10272-1:2006 method (Anon 2006; Silva *et al.* 2011).

In this study, the Cape Town Protocol was not successful in isolating any *Campylobacter* species from the same chicken carcasses and neck skin samples from which *Campylobacter*

was isolated using the ISO 10272-1:2006 method (Figure 3.4). This was not in agreement with Jacob *et al.* (2011) and could be attributed to the very low numbers of campylobacters that may be present on the raw chicken meat or cells that are damaged due to exposure to harsh processing or environmental (packaging) conditions that the chickens undergo from the abattoir to the retail level (Lynch *et al.* 2010). There must be at least 900 bacterial cells present on the filter in order for one cell to pass through to the TBA plate and form a single colony (A.J. Lastovica, personal communication). If it is suspected that low numbers of bacterial cells or damaged cells are present in a sample type, a portion of the sample would have to be incubated with an enrichment broth that will allow for the recovery and multiplication of *Campylobacter* cells (Lynch *et al.* 2010).

Figure 3.1 shows the analysis of *Campylobacter* species in retail chicken. All the isolates from the retail chicken samples were obtained using the ISO 10272-1:2006 method. *Campylobacter* species were present in 34% (22/64) of free range chicken samples while present at a much lower number (5%, 1/20) in commercial chicken samples. The overall prevalence of *Campylobacter* in retail poultry was 27% (23/84) (Figure 3.1a). Samples obtained from the Western Cape province contributed significantly to the total prevalence of *Campylobacter* in South African retail poultry. This is due to the fact that the majority of the samples originated from this province. The prevalence in free range carcasses were 25% (6/24) while 50% of the free range neck skin samples were positive for *Campylobacter*. No commercial chicken samples were positive for the presence of *Campylobacter*, however, only six commercial samples were tested (Figure 3.1b). For a better comparison between free range and commercial chicken, future studies should include more retail samples of commercial farming origin. In the KwaZulu Natal province, 25% (2/8) of free range carcasses and neck skins were positive for *Campylobacter* while only 1/4 (25%) of

commercial neck skin samples were positive. No commercial carcasses were positive for the presence of *Campylobacter* species (Figure 3.1c).

3.5.2 *Campylobacter* isolation analysis of free range and commercial abattoir samples using the Cape Town (CPT) Protocol and the ISO 10272-1:2006 methods

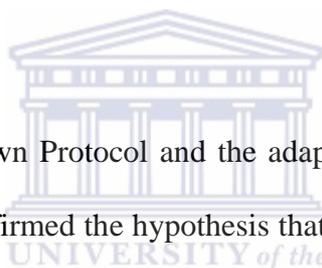
The chicken samples received directly from the abattoir was also intended for sale at the retail level but for simplicity in the analysis of results, these samples were not combined with the previously described retail samples as these samples originated only from the Western Cape and was received entirely in the spring and summer months of the year. These samples were also analysed using the adapted CPT Protocol and adapted ISO 10272-1:2006 methods described.

Three batches of free range samples were received (Figure 3.2). In batch one, 100% (15/15) of samples were positive for *Campylobacter* using the adapted CPT Protocol while 87% (13/15) were positive using the ISO 10272-1:2006 method. In batch two, 41% (7/17) of samples were positive using the adapted CPT Protocol while 53% (9/17) were positive using the ISO 10272-1:2006 method. In batch three, both analysis protocols showed that 96% (26/27) of samples were positive for *Campylobacter* species.

Four different commercial farms within the Western Cape region supplied 12 chicken leg samples each while one farm supplied three chicken leg samples. From the total number of commercial samples received, only 32/51 were analysed for the presence of *Campylobacter* species. The prevalence of *Campylobacter* in commercial chicken was 53% (17/32) using the adapted CPT Protocol and 59% (19/32) using the ISO 10272-1:2006 method (Figure 3.3). The prevalence of *Campylobacter* in the abattoir samples, including both the free range and

commercial samples, was 71% using the adapted CPT Protocol and 74% using the ISO 10272-1:2006 method (Figure 3.5).

The two isolation protocols used differed by 3% in its isolation efficiency. The CPT Protocol differed by not detecting campylobacters in only 2 samples in the second free range batch and the commercial chicken sample batch. It had shown superiority to the ISO 10272-1:2006 method where it had a 100% prevalence in the first free range batch of chicken samples. The isolation rate is very high for all samples (71% and 74%) and these prevalence are in agreement with previous studies where it showed up to 85% of chickens intended for human consumption are *Campylobacter* positive (Sampers *et al.* 2008; El-Shibiny *et al.* 2009a; Horrocks *et al.* 2009; Ligowska *et al.* 2011). This high prevalence are definitely a high risk for contracting campylobacteriosis if contaminated meat is mishandled or consumed when undercooked.



The use of the original Cape Town Protocol and the adapted Cape Town Protocol (Figures 3.4 and 3.5) in this study has confirmed the hypothesis that the numbers of *Campylobacter* in food samples are low and that cells may be injured. The use of Bolton broth was an efficient tool for the recovery and enrichment of campylobacters in food samples as shown by the results obtained where no campylobacters were isolated using the original protocol and the high prevalence obtained when using Bolton broth. This comparison shows that the adapted CPT Protocol can be implemented as an alternative to the ISO 10272-1:2006 method. The advantage that it boasts, is that the time taken to obtain results are reduced by at least 48 h and it is more cost effective than the inclusion of chemical selective agents as in the ISO 10272-1:2006 method. However, a disadvantage of using Bolton broth is that it contains antibiotics that suppresses the growth of the more fastidious emerging *Campylobacter* species (Lastovica 2006; Lynch *et al.* 2011) and enhances the recovery of the thermotolerant

Campylobacter species; *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis* (Anon 2006). It is for this reason, that a non-selective enrichment broth for the efficient recovery of all *Campylobacter* species from food samples be sought.

The differences in isolation between the CPT Protocol and the ISO 10272-1:2006 method could possibly be attributed to the type of filter used. The CPT Protocol recommends the use of the mixed cellulose ester membrane filter which is more suited to microbiological applications such as isolation and enumeration of microorganisms. The filter efficiency test has also shown that this type of filter had an added advantage\ to the nitrocellulose filter used as the flow-through obtained using the mixed cellulose ester membrane filter was much more than the nitrocellulose filters (data not shown). It can be noted that *Campylobacter* was isolated in 100% of the chicken samples analysed with the use of the mixed cellulose ester filter as compared with the 87% isolation rate with the ISO 10272-1:2006 method analysing the same samples (Figure 3.2). The nitrocellulose filter was used for the rest of the study because the supply of the mixed cellulose ester into South Africa was discontinued and the specialized importing of the filter would not have been cost effective. It has now been determined that the mixed cellulose ester filter is once again being supplied in South Africa.

The lower prevalence obtained in the retail chicken samples (Figure 3.1 a, b and c) compared to the abattoir samples (Figure 3.5) could be explained by the seasons in which the samples were supplied. The retail samples were supplied in the cooler months of the year (April to July) while the abattoir samples were supplied in the spring and summer months of the year. The prevalence of *Campylobacter* in chicken has been reported to be lower in the winter months of the year whereas the prevalence increases in the summer months (Humphrey *et al.* 2007).

3.5.3 PCR confirmation of *Campylobacter* isolates

The presumptive positive isolates were initially confirmed to belong to the genus *Campylobacter* by performing biochemical tests as outlined in the CPT Protocol and the ISO 10272-1:2006 method (data not shown). The PCR was performed as a further confirmation of the assignment of the isolates to the genus *Campylobacter*. The primer pair was genus specific and amplified the 16S rRNA gene of *Campylobacter*. It could not discriminate between the different species of the *Campylobacter* isolates.

Upon selection, the primers were confirmed to be selective solely for the amplification of isolates belonging to the genus *Campylobacter* by performing a BLASTn search on the NCBI website. The primers showed 100% homology to species within the genus *Campylobacter* (data not shown). Furthermore, reference strains of *Campylobacter* species and other pathogenic foodborne bacteria were amplified by the primers (Fig. 3.6). The primers showed that it only amplified the *Campylobacter* reference strains while the closely related *Arcobacter butzleri* and *Helicobacter pylori* strains were not amplified. The primers could also not detect the methicillin resistant *Staphylococcus aureus*, *Salmonella* spp and *Listeria monocytogenes* strains used as a negative control.

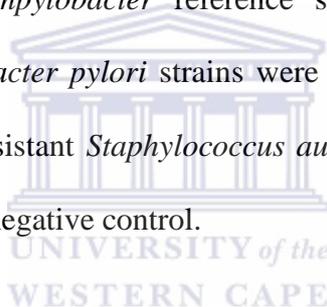


Figure 3.7 refers to the amplification of the DNA belonging to *Campylobacter* reference strains and isolates obtained in the present study. This amplification was performed strictly according to the PCR conditions and amplification parameters as specified by Inglis and Kalischuk (2003). Four non-specific amplicon bands were obtained. Thereafter, a series of optimization reactions were carried out, including the titration of the MgCl₂ concentrations, performing a gradient PCR of the annealing temperature, altering the lengths of the denaturation, annealing and extension steps in the PCR cycling parameters as well as performing titrations of all other reagents used in the PCR. Different amplification kits were also purchased from leading companies (KapaBiosystems, South Africa and Qiagen, GmbH,

Germany). Throughout the optimization process, the 816 bp and 475 bp IAC amplicons were always obtained but together with the different optimizations conditions, different sizes of non specific bands were obtained concurrently. The best that the PCR could be optimized is shown in Figures 3.6, 3.8 and 3.9. The nonspecific bands were sent for sequencing and a BLASTn search was performed once again. Both the non-specific bands (~1400 bp and ~700 bp) in Figure 3.6 was shown to belong to *Campylobacter* and therefore it can be deduced that the primers amplified these different regions in the 16S rRNA gene in addition to the target regions.

The 816 bp and 475 bp amplicons were also sent for sequencing and was confirmed to belong to species of the genus *Campylobacter*. Therefore, the isolates obtained were further confirmed by PCR analysis to belong to the genus *Campylobacter* (Figures 3.8 and 3.9).

3.6 CONCLUSION

Campylobacter species are most commonly associated with chicken and chicken is therefore said to be the primary reservoir of the pathogen. It is present in chicken flocks across the globe at high prevalence levels. To the best of my knowledge, this present study was the first of its kind to be undertaken where the prevalence of *Campylobacter* in South African broiler chickens were determined on a large scale. In addition, this study determined the prevalence levels of *Campylobacter* in free range chicken and commercial chicken concurrently where separate studies of the differently reared chicken have dominated across the globe. The study showed that there is a high prevalence of *Campylobacter* species in South African chicken and that the most common species is *C. jejuni*. These high prevalence are indicative of a high risk of contracting campylobacteriosis associated with the handling and consumption of contaminated chicken. It is also noted that free range chicken that are marketed as the healthier alternative to commercially-bred chicken have higher prevalence of *Campylobacter*

and is therefore not always the safer option for human consumption. The South African community should be made aware of the risks associated with the consumption of poultry and should be educated and reminded to cook food thoroughly at, at least 60°C or preferably at 72°C to prevent the acquisition of campylobacteriosis.

The CPT Protocol originally designed for the cost effective isolation of all *Campylobacter* species was effectively optimized to isolate *Campylobacter* from the raw chicken samples. However, since the recovery of *Campylobacter* in food samples depends on the inclusion of an enrichment broth, further research needs to be done to obtain an enrichment broth that will not suppress the growth of emerging campylobacters while enhancing the growth of thermotolerant campylobacters only. Both methods proved to be reliable methods of detection of *Campylobacter* from chicken meat samples and to further confirm the reliability of the protocols, future studies, although laborious and time consuming, should include performing the study in duplicate or triplicate.



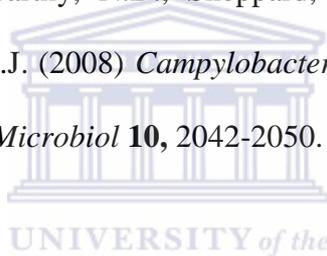
3.7 REFERENCES

Allos, B.M. and Lastovica, A.J. (2011) *Campylobacter* Infections. In Tropical Infectious Diseases: Principles, Pathogens and Practise ed. Guerrant, R.L., Walker, D.H. and Weller, P.F. pp.145-149. Philadelphia: Saunders/Elsevier.

Anonymous (2006) The Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp.: Part 1 – Detection method. ISO 10272-1:2006, International Organisation for Standardisation. SANS, Pretoria, South Africa.

Avrain, L., Humbert, F., L'Hospitalier, R., Sanders, P., Vernozy-Rozand, C. and Kempf, I. (2003) Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. *Vet Microbiol* **96**, 267-276.

Colles, F.M., Jones, T.A., McCarthy, N.D., Sheppard, S.K., Cody, A.J., Dingle, K.E., Dawkins, M.S. and Maiden, M.C.J. (2008) *Campylobacter* infection of broiler chickens in a free range environment. *Environ Microbiol* **10**, 2042-2050.



Cui, S., Ge, B., Zheng, J. and Meng, J. (2005) Prevalence and Antimicrobial Resistance of *Campylobacter* spp. and *Salmonella* Serovars in Organic Chickens from Maryland Retail Stores. *Appl Environ Microbiol* **71**, 4108-4111.

El-Shibiny, A., Connerton, P. and Connerton, I. (2009) Survival at refrigeration and freezing temperatures of *Campylobacter coli* and *Campylobacter jejuni* on chicken skin applied as axenic and mixed inoculums. *Int J Food Microbiol* **131**, 197-202.

Habib, I., Berkvens, D., De Zutter, L., Dierick, K., Van Huffel, X., Speybroeck, N., Geeraerd, A.H. and Uyttendaele, M. (2012) *Campylobacter* contamination in broiler carcasses and

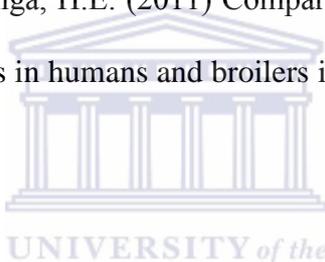
correlation with slaughterhouses operational hygiene inspection. *Food Microbiol* **29**, 105-112.

Horrocks, S.M., Anderson, R.C., Nisbet, D.J. and Ricke, S.C. (2009) Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* **15**, 18-25.

Humphrey, T., O'Brien, S. and Madsen, M. (2007) Campylobacters as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* **117**, 237-257.

Inglis, D.G. and Kalischuk, L.D. (2003) Use of PCR for Direct Detection of *Campylobacter* species in Bovine Feces. *Appl Environ Microbiol* **69**, 3435-3447.

Jacob, P., Mdegela, R.H. and Nonga, H.E. (2011) Comparison of Cape Town and Skirrow's *Campylobacter* isolation protocols in humans and broilers in Morogoro, Tanzania. *Trop Anim Health Prod* **43**, 1007-1013.



Lastovica, A. J. (2006) Emerging *Campylobacter* spp.: the Tip of the Iceberg, *Clin Microbiol Newslett* **28**, 49-56.

Lastovica, A. J. and le Roux, E. (2003) Optimal detection of *Campylobacter* spp in stools. *J Clin Pathol* **56**, 480.

Le Roux, E. and Lastovica, A.J. (1998) The Cape Town Protocol: how to isolate the most *Campylobacter* for your dollar, pound, franc, yen, etc. In *Campylobacter, Helicobacter &*

related organisms. ed. Lastovica, A.J., Newell, D.G. and Lastovica, E.E. pp.30-33. Cape Town, South Africa: Institute of Child Health.

Ligowska, M., Cohn, M.T., Stabler, R.A., Wren, B.W. and Brønsted, L. (2011) Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food. *Int J Food Microbiol* **145**, 111-115.

Linton, D., Owen, R.J. and Stanley, J. (1996) Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res Microbiol* **147**, 707-718.

Lynch, O.A., Cagney, C., McDowell, D.A. and Duffy, G. (2010) A method for the growth and recovery of 17 species of *Campylobacter* and its subsequent application to inoculated beef. *J Microbiol Meth* **83**, 1-7.

Lynch, O.A., Cagney, C., McDowell, D.A. and Duffy, G. (2011) Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol. *Int J Food Microbiol* **150**, 171-177.

Oyarzabal, O.A., Macklin, K.S., Barbaree, J.M. and Miller, R.S. (2005) Evaluation of Agar Plates for Direct Enumeration of *Campylobacter* spp. from Poultry Carcass Rinses. *Appl Environ Microbiol* **71**, 3351-3354.

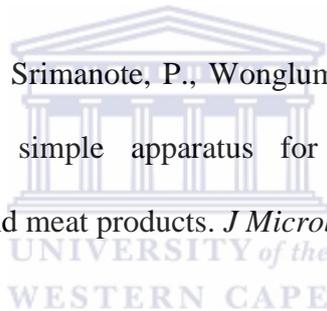
Rosenquist, H., Sommer, H.M., Nielsen, N.L. and Christensen, B.B. (2006) The effect of slaughter operations on the contamination of chicken carcasses with thermotolerant *Campylobacter*. *Int J Food Microbiol* **108**, 226-232.

Sampers, I., Habib, I., Berkvens, D., Dumoulin, A., De Zutter, L. and Uyttendaele, M. (2008) Processing practises contributing to *Campylobacter* contamination in Belgian chicken meat preparations. *Int J Food Microbiol* **128**, 297-303.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. and Teixeira, P. (2011) *Campylobacter* spp. as a foodborne pathogen: a review. *Front Microbiol* **2**, 1-12.

Wilson, K. (1994) Preparation of genomic DNA from bacteria. In Current protocols in molecular biology ed. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. pp.2.4.1-2.4.2. New York: Greene Publishing and Wiley-Interscience.

Wisessombat, S., Kittiniyom, K., Srimanote, P., Wonglumsom, W., Voravuthikunchai, S.P. (2009) A novel method and simple apparatus for the detection of thermophilic *Campylobacter* spp. in chicken and meat products. *J Microbiol Meth* **76**, 169-173.



Chapter 4 - The survival of *Campylobacter* species in chicken meat

4.1 ABSTRACT

Campylobacter species are naturally found in the environment yet the sporadic nature of campylobacteriosis cases suggest that its main source is in the domestic setting where the handling of raw chicken during food preparation practices are likely to contaminate the preparer's hands, utensils used as well as any surfaces that come into contact with contaminated chicken. *Campylobacter* has previously been documented to survive for extended periods in chicken meat at refrigeration and freezing temperatures. The aim of this study was to determine the survival period of *C. coli* and *C. jejuni* previously isolated from chicken meat and the neck skin of South African commercial and free range chicken farms. The survival of the strains were tested in chicken meat of commercial farming origin at 4°C and -20°C, thus simulating refrigeration and freezing temperatures in the domestic setting. It was found that the four *Campylobacter* strains used in the experiment was able to survive at 4°C in the refrigerator for eight days with no noticeable change in the initial bacterial count inoculated onto the chicken meat. The survival of the strains at -20°C proved to be more variable. Initially, there was no immediate reduction of bacterial load upon visual examination. The *C. jejuni* subsp. *jejuni* biotype 1 NS24a strain of free range origin lost its culturability at day eight of the sampling period. *C. coli* CF10a was able to survive for 25 days, while *C. coli* CF9a and *C. jejuni* S1b were still viable on day 30 of the sampling period. The results indicate that strain viability is variable but still show that campylobacters are able to survive on chicken meat for a significant period of time. Therefore the consumers' food preparation practices should be of a very strict hygienic standard while ensuring that their food, especially those containing raw poultry, should be thoroughly cooked at high temperatures, to prevent the risk of contracting campylobacteriosis.

4.2 INTRODUCTION

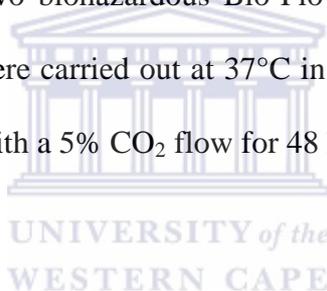
Campylobacter species are ubiquitous in the environment (Allos and Lastovica 2011). The sporadic nature of campylobacteriosis cases (Silva *et al.* 2011; Calciati *et al.* 2012; Habib *et al.* 2012) suggest that the majority of these cases originate from the domestic kitchen (Melero *et al.* 2012). The most important risk factors for acquiring the illness are the handling of and consumption of contaminated chicken, chicken products and ready-to-eat (RTE) foods (Simmons *et al.* 2008; Alonso *et al.* 2011; Melero *et al.* 2012). Studies have also shown that the cooking utensils such as dishes, knives and cutting boards as well as food contact surfaces and hands are readily contaminated if it comes into contact with contaminated raw chicken. Cross-contamination of RTE foods (Lindqvist and Lindblad 2008; Melero *et al.* 2012) and fresh produce (Humphrey *et al.* 2007) occur when the above-mentioned contaminated utensils come into direct contact with the food. The above factors increases the risk of acquiring campylobacteriosis (Lindqvist and Lindblad 2008; Melero *et al.* 2012). Contaminated RTE foods and fresh produce does not undergo any further cooking and therefore a greater risk of acquiring campylobacteriosis is identified if consumed (Humphrey *et al.* 2007).

Campylobacter has the ability to survive in water for 14 to 60 days at room temperature but the period of viability differs and is strain dependent. It was also demonstrated by Talibart *et al.* (2000) that *Campylobacter* strains have the ability of being resuscitated when the non-culturable strains are injected into fertilized chicken eggs. Separate studies have shown that *Campylobacter* species are more susceptible to freezing than refrigeration temperatures as the bacterial load is significantly reduced upon initial freezing (Georgsson *et al.* 2006; Habib *et al.* 2010; Sampers *et al.* 2010). However, strains in the respective studies could still be

detected after 84 (Sampers *et al.* 2010) to 220 days (Georgsson *et al.* 2006). *Campylobacter* numbers remain relatively invariable over a period of 14 days at 4°C (Sampers *et al.* 2010). This study aimed to qualitatively determine the survival period of *C. coli* and *C. jejuni* in chicken meat through the artificial contamination of the chicken with different strains of the bacteria.

4.3 MATERIALS AND METHODS

Tryptose Blood Agar Base (CM0233 – Oxoid, Basingstoke, UK) supplemented with 10% unlysed citrated horse blood (MRC, Delft, South Africa) (hereafter wholly referred to as TBA plates) was used for the routine isolation and subculturing of *Campylobacter* species, unless otherwise stated. The detailed preparation procedure of the growth media plates are described in Appendix A. Excess moisture was removed from the TBA plates prior to use by drying the plates for one hour in a class two biohazardous Bio-Flow laminar flow cabinet (Labotec, South Africa). All incubations were carried out at 37°C in a carbon dioxide (CO₂) incubator (Nu-Aire, Lasec, South Africa) with a 5% CO₂ flow for 48 h, ± 4 h, unless otherwise stated.



4.3.1 Reference strains

Strains that were used in the survival study of *Campylobacter* species in chicken meat were originally isolated from commercial and free range chicken in a separate study. No clinical strains were used in this study for a comparison as they were unable to be revitalized. Four *Campylobacter* strains were used for this study; *C. coli* CF9a, *C. coli* CF10a, *C. jejuni* S1b (of commercial farming origin) and *C. jejuni* subsp. *jejuni* biotype 1 NS24a (of free range farming origin). The strains were viable after its isolation and characterization from chicken meat and immediately used in the survival study.

4.3.2 Inoculation of chicken meat with *Campylobacter* species

Fresh chicken breasts with skin (of commercial farming origin - hereafter referred to as chicken meat) portions were purchased from a local retailer and kept refrigerated until transported to the laboratory, where it was refrigerated once again upon arrival until further processed. The chicken meat was aseptically divided into 16 × 25 g portions and placed in Whirl-Pak bags (Nasco, USA). The *Campylobacter* inoculums were prepared by resuspending a 10µl loopful of the respective *Campylobacter* strain, grown on TBA plates in 990 µl of sterile distilled water. The culture was resuspended completely into solution by repeatedly pipetting the mixture up and down and finally vortexing the solution on the highest setting for 15 s. From the respective inoculums, 100 µl was applied to the chicken meat (underneath and above the skin as well as on the meat). This was done in duplicate. Each bag was then carefully closed displacing as much air from the headspace as possible and placed in a refrigerator at 4°C. The same procedure was followed for the inoculation of the chicken meat that was stored at -20°C. Therefore the survival study of each *Campylobacter* strain was performed in duplicate at both refrigeration and freezer temperatures.

A 1000-fold serial dilution from each inoculum was performed using sterile distilled water as the diluent and plated in duplicate using the standard plating method on TBA plates and incubated. This was done to determine the original concentration of bacteria inoculated on the chicken meat. The concentration is expressed as CFU.ml⁻¹ and is calculated using the following formula: CFU.ml⁻¹ = average number of colonies ÷ dilution factor.

4.3.3 Determination of the survival period of *Campylobacter* species in chicken

4.3.3.1 *The survival of Campylobacter species in chicken at refrigeration temperature*

The survival of the respective *Campylobacter* strains in refrigerated chicken was determined over a period of eight days. Sampling was performed at 48 h intervals for the eight day period after the initial inoculation time. The bags containing the artificially contaminated chicken were removed from the refrigerator and the chicken was sampled immediately. The best manner in which to sample the chicken was not previously established and therefore two sampling methods were used in the initial sampling periods.

- 1. Standard streaking technique** – A sterile 10 µl plastic loop (LP Italian Spa, Italy) was drawn over the surface and beneath the skin of the chicken meat and streaked across a fresh TBA plate using the standard streaking technique.
- 2. Filtration technique** – A sterile swab (Sterilin, UK) was wiped across the surface and beneath the skin of the chicken meat. The head of the swab was transferred to a microcentrifuge tube containing one ml sterile distilled water. The contents of the microcentrifuge tube were vortexed at the highest setting for 15 s. A 0.65 µm cellulose nitrate filter (Sartorius Stedim Biotech, Germany) was aseptically placed on the surface of a fresh TBA plate and left to adhere to the surface of the plate for five min. One hundred microlitres of the solution was placed drop wise on the filter paper and left to stand for 15 min. The filter paper was then removed and the plate was incubated in the inverted position. This was done for each artificially contaminated chicken portion. The chicken portions were placed back in the refrigerator until the next sampling time.

Both sampling techniques were applied at the first and second sampling intervals. Thereafter the filtration technique was used for the rest of the sampling period.

It was noted that a lawn of *Campylobacter* growth was recovered after each sampling period using the filtration technique and therefore no difference in the *Campylobacter* load was recorded.

Albeit being a qualitative study, a method to quantitatively determine the survival of *Campylobacter* at each sampling period would have been essential to the construction of the survival curve (Figure 4.1). However, the limitation of time did not allow for the development of a quantitative method for the survival of *Campylobacter* at refrigeration temperatures. Therefore, the construction of the survival curve (Figure 4.1) was based on the assumption that the original *Campylobacter* load had remained constant even though, in reality, some bacterial cells must have died. The constant value assigned to the concentration of the respective bacteria isolated at the sampling intervals, was recorded as the initial respective concentrations that were used to contaminate the chicken for the construction of the curve. Since there was no difference between trial 1 and trial 2 of the study, no standard deviation was observed and therefore, no standard deviation is shown on the curve (Figure 4.1).

4.3.3.2 *The survival of Campylobacter species in frozen chicken*

The survival of the respective *Campylobacter* strains on frozen chicken was determined for a period of 30 days. Sampling was performed at 48 h intervals for the first 10 days of the survival experiment period. Thereafter, sampling was performed every fifth day until the end of the sampling period. The artificially contaminated chicken portions were allowed to thaw at room temperature until the meat was completely soft. Both the streaking and filtration techniques described in 4.3.3.1 above were employed to sample the chicken for the first and

second sampling interval. Thereafter, the filtration technique was used for the rest of the sampling period.

It was noted that, after the initial sampling periods had elapsed, isolated *Campylobacter* colonies were recovered. These colonies were counted and the concentration of the surviving bacterial load calculated albeit not being a quantitative study. This was done to enable the construction of a survival curve of each bacterial strain (Figure 4.2).

4.4 RESULTS

4.4.1 Respective inoculum concentrations used to artificially contaminate chicken meat samples

Table 4.1 Concentrations (CFU.ml⁻¹) of the respective *Campylobacter* inoculums used to artificially contaminate fresh chicken meat portions

<i>Campylobacter</i> strain	Concentration (*CFU.ml ⁻¹)
<i>Campylobacter coli</i> CF9a	8.75×10^{24}
<i>Campylobacter coli</i> CF10a	7.35×10^{24}
<i>Campylobacter jejuni</i> S1b	1.56×10^{24}
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> biotype 1	2.1×10^{24}

*CFU.ml⁻¹ : Colony forming units per millilitre



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4.4.2 Effect of refrigeration temperatures on the survival of *C. coli* and *C. jejuni* in artificially contaminated chicken meat

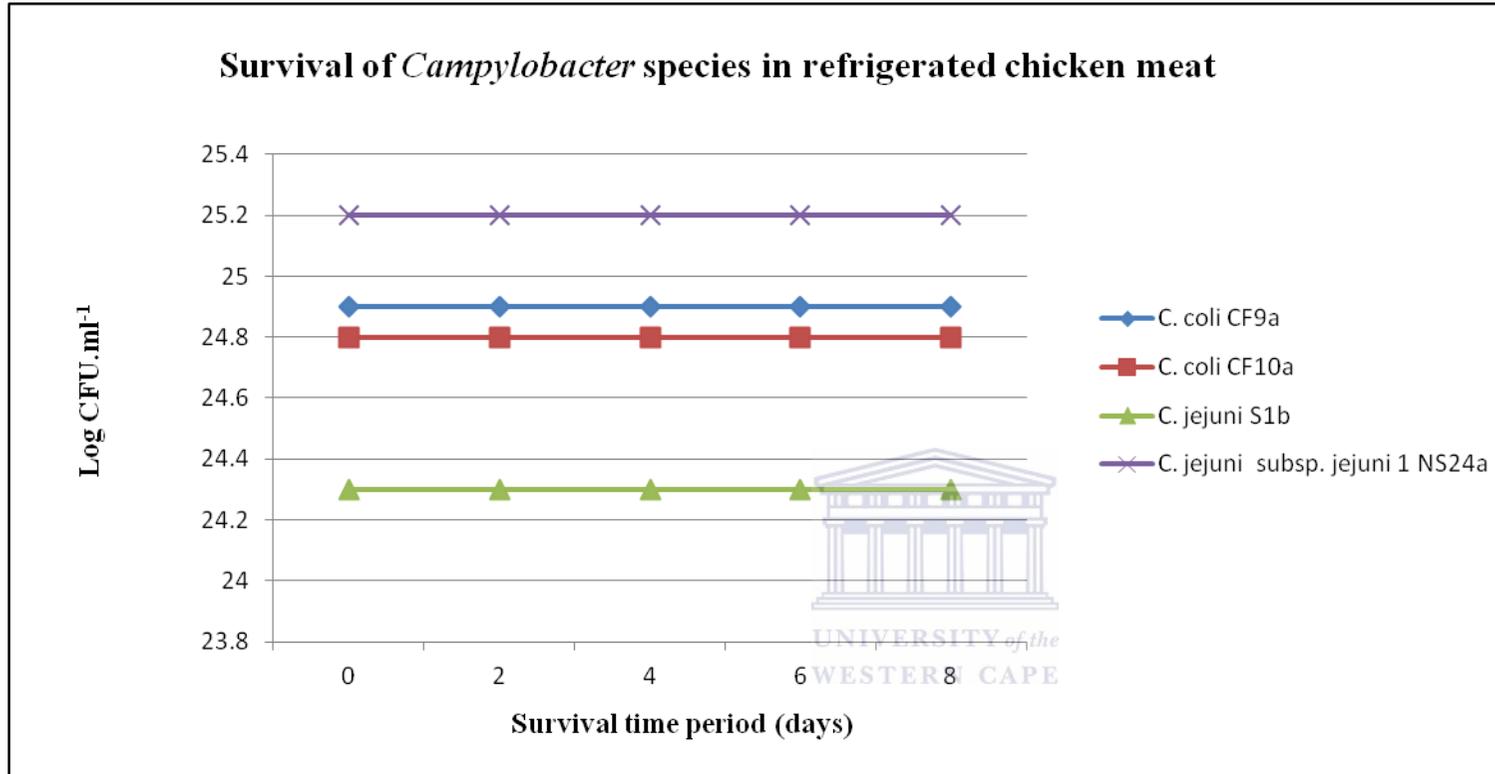


Fig. 4.1 Survival curve of *Campylobacter* species in refrigerated chicken meat for a period of 8 days

4.4.3 The effect of freezing on the survival of *C. coli* and *C. jejuni* in artificially contaminated chicken meat - next page

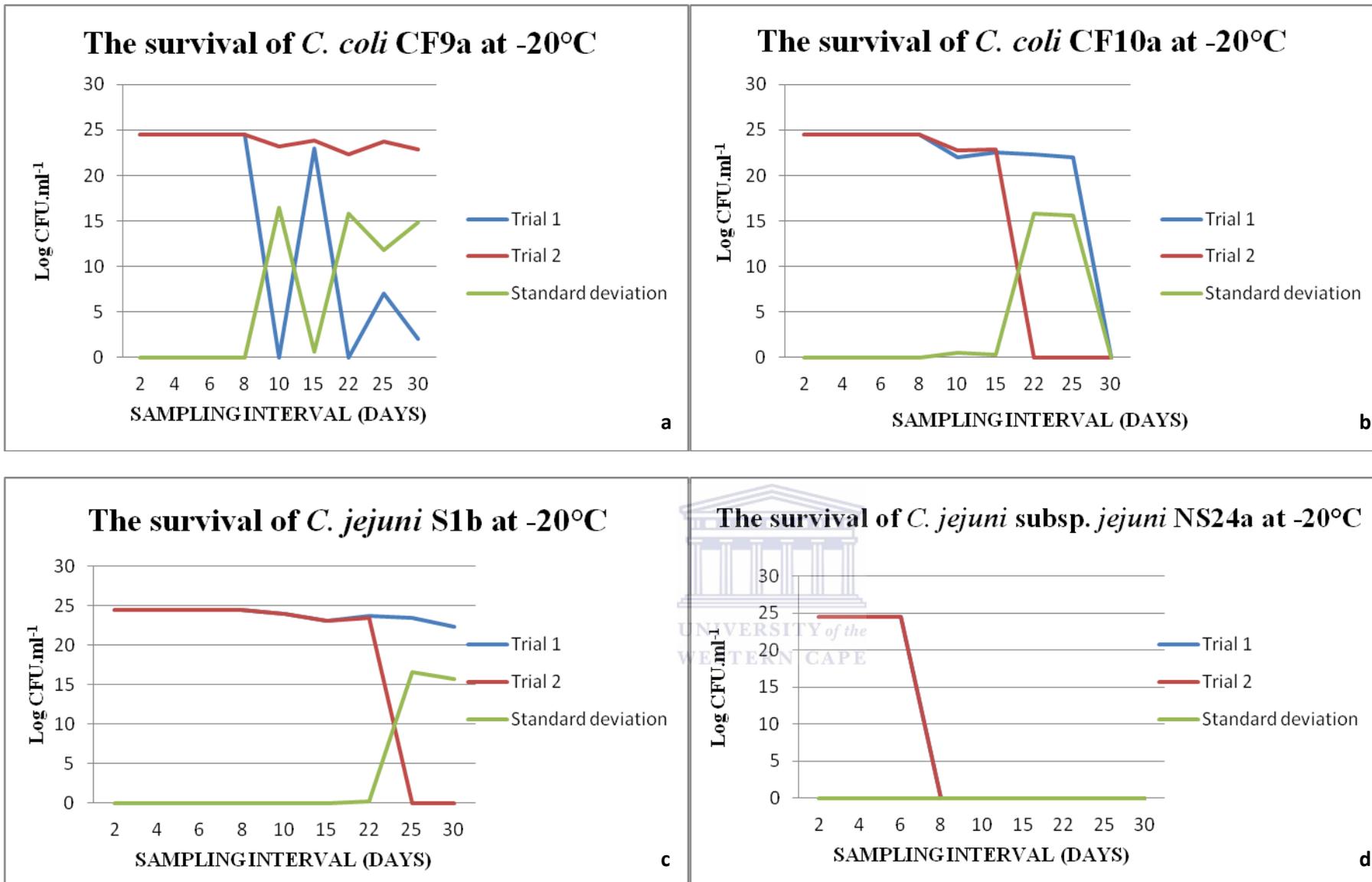


Fig. 4.2 The effect of freezing on the survival of *C. coli* (a, b) and *C. jejuni* (c, d) in artificially contaminated chicken meat over 30 days (standard deviations varied from 0 and 16.6 log₁₀ CFU.ml⁻¹).

4.5 DISCUSSION

This study qualitatively determined the survival period of *C. coli* and *C. jejuni* in fresh chicken refrigerated at 4°C and frozen chicken at -20°C. These temperatures were chosen as fresh chicken is kept refrigerated at 4°C in supermarkets and in the domestic setting and chicken are usually frozen for long term storage. This study therefore simulates the practices of the supermarket and domestic setting.

A high concentration of bacteria was used to inoculate the chicken portions (Table 4.1). This study was of a qualitative nature and therefore this high concentration of the inoculums were not reduced to a concentration level of naturally contaminated chicken ($\sim 10^5$ to 10^9 CFU g⁻¹ - Corry and Atabay 2001; Zweifel *et al.* 2008). The main aim was to determine the period of survival of the respective *Campylobacter* strains at the respective temperatures.

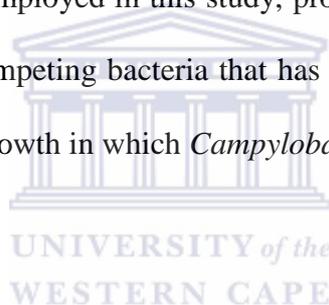
The *Campylobacter* strains used to artificially contaminate the chicken meat portions were initially isolated from free range (*C. jejuni* subsp. *jejuni* biotype 1 NS24a) and commercial (*C. coli* CF9a and CF10a, *C. jejuni* S1b) chicken. The chicken meat portions used in the study was of commercial farming origin. Antibiotics and Antibiotic Growth Promoters (AGPs) are still extensively used in commercial farming in South Africa at both therapeutic and sub-therapeutic levels respectively (Xingwana 2008). This means that it is probable that the *Campylobacter* strains of commercial chicken origin have previously been exposed to antibiotics and it is likely that the strains were resistant to the antibiotic present in the chicken meat portions used in this study. It is unknown which antibiotics were used in the rearing of the commercial chicken. The opposite is true for the *Campylobacter* strain of free range chicken origin. These chickens are not exposed to antibiotics during rearing and therefore the *Campylobacter* strains isolated from free range chicken are not likely to be resistant to antibiotics.

The four *Campylobacter* strains used in this study to artificially contaminate the chicken meat portions were able to survive for 8 days at 4°C (Figure 4.1) after which the chicken was discarded as it had exceeded its shelf life at refrigeration temperatures. The bacterial counts did not appear reduced in number as smears of growth were evident at each sampling interval. *C. jejuni* subsp. *jejuni* biotype 1 NS24a, an isolate of free range origin, also showed no decline in number upon visual examination, in comparison with the rest of the strains used in the study. These results are in agreement with a study done by Sampers *et al.* (2010) as their results also demonstrated that *Campylobacter* were able to survive for 14 days at 4°C without a noticeable reduction in the bacterial count. This present study has an added advantage whereby a direct detection method was used in comparison with the use of the enrichment protocol, ISO10272-1:2006, to detect survival of *Campylobacter* in frozen chicken meat samples (Sampers *et al.* 2010).

The study of the effect of freezing on *Campylobacter* species was done in duplicate and each strain displayed a different trend in its respective survival curve (Figure 4.2). All strains survived the first six days with no noticeable reduction in bacterial numbers. This is not in agreement with previous studies as those studies showed an immediate (within ~2 days) reduction in bacterial numbers (Georgsson *et al.* 2006; Habib *et al.* 2010; Sampers *et al.* 2010). All strains beside the strain *C. jejuni* subsp. *jejuni* biotype 1 NS24a, also survived until the eighth day with no noticeable reduction in bacterial count. *C. jejuni* subsp. *jejuni* biotype 1 NS24a was not culturable at all from day eight. This is not in agreement with other studies as there was no gradual reduction in bacterial numbers before losing its culturability. A possible reason for this could be that the strain is more susceptible to antibiotic residues that may be left on the chicken than the other strains, as it is of free range chicken origin. Strain *C. coli* CF10 only survived for 25 days where only one colony had grown. Strains *C. coli* CF9 and *C. jejuni* S1b both survived for 30 days where counts <10 colonies were still obtained. A gradual decrease in numbers was observed throughout the survival experimental period of these two *Campylobacter* strains. The standard deviations varied from 0 to 16.6. The

higher standard deviations (for all strains) at some of the the respective sampling intervals (Figure 4.2) are due to no colony count observed in one trial while colonies were observed for the second trial of each respective strain (experiments performed in duplicate). These standard deviations would improve if the experiment is repeated several more times. The experiments were stopped at day 30 and this means that these two strains may have survived past the 30 day experimental period. Previous studies have shown that *Campylobacter* strains are able to survive >84 (Sampers *et al.* 2010) to 220 days (Georgsson *et al.* 2006). The commentary on the reduction in bacterial count in this present study is not for quantitative purposes but rather described for comparison with previous studies.

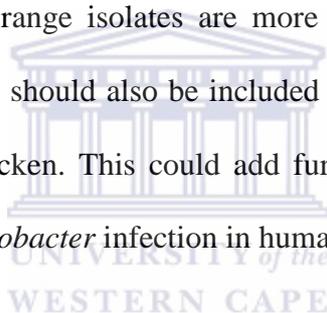
The filtration technique is based on the motility ability of *Campylobacter* organisms. This method could promote a more rapid and reliable detection method of viable *Campylobacter* (Wisessombat *et al.* 2009). This filtration technique employed in this study, proved to be superior to the standard streaking technique as it eliminated competing bacteria that has the potential to inhibit the growth of *Campylobacter* or to cause an overgrowth in which *Campylobacter* will not be identified.



4.6 CONCLUSION

The results obtained in the survival of *Campylobacter* in chicken meat demonstrate the risk associated with handling chicken in the supermarket and in the home during food preparation. These results show that it is very likely that chicken naturally contaminated with *Campylobacter* may retain *Campylobacter* levels if stored in the refrigerator or a reduced count if frozen due to the susceptibility of *Campylobacter* to multiple freeze-thaw cycles. Handling raw chicken and the consumption of undercooked contaminated chicken meat products has the ability to cause campylobacteriosis if proper hygiene and cooking practices are not adhered to in the home.

The variability of the survival period of each *Campylobacter* strain used in the study demonstrates that the survival of *Campylobacter* species in chicken meat is strain dependent. This study could be improved by increasing the number of strains used in the study, especially those of free range origin to determine the hypothesis that free range isolates are more susceptible to antibiotic residues present. Clinical *Campylobacter* strains should also be included in the study to determine whether they have the ability to survive in chicken. This could add further proof that chicken meat and products are the main source of *Campylobacter* infection in humans.



4.7 REFERENCES

Alonso, R., Girbau, C. and Astorga, A.F. (2011) *Campylobacter*. In Molecular Detection of Human Bacterial Pathogens ed. Liu, D. pp 1125-1139. Boca Raton, Florida: CRC Press, Taylor & Francis Group.

Allos, B.M. and Lastovica, A.J. (2011) *Campylobacter* Infections. In Tropical Infectious Diseases: Principles, Pathogens and Practise ed. Guerrant, R.L., Walker, D.H. and Weller, P.F. pp.145-149. Philadelphia: Saunders/Elsevier.

Calciati, E., Lafuente, S., De Simó, M., Balfagon, P., Bartolomé, R. and Caylá, J. (2012) A *Campylobacter* outbreak in a Barcelona school. *Enferm Infecc Microbiol Clin.* **30**, 243-245.

Corry, J.E.L. and Atabay, H.I. (2001) Poultry as a source of *Campylobacter* and related organisms. *J Appl Microbiol* **90**, 96-114.

Georgsson, F., Þorkelsson, A.E., Geirsdóttir, M., Reiersen, J. and Stern, N.J. (2006) The influence of freezing and duration of storage on *Campylobacter* and indicator bacteria in broiler carcasses. *Food Microbiol* **23**, 677-683.

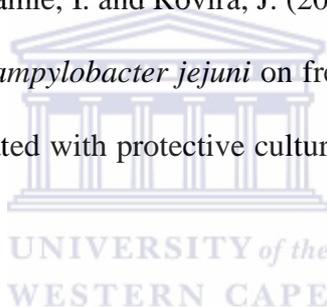
Habib, I., Uyttendaele, M. and De Zutter, L. (2010) Survival of poultry-derived *Campylobacter jejuni* of multilocus sequence type clonal complexes 21 and 45 under freeze, chill, oxidative, acid and heat stresses. *Food Microbiol* **27**, 829-834.

Habib, I., Berkvens, D., De Zutter, L., Dierick, K., Van Huffel, X., Speybroeck, N., Geeraerd, A.H. and Uyttendaele, M. (2012) *Campylobacter* contamination in broiler carcasses and correlation with slaughterhouses operational hygiene inspection. *Food Microbiol* **29**, 105-112.

Humphrey, T., O'Brien, S. and Madsen, M. (2007) Campylobacters as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* **117**, 237-257.

Lindqvist, R. and Lindblad, M. (2008) Quantitative risk assessment of thermophilic *Campylobacter* spp. and cross-contamination during food handling of raw broiler chickens evaluating strategies at the producer level to reduce human campylobacteriosis in Sweden. *Int J Food Microbiol* **121**, 41-52.

Melero, B., Diez, A.M., Rajkovic, A., Jamie, I. and Rovira, J. (2012) Behaviour of non-stressed and stressed *Listeria monocytogenes* and *Campylobacter jejuni* on fresh chicken burger meat packaged under modified atmosphere and inoculated with protective culture. *Int J Food Microbiol* **158**, 107-112.



Sampers, I., Habib, I., De Zutter, L., Dumoulin, A. and Uyttendaele, M. (2010) Survival of *Campylobacter* spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *Int J Food Microbiol* **137**, 147-153.

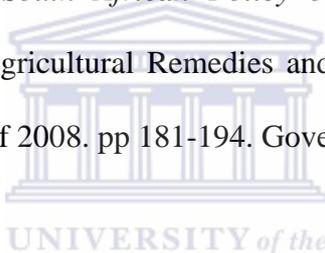
Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. and Teixeira, P. (2011) *Campylobacter* spp. as a foodborne pathogen: a review. *Front Microbiol* **2**, 1-12.

Simmons, M., Hiett, K.L., Stern, N.J. and Frank, J.F. (2008) Comparison of poultry exudates and carcass rinse sampling methods for the recovery of *Campylobacter* spp. subtypes demonstrates unique subtypes recovered from exudates. *J Microbiol Meth* **74**, 89-93.

Talibart, R., Denis, M., Castillo, A., Cappelier, J.M. and Ermel, G. (2000) Survival and recovery of viable but noncultivable forms of *Campylobacter* in aqueous microcosm. *Int J Food Microbiol* **55**, 263-267.

Wisessombat, S., Kittiniyom, K., Srimanote, P., Wonglumsom, W., Voravuthikunchai, S.P. (2009) A novel method and simple apparatus for the detection of thermophilic *Campylobacter* spp. in chicken and meat products. *J Microbiol Meth* **76**, 169-173.

Xingwana, L. (2008) *Publication of South African Policy on Animal Feeds*. Department of Agriculture, Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act no.36 of 1947). No. 31005 Notice 511 of 2008. pp 181-194. Government Gazette.



Zweifel, C., Scheu, K.D., Keel, M., Renggli, F. and Stephan, R. (2008) Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. *Int J Food Microbiol* **125**, 182-187.

Chapter 5 – Antimicrobial profiling of *Campylobacter* isolates

5.1 ABSTRACT

Antibiotics are chemical compounds that inhibit or destroys the growth of microorganisms and are used for the therapeutic treatment of infections in humans and animals. They are also used sub-therapeutically for the promotion of growth and performance in food animals. Incorrect usage and exposure to antibiotics increases the risk of the bacteria acquiring resistance to a specific antibiotic. Antibiotic growth promoters (AGPs) are still used intensively in the South African poultry industry and therefore contribute to the increase in resistance patterns of certain antibiotics. Antibiotic susceptibility testing was carried out for nalidixic acid, tetracycline, erythromycin and ciprofloxacin according to the disk diffusion method of the NHLS. The percentages of resistant *C. coli* strains (n = 33) of free range chicken were 18, 39, 61 and 85% respectively, while *C. jejuni* strains (n = 56) isolated from free range chicken were resistant to the above-mentioned antibiotics by 4, 11, 50 and 64%, respectively. *C. coli* isolates (n = 4) of commercial chicken origin were 100, 50, 100 and 25% resistant to the antibiotics, respectively, while *C. jejuni* strains (n = 29) showed 100, 86, 97 and 79% resistance to the antibiotics, respectively. The resistance prevalence is very high and is suggestive that previous exposure of the bacterial isolates to antibiotics is the main reason of the high resistance levels. Alternative strategies to reduce bacterial infections in poultry should be sought. More research, however, into bacteriophage therapy, the use of bacteriocins and competitive exclusion is needed as the methods show a great potential as a substitution to antibiotic therapy. Since the implementation of an appropriate measure of control for the successful reduction of *Campylobacter* on the farm is not in place, the focus should be to limit the transmission of infection from animals, animal products and the environment to humans.

5.2 INTRODUCTION

Antibiotics were originally isolated from microorganisms and can be defined as chemical compounds that inhibit the growth of other microbes in its surrounding environment. The classes of antibiotics are classified by its structural type as well as its mechanism of action in inhibiting or killing the target cell(s) (Davies 2006). In the early 20th century, it was discovered that not only were antibiotics beneficial to the health of humans, but was also beneficial to the health of food animals and enhanced its growth and performance at sub-therapeutic levels (van den Bogaard and Stobberingh 2000; Castanon 2007; Martinez 2009; Silva *et al.* 2011). However, a clear correlation of the acquired resistance of bacteria to the antibiotics used in the animal feed became apparent (Alanis 2005; Dibner and Richards 2005).

The use of antibiotics as growth promoters led to the vast emergence of antibiotic resistant pathogens globally in the 1980s and this led to the recommendation that all antibiotic growth promoters (AGPs) should be banned. The next decade saw the banning of AGPs in several European countries (Aarestrup *et al.* 1998; Casewell *et al.* 2003; Dibner and Richards 2005; Castanon 2007). South Africa (SA), however, continues to use antibiotics intensively in the 21st century but acknowledges that this practise may lead to the development of antibiotic resistant bacteria, which in turn, could compromise human therapy (Xingwana 2008). This use of antibiotics at sub-therapeutic levels apply to the commercial chicken farming industry in SA, while free range chicken are bred with limited exposure to antibiotics and are only administered to the chicken for therapeutic treatment of diseases (Humphrey *et al.* 2007).

Campylobacteriosis is considered to be a zoonotic illness as it is mainly acquired from the handling and consumption of raw and inadequately cooked contaminated chicken (Bester and Essack 2010). Patients with campylobacteriosis that require antibiotic therapy are primarily treated with macrolides and fluoroquinolones (Avrain *et al.* 2003; Ishihara *et al.* 2004; Moore *et al.* 2006; Bester

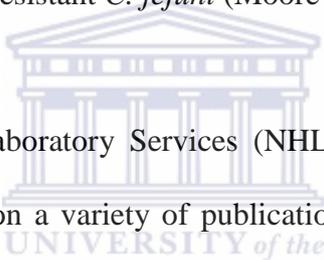
and Essack 2008) while tetracyclines, although rarely used, have been suggested as an alternative choice of treatment. Serious cases of *Campylobacter* infections, such as bacteremia or other systemic infections may necessitate the administration of intravenous aminoglycoside (gentamicin) therapy (Aarestrup and Engberg 2001).

Globally, there have been increased reports of *Campylobacter* of broiler (chicken) origin being resistant to antibiotics within the fluoroquinolone and macrolide classes of antibiotics (Luangtongkum *et al.* 2009). Various food safety organizations across the globe have highlighted the importance of monitoring the trend of antibiotic resistance and subsequently, organizations such as the Clinical and Laboratory Standards Institute (CLSI) and British Society for Antimicrobial Chemotherapy (BSAC) were established to set standards for the detection of antibiotic resistance in pathogens of clinical and food origin.

Many methods exist for the detection of antibiotic resistance in bacterial isolates. The earliest method available was the broth macrodilution method which involved the preparation of two-fold dilutions of antibiotic in an appropriate liquid growth medium. The tubes are inoculated with a standardized suspension of bacterial culture and the lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The macrodilution protocol can now be performed on a micro scale where the assay is performed in a 96 well tray and eliminates the laborious disadvantages of the former method. The possibility of errors in the preparation of the antibiotic solutions and the relatively large amount of reagents used is also reduced. The antimicrobial gradient diffusion method establishes an antibiotic concentration gradient in a solid growth medium as a means of detecting susceptibility. The method has been commercialized with the advent of the Epsilometer-test (E-test). The E-test strips are impregnated on the underside with a dried antibiotic concentration gradient and the MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. A

disadvantage of this method is that it is expensive especially when a few different types of antibiotics are to be tested (Jorgenson and Ferraro 2009). The disk diffusion method is simple, practical and cost efficient yielding qualitative results that can be interpreted as susceptible, intermediate resistance or resistant (Anon 2005; Jorgenson and Ferraro 2009; Anon 2011; Anon 2012).

The above-mentioned methods have been employed to determine the antimicrobial susceptibility of *Campylobacter* to a range of antibiotics. However, no internationally accepted method is available as a susceptibility testing method for *Campylobacter*. As a result, there is much debate about the method and interpretation of results used for susceptibility testing of campylobacters. Many studies have investigated the variation of results when the above-mentioned methods were compared. It was agreed upon, amongst authors, that the disk diffusion method was the most reliable for monitoring the prevalence of antibiotic resistant *C. jejuni* (Moore *et al.* 2006).



The South African National Health Laboratory Services (NHLS) have compiled disk diffusion susceptibility testing guidelines based on a variety of publications comparing the MICs obtained using the agar dilution method to the susceptibility testing results obtained using the disk diffusion method (A. Whitelaw, personal communication).

The aim of this study was to establish the antimicrobial susceptibility patterns of *C. coli* and *C. jejuni* from South African free range and commercial chicken origin using reliable, locally established disk diffusion testing parameters.

5.3 MATERIALS AND METHODS

5.3.1 Control and reference strains

The appropriate susceptible control strains should be used to monitor the performance of antimicrobial susceptibility tests (not for the interpretation of susceptibility) while resistant strains such as *Haemophilus influenzae* ATCC 49247, which is a β -lactamase negative, ampicillin resistant strain, should be used to determine whether the method will detect mechanisms of resistance against certain antibiotics (Anon 2012). Due to the lack of availability of control strains, a clinical reference strain, *C. jejuni* GSH 5953887, was used to monitor the performance of the technique used. The reproducibility of the antibiotic susceptibility test technique was determined in triplicate using the disk diffusion method outlined below.

5.3.2 Antibiotic susceptibility testing of *Campylobacter* isolates using the disk diffusion method

Tryptose Blood Agar Base (Oxoid, Basingstoke, UK) supplemented with 10% unlysed citrated horse blood (MRC, Delft, South Africa) (hereafter wholly referred to as TBA plates) was used for the routine isolation and subculturing of *Campylobacter* species, unless otherwise stated. Mueller Hinton Agar (Oxoid, Basingstoke, UK) supplemented with 5% unlysed citrated horse blood (hereafter wholly referred to as MHA) plates were used for the antibiotic susceptibility testing using the disk diffusion method. The detailed preparation procedure of the growth media plates are described in Appendix A. Excess moisture was removed from the plates prior to use by drying the plates for one hour in a class two biohazardous Bio-Flow laminar flow cabinet (Labotec, Cape Town, South Africa). All incubations were carried out at 37°C in a carbon dioxide (CO₂) incubator (Nu-Aire, Lasec, South Africa) with a 5% CO₂ flow for 48 h, \pm 4 h, unless otherwise stated.

The *Campylobacter* isolates (n = 122) were isolated from South African free range and commercial chicken using the Cape Town Protocol and ISO 10272-1:2006 method outlined in Chapter 2. After biochemical characterization and PCR confirmation of the isolates as belonging to the genus *Campylobacter*, the disk diffusion method was used to determine the antibiogram of each isolate. Free range chickens yielded 89 *Campylobacter* isolates, of which 56 were *C. jejuni* and 33 were *C. coli*. Commercial chickens yielded 34 isolates, of which 29 were *C. jejuni* and 4 were *C. coli*.

The antibiotics and concentrations thereof used in this study as well as the interpretation of results obtained are listed in Table 5.1. All antibiotics were purchased from Oxoid, Basingstoke, UK.

Table 5.1 Concentrations of antibiotics and the corresponding zone diameters of the disk diffusion susceptibility test according to the NHLS

Antibiotic Class	Antibiotic	Concentration (μg)	Interpretation of zone diameters (mm)		
			R \leq	I	S \geq
Cephalosporin	Cephalothin	30	For identification only		
Fluoroquinolone	Ciprofloxacin	1	19	20-22	23
Quinolone	Nalidixic acid	30	6	-	6
Macrolide	Erythromycin	15	15	16-18	19
Tetracyclines	Tetracycline	10	17	18-28	29

R – Resistant; I – Intermediate resistance; S – Susceptible

The batch of *Campylobacter* isolates (n = 34) of commercial chicken origin were tested with the same antibiotics as listed in Table 5.1 except that the concentrations of the ciprofloxacin disks used were 5 μg and the concentration of tetracycline used was 30 μg . These concentrations were used as part of the guidelines set forth by the CLSI. The CLSI has no guidelines for the interpretation of disk diffusion susceptibility for *Campylobacter*. Therefore, the CLSI concentrations were used yet the zones of inhibition were interpreted according to the NHLS guidelines (Table 5.1).

5.3.2.1 Preparation of the inoculum

The inoculum should give a semi-confluent growth of colonies after $48 \text{ h} \pm 4 \text{ h}$ of incubation. The density of the inoculum was matched to the 0.5 McFarland standard within the absorbance range of 0.08 – 0.13 at 625 nm. The McFarland standard was prepared by adding 0.05 ml of 0.048 M BaCl_2 (Merck, South Africa) to 9.5 ml of 0.18 M H_2SO_4 (Merck, South Africa) with constant stirring. The suspension was thoroughly mixed by vortexing for 15 s and single-use aliquots (1.5 ml) was dispensed into 2 ml microcentrifuge tubes and stored in the dark at room temperature for up to six months. Before use, the McFarland standard was thoroughly mixed using a vortex and transferred to a cuvette (Lasec, South Africa) with a one cm light path. Using sterile, filtered distilled water as a blank standard, the absorbance of the McFarland standard was measured using a Spectronic Helios Epsilon spectrophotometer (Thermo Scientific, USA) at 625 nm. The absorbance of a 0.5 McFarland standard should be between 0.08 – 0.13.

The *Campylobacter* inoculums were prepared using the direct colony suspension method where each *Campylobacter* isolate was sub-cultured on a fresh TBA plate and incubated. The colonies were then transferred to one millilitre of sterile, filtered distilled water, vortexed to ensure an even suspension and the absorbance was read. The density of the inoculum matched that of the 0.5 McFarland standard absorbance range (0.08 to 0.13) at 625 nm. When needed, the density of the inoculum was adjusted to fall within the absorbance range using sterile, filtered distilled water or with the addition of more colonies to the inoculum and mixed to ensure an even suspension before the absorbance was read again.

The inoculum suspension was used within 15 min of preparation. A sterile cotton-wool swab (Sterilin, UK) was dipped into the suspension and excess liquid was removed by turning the head of the swab against the side of the microcentrifuge tube. The inoculum was spread over the entire surface of the MHA plate by swabbing in three directions. The plate was allowed to dry at room

temperature before the antibiotic disks were applied. An antibiotic disk dispenser (Oxoid, Basingstoke, UK) was used to apply the disks to the plates. No more than three antibiotic disks were applied to one plate.

Following incubation, the diameter of the zones were measured in millimetres and interpreted as susceptible, intermediate or resistant for each antibiotic disk.



5.4 RESULTS

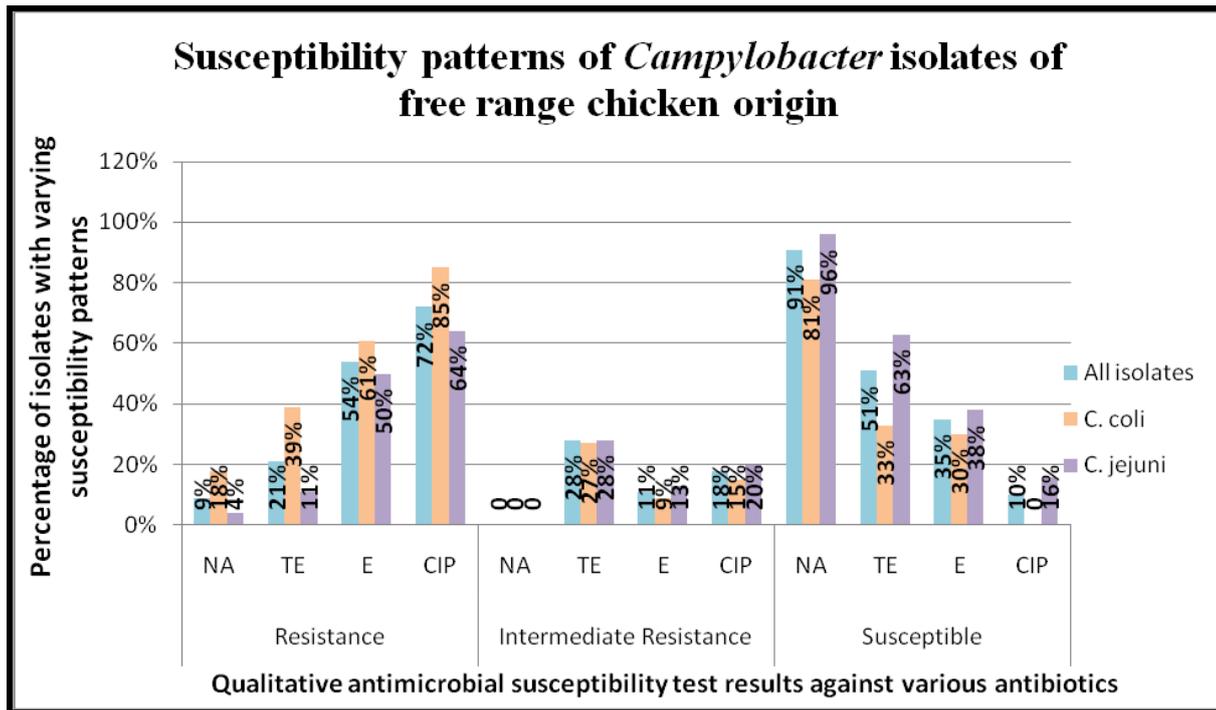


Fig. 5.1 The antimicrobial susceptibility patterns of *Campylobacter* isolates of free range chicken farming origin (NA – Nalidixic acid; TE – Tetracycline; E – Erythromycin; CIP – Ciprofloxacin)

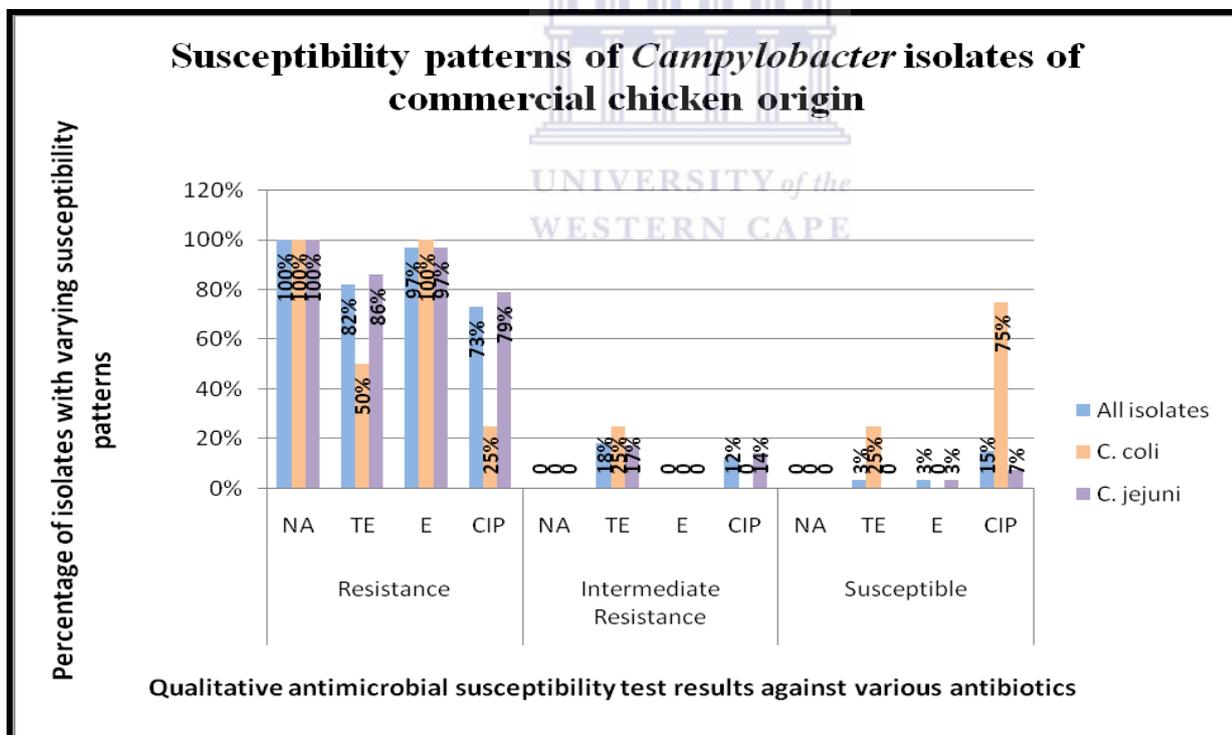


Fig. 5.2 The antimicrobial susceptibility patterns of *Campylobacter* isolates of commercial chicken farming origin (NA – Nalidixic acid; TE – Tetracycline; E – Erythromycin; CIP – Ciprofloxacin)

5.5 DISCUSSION

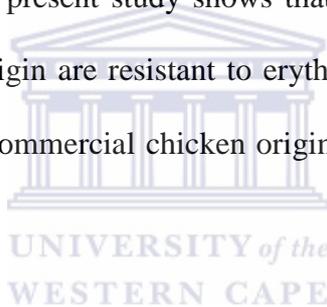
The use of antimicrobials as therapeutic agents in food animals has resulted in the emergence of antibiotic resistant bacteria, including antibiotic resistant *Campylobacter* (Aarestrup *et al.* 1998; Aarestrup & Engberg 2001; The *Campylobacter* Sentinel Surveillance Scheme Collaborators 2002; Rahimi and Ameri 2011). This could have a potentially serious impact on human health as antimicrobials are used to decrease the morbidity and mortality associated with serious and life-threatening infections. Resistance to antimicrobials decreases the effectiveness of the drugs and results in human health being compromised (Collignon *et al.* 2009).

In recent years, campylobacters have developed resistance to antibiotics, particularly fluoroquinolones and macrolide antibiotics globally (Moore *et al.* 2006; Rahimi and Ameri 2011). A study in the UK has shown that 55% of campylobacteriosis cases acquired abroad were resistant to ciprofloxacin compared to 10% of locally acquired infections that were resistant to the antibiotic (The *Campylobacter* Sentinel Surveillance Scheme Collaborators 2002). In the period 1996-2003, a decreased rate of ciprofloxacin resistance was reported in *C. jejuni* isolated from chicken meat in Denmark (Andersen *et al.* 2006). In South Africa, ciprofloxacin resistance in clinical *C. jejuni* isolates increased from 1.4% to 31.0% in a 14 year period between 1998 and 2011 (Bester *et al.* 2011). In the present study, *C. coli* and *C. jejuni* of free range chicken origin showed 85% and 64% resistance to ciprofloxacin respectively (Figure 5.1). The same isolates showed a low level of resistance to nalidixic acid (quinolone antibiotic), with 18% and 4% respectively. *C. coli* and *C. jejuni* isolates of commercial farming origin showed a 25% and 79% level of resistance to ciprofloxacin and both species showing 100% resistance to nalidixic acid.

The use of tetracyclines for therapeutic use in humans with campylobacteriosis has decreased in recent years (Andersen *et al.* 2006; Gibreel and Taylor 2006; Moore *et al.* 2006) and is used as an alternative choice of treatment to macrolides and fluoroquinolones. However, tetracycline resistant

campylobacters were prevalent in poultry in the KwaZulu Natal province of SA at a rate of 98% while tetracycline resistance in clinical *C. jejuni* isolates increased from 14.2% to 78.0% from 1998 to 2011 (Bester *et al.* 2011). The present study showed that 39% and 11% of *C. coli* and *C. jejuni* isolates from free range chicken were resistant to tetracycline respectively while 50% and 86% of the respective isolates of commercial farming origin were tetracycline resistant (Figure 5.1).

Macrolide antibiotics such as erythromycin are considered as the first drug of choice for the treatment of human campylobacteriosis cases. However, a significant increase in resistance to macrolides among campylobacters have been reported since the 1990s (Andersen *et al.* 2006; Gibreel and Taylor 2006; Collignon *et al.* 2009; Luangtongkum *et al.* 2009) while some studies report 0% resistance to erythromycin (Avrain *et al.* 2003; Wieczorek *et al.* 2012). The resistance of clinical *C. jejuni* isolates to erythromycin has increased from 3.4% to 7.2% from 1998-2011 in South Africa (Bester *et al.* 2011). The present study shows that 61% and 50% of *C. coli* and *C. jejuni* isolates of free range chicken origin are resistant to erythromycin respectively while 100% and 97% of the respective isolates of commercial chicken origin was resistant to the drug (Figure 5.2).



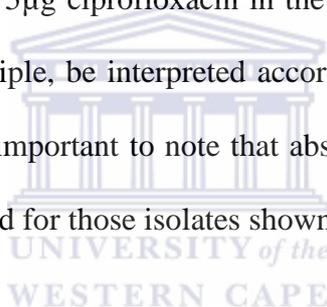
Many of the isolates showed resistance to two or more antibiotics, confirming that there is an increase in the emergence of multi-drug resistant *Campylobacter* (Moore *et al.* 2006; Bester *et al.* 2011; Rahimi and Ameri 2011). This is also in agreement with studies done in Poland by Wieczorek *et al.* (2012) where 60.9% of 321 *Campylobacter* isolates from poultry were resistant to two or more while one strain showed resistance to four different classes of antibiotics.

Cephalothin is an antibiotic that is used for the identification of *Campylobacter* species only. All *C. coli* and *C. jejuni* strains are resistant to this antibiotic (Lastovica and Skirrow 2000; Lastovica 2006) and therefore the analysis of the antibiogram results for this antibiotic was not discussed in

detail. The reference strain, *C. jejuni* GSH 5953887, was used to monitor the performance of the technique used. The reproducibility of the antibiotic susceptibility disk diffusion technique was determined in triplicate. The zone diameters for all antibiotics in triplicate corresponded well and therefore the method was shown to be reproducible.

Tetracycline (10 µg) was used for the antibiotic susceptibility testing of the free range isolates. This concentration used was an oversight as when initial contact was made with the NHLS, this information was erroneously captured. The correct concentration that should have been used was 30 µg. This error was only communicated at the end of the study and therefore the antibiotic susceptibility tests could not be repeated. The interpretation of the results was still done according to the guidelines of the NHLS.

The results obtained from the usage of 5µg ciprofloxacin in the antibiotic susceptibility testing of commercial isolates could not, in principle, be interpreted according the diameter zone guidelines set forth by the NHLS. However, it is important to note that absolute resistance was obtained and no visual zone of inhibition was obtained for those isolates shown to be resistant to ciprofloxacin.



The high levels of resistance obtained in this study is indicative that high levels of antibiotics are still being used in the South African poultry farming industry whether as therapeutic agents or as AGPs. This poses a serious risk for those persons who will acquire an infection from contaminated chicken meat (Collignon *et al.* 2009). The antibiotics erythromycin, tetracycline and ciprofloxacin will not be an effective remedy in the treatment of human campylobacteriosis cases and other alternative antibiotics such as gentamicin could be used therapeutically (Aarestrup and Engberg 2001; Wiczorek *et al.* 2012).

5.6 CONCLUSION

The high prevalence of resistance to ciprofloxacin, nalidixic acid, tetracycline and erythromycin, the antibiotics used in the therapeutic treatment of humans that have acquired serious cases of campylobacteriosis, is indicative that antibiotics are still used in the South African poultry industry. The antibiotic resistant trends suggest that exposure of bacteria to antibiotics increases its resistance to those specific drugs. Resistance could be acquired by the transfer of antibiotic resistance genes in the environment as well as the transfer of resistance genes within the gut of the bird.

Alternative treatment methods to prevent infection of the chicken should be further explored as a means of eradication of the rise of antibiotic resistant bacteria. Methods such as bacteriophage treatment, usage of bacteriocins and competitive exclusion prove to be promising methods for the treatment of infections. The former method has already been shown to reduce *Campylobacter* levels immediately but the mechanism of the competitive exclusion strategy has not been determined and therefore this approach has not yet been successful in the treatment of *Campylobacter* infection.

After years of research, there are still no effective, reliable and practical control measures in place to reduce or to completely prevent *Campylobacter* colonisation and therefore it is imperative that the transmission of infection from animals, animal products and the environment to humans must be prevented.

5.7 REFERENCES

Aarestrup, F.M., Bager, F., Jensen, N.E., Madsen, M., Meyling, A. and Wegener, H.C. (1998) Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. *APMIS* **106**, 606-622.

Aarestrup, F.M. and Engberg, J. (2001) Antimicrobial resistance of thermophilic *Campylobacter*. *Vet Res* **32**, 311-321.

Alanis, A.J. (2005) Resistance to Antibiotics: Are We in the Post-Antibiotic Era? *Arch Med Res* **36**, 697-705.

Andersen, S.R., Saadbye, P., Shukri, N.M., Rosenquist, H., Nielsen, N.L. and Boel, J. (2006) Antimicrobial resistance among *Campylobacter jejuni* isolated from raw poultry meat at retail level in Denmark. *Int J Food Microbiol* **107**, 250-255.

Anonymous (2005) *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Proposed Guideline*. Clinical and Laboratory Standards Institute CLSI document M45-P. Pennsylvania, USA: Clinical and Laboratory Standards Institute.

Anonymous (2011) *BSAC Methods for Antimicrobial Susceptibility Testing*. British Society for Antimicrobial Chemotherapy Version 10.2 May 2011. Birmingham, NJ: British Society for Antimicrobial Chemotherapy.

Anonymous (2012) *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement*. Clinical and Laboratory Standards Institute CLSI document M100-S22 Volume 32, No.3. Pennsylvania, USA: Clinical and Laboratory Standards Institute.

Avrain, L., Humbert, F., L'Hospitalier, R., Sanders, P., Vernozzy-Rozand, C. and Kempf, I. (2003) Antimicrobial resistance in *Campylobacter* from broilers: association with production type and antimicrobial use. *Vet Microbiol* **96**, 267-276.

Bester, L.A. and Essack, S.Y. (2008) Prevalence of antibiotic resistance in *Campylobacter* isolates from commercial poultry suppliers in KwaZulu-Natal, South Africa. *J Antimicrob Chemother* **62**, 1298-1300.

Bester, L.A. and Essack, S.Y. (2010) Antibiotic resistance via the food chain: Fact or Fiction? *S Afr J Sci* **106**, 1-5.

Bester, L., Basardien, L. and Lastovica, A.J. (2011) Antibiotic Resistance Patterns of *Campylobacter jejuni*, *C. concisus* and *C. upsaliensis* Isolated from Paediatric Patients in Cape Town, South Africa, 1998-2011. In Final Program and Abstract Book, Abstract A150, pp. 90. 16th International Workshop on *Campylobacter*, *Helicobacter*, and Related Organisms. Aug 28 – Sept 1, 2011, Vancouver, BC, Canada.

Casewell, M., Friis, C., Marco, E., McMullin, P. and Phillips, I. (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J Antimicrob Chemother* **52**, 159-161.

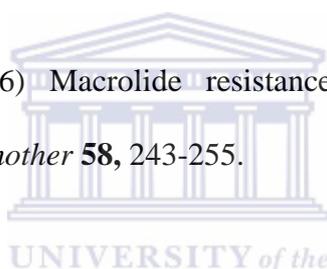
Castanon, J.I.R. (2007) History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poult Sci* **86**, 2466-2471.

Collignon, P., Powers, J.H., Chiller, T.M., Aidara-Kane, A. and Aarestrup, F.M. (2009) World Health Organization Ranking of Antimicrobials According to Their Importance in Human Medicine: A Critical Step for Developing Risk Management Strategies for the Use of Antimicrobials in Food Production Animals. *Food Safety* **49**, 132-141.

Davies, J. (2006) Are antibiotics naturally antibiotics? *J Ind Microbiol Biotechnol* **33**, 496-499.

Dibner, J.J. and Richards, J.D. (2005) Antibiotic Growth Promoters in Agriculture: History and Mode of Action. *Poult Sci* **84**, 634-643.

Gibreel, A. and Taylor, D.E. (2006) Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J Antimicrob Chemother* **58**, 243-255.



Humphrey, T., O'Brien, S. and Madsen, M. (2007) Campylobacters as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* **117**, 237-257.

Ishihara, K., Kira, T., Ogikubo, K., Morioka, A., Kojima, A., Kijima-Tanaka, M., Takahashi, T. and Tamura, Y. (2004) Antimicrobial susceptibilities of *Campylobacter* isolated from food-producing animals on farms (1999–2001): results from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. *Int J Antimicrob Agents* **24**, 63-69.

Jorgenson, J.H. and Ferraro, M.J. (2009) Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practises. *Med Microbiol* **49**, 1749-1755.

Lastovica, A. J. (2006) Emerging *Campylobacter* spp.: the Tip of the Iceberg, *Clin Microbiol Newslett* **28**, 49-56.

Lastovica, A.J. and Skirrow, M.B. (2000) Clinical Significance of *Campylobacter* and Related Species Other than *Campylobacter jejuni* and *C. coli*. In *Campylobacter* ed Nachamkin, I. and Blaser, M.J. pp. 89-120. Washington, D.C.: ASM Press.

Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C.M. and Zhang, Q. (2009) Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol* **4**, 189-200.

Martinez, J.L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* **157**, 2893-2902.

Moore, J.E., Barton, M.D., Blair, I.S., Corcoran, D., Dooley, J.S.G., Fanning, S., Kempf, I., Lastovica, A.J., Lowery, C.J., Matsuda, M., McDowell, D.A., McMahon, A., Millar, B.C., Rao, J.R., Rooney, P.J., Seal, B.S., Snelling, W.J. and Tolba, O. (2006) The epidemiology of antibiotic resistance in *Campylobacter*. *Microbe Infect* **8**, 1955-1966.

Rahimi, E. and Ameri, M. (2011) Antimicrobial resistance patterns of *Campylobacter* spp. isolates from raw chicken, turkey, quail, partridge, and ostrich meat in Iran. *Food Control* **22**, 1165-1170.

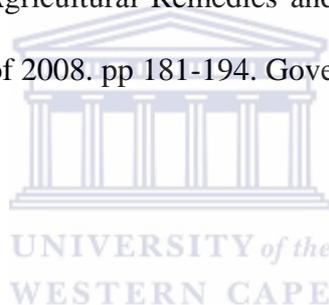
Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. and Teixeira, P. (2011) *Campylobacter* spp. as a foodborne pathogen: a review. *Front Microbiol* **2**, 1-12.

The *Campylobacter* Sentinel Surveillance Scheme Collaborators (2002) Ciprofloxacin resistance in *Campylobacter jejuni*: case-case analysis as a tool for elucidating risks at home and abroad. *J Antimicrob Chemother* **50**, 561-568.

van den Bogaard, A.E. and Stobberingh, E.E. (2000) Epidemiology of resistance to antibiotics Links between animals and humans. *Int J Antimicrob Agents* **14**, 327-335.

Wieczorek, K., Szewczyk, R. and Osek, J. (2012) Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter jejuni* and *C. coli* isolated from retail raw meat in Poland. *Vet Med* **57**, 293-299.

Xingwana, L. (2008) *Publication of South African Policy on Animal Feeds*. Department of Agriculture, Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act no.36 of 1947). No. 31005 Notice 511 of 2008. pp 181-194. Government Gazette.



Chapter 6 – DNA fingerprinting analysis of *Campylobacter* species using Amplified Fragment Length Polymorphism

6.1 ABSTRACT

Campylobacter species and particularly the thermotolerant *C. coli* and *C. jejuni* strains are the causative agents of the world's leading foodborne infection, campylobacteriosis. Cases of campylobacteriosis mainly occur sporadically while outbreaks have been reported. The current phenotypic methods for characterization of isolates are unreliable as they do not have a high degree of discriminatory power while some strains are untypeable. Therefore, genetic-based typing techniques have been designed as a more reliable tool with a greater discriminatory power between strains. The AFLP technique has been shown to have the greatest discriminatory power when compared to other leading DNA typing techniques and it was applied to determine the genetic and epidemiological relatedness between *C. coli* and *C. jejuni* strains isolated from free range and commercial chicken farms in the Western Cape province. A genetic similarity of 90% or more was observed for five phenons of *C. jejuni* strains from different farms and even between free range and commercial farms. All *C. coli* and *C. jejuni* strains displayed high genetic diversity indicating that there were various sources of *Campylobacter* infections of the chickens, even within the same farm. A few strains isolated from chicken bred on different farms were found to be identical and this indicates that a source common to both farms such as the feed or the hatchery could be implicated as the source of a diversity of *Campylobacter* strains. Clinical *C. jejuni* strains and *C. coli* strains from chicken generated completely different AFLP fingerprints to that of the *C. jejuni* strains of chicken origin and were clustered accordingly. The genetic fingerprints showed that four of the seven strains initially characterized biochemically as *C. coli* was clustered as *C. jejuni*. The results indicate that the relatedness between strains at both the genetic and epidemiological levels can be evaluated using AFLP analysis and subsequent computerized data analysis. Further AFLP analysis of a larger selection of clinical *Campylobacter* strains and more strains from within and from

different chicken farms in the Western Cape region and possibly from South Africa (SA) should be done in order to determine whether chicken can be implicated as the main source of human campylobacteriosis cases in SA. The AFLP tool exhibits high discriminatory power and promises to enable the tracking of the source and typing of *Campylobacter* infections.



6.2 INTRODUCTION

Campylobacter species are regarded as the most common agent of bacterial gastroenteritis worldwide (Kokotovic and On 1999; Lindstedt *et al.* 2000; Messens *et al.* 2009). These bacteria naturally occur in food animals, household pets and from environmental sources (Kokotovic and On 1999). The identification and subtyping of campylobacters are however considered to be problematic since traditional phenotypic methods have inadequate differential powers and a high proportion of strains are untypeable (Duim *et al.* 1999; Kokotovic and On 1999; Lindstedt *et al.* 2000; On and Harrington 2000). Epidemiological studies are further complicated by the sporadic nature of campylobacteriosis cases (Kokotovic and On 1999). Additional methods are therefore needed to correctly identify the source of *Campylobacter* infections and phylogenetic studies. The genomic ‘fingerprint’ of a *Campylobacter* strain has the ability to distinguish different strains from one another and to trace the source and routes of cases and/or outbreaks (Newell *et al.* 2000; Humphrey *et al.* 2007). DNA typing methods have gained popularity in recent years yet it has been difficult to combine speed and simplicity with high discriminatory power and reproducibility (Kokotovic and On 1999; Lindstedt *et al.* 2000).

Amplified Fragment Length Polymorphism (AFLP) is a high resolution genotyping method that was originally developed by Keygene BV, Wageningen, The Netherlands, for the genotyping of plants and to a lesser extent, animals (Vos *et al.* 1995; Savelkoul *et al.* 1999; Meudt and Clarke 2007). It has also been adapted for the genotyping of bacteria (Duim *et al.* 1999; Kokotovic and On 1999; Duim *et al.* 2000; Duim and Savelkoul 2003). The technique cannot be used for the identification of the genus or family to which a specific bacterium belongs but can be used for the typing of species, subspecies and strains (Savelkoul *et al.* 1999). The powerful advantage of the AFLP technique is that it requires no prior knowledge of the target organism’s genomic sequence (Vos *et al.* 1995; Savelkoul *et al.* 1999; Duim *et al.* 2000; Messens *et al.* 2009).

AFLP analysis requires a small amount of genomic DNA which is digested with two restriction enzymes; a rare cutter enzyme which cleaves a six bp recognition site and will cut on average, at every 4096 bp in the genome and a frequent cutter enzyme which cleaves a four bp recognition site and will have an average cutting frequency once every 256 bp (Vos *et al.* 1995). Several enzyme combinations have been used since the original publication by Vos *et al.* (1995) (Savelkoul *et al.* 1999), and the combination of *Hind*III and *Hha*I have been found to cleave *Campylobacter* DNA optimally (B. Duim, personal communication). Double stranded adaptors are ligated to the restriction fragments generated. The adaptors are designed in such a way that the initial restriction site is not restored after ligation which allows for simultaneous restriction and ligation. This creates a DNA template for the PCR amplification by adaptor-specific primers containing one or more selective nucleotides, ensuring that only a subset of fragments will be amplified under stringent PCR conditions. An extension of one selective nucleotide amplifies one of four of the ligated fragments whereas the incorporation of three selective nucleotides will amplify one in 4096 of the ligated fragments (Vos *et al.* 1995; Duim *et al.* 1999; Savelkoul *et al.* 1999; On and Harrington 2000; Messens *et al.* 2009). The selective primer which spans the average-frequency restriction site is fluorescently labelled. The generated fragments are detected by this fluorescently labelled primer and analyzed on gel or capillary based automated DNA sequencers (Duim *et al.* 2001; Messens *et al.* 2009). The AFLP technique is rapid and easily standardized and has become feasible due to the increasing availability of automated DNA sequencers. However, this equipment is expensive but the digitization of AFLP results allows accurate interpretation, ease of data storage and ready data exchange between laboratories (Newell *et al.* 2000).

The aim of this study was to discriminate and determine the relatedness between *C. coli* and *C. jejuni* strains from different farming regions in the Western Cape, South Africa and from different farm types, i.e. from free range and commercial chicken farms using the AFLP technique.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial strains and culture conditions

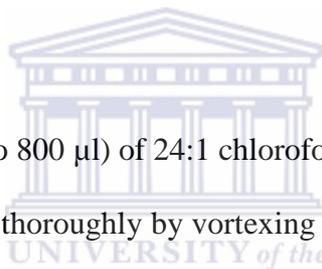
Campylobacter species (n = 20) were obtained from a separate study that aimed to determine the prevalence of *Campylobacter* in South African free range and commercial chicken. AFLP analysis was performed on these strains of which seven were *C. coli* strains and 13 were *C. jejuni* strains. Clinical *C. jejuni* isolates (n = 2) were used as a comparison to the *Campylobacter* isolates from the chicken. The chicken isolates were randomly selected for AFLP analysis; seven isolates were of free range chicken origin and 13 were of commercial chicken origin.

Tryptose Blood Agar Base (Oxoid, Basingstoke, UK) supplemented with 10% unlysed citrated horse blood (MRC, Delft, South Africa) (hereafter wholly referred to as TBA plates) was used for the routine isolation and subculturing of *Campylobacter* species, unless otherwise stated. The detailed preparation procedure of the growth media plates are described in Appendix A. Excess moisture was removed from the plates prior to use by drying the plates for one hour in a class two biohazardous Bio-Flow laminar flow cabinet (Labotec, South Africa). All incubations were carried out at 37°C in a carbon dioxide (CO₂) incubator (Nu-Aire, Lasec, South Africa) with a 5% CO₂ flow for 48 h, ± 4 h, unless otherwise stated. *Campylobacter* isolates were aseptically stored at -80°C in Microbank™ beads (Pro-lab Diagnostics, USA).

6.3.2 Isolation of bacterial genomic DNA

The detailed preparation of all buffers and chemicals used in the extraction of the genomic DNA is listed in Appendix B. All mixing by use of the vortex was done at the highest setting for 15 s. All centrifugation steps were done at 15 000 × g for 5 min using a Spectrafuge (Labnet, USA).

The genomic DNA of the isolates was extracted using an adaptation of the miniprep of bacterial genomic DNA protocol (Wilson 1994). The bacterial isolates were sub-cultured on a fresh TBA plate and incubated for 48 h in a microaerobic atmosphere. The resulting bacterial growth (10-20 μl) was removed from the plate using a sterile 10 μl plastic loop (LP Italian Spa, Italy) and completely resuspended in 567 μl Tris-EDTA (TE) buffer by repeated pipetting. Thereafter, 30 μl of 10% sodium dodecyl sulfate (SDS) (Merck, South Africa) and 3 μl of 20 $\text{mg}\cdot\text{ml}^{-1}$ proteinase K (Roche Diagnostics, South Africa) to give a final concentration of 100 $\mu\text{g}\cdot\text{ml}^{-1}$ proteinase K in 0.5% SDS added. The mixture was thoroughly mixed by vortexing and placed in a Scientific Series 9000 incubator (Lasec, South Africa) at 37°C for 1 h or overnight (~16 h). Thereafter, 100 μl of 5 M NaCl (Merck, South Africa) was added to give a final concentration of 0.7 M and mixed thoroughly by vortexing. The CTAB/NaCl solution (80 μl) (preheated in a water bath (Mettler, Lasec, South Africa) at 65°C for 15 – 30 min) was added and thoroughly mixed by vortexing and incubated in a water bath at 65°C for 10 min.



An approximate equal volume (700 μl to 800 μl) of 24:1 chloroform:isoamyl alcohol (Merck, South Africa) was subsequently added, mixed thoroughly by vortexing and then centrifuged. The aqueous viscous supernatant was transferred to a fresh microcentrifuge tube and an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Merck, South Africa) was added. The mixture was vortexed and then centrifuged. The resulting supernatant was transferred to a fresh microcentrifuge tube and 0.6 volume (of the volume transferred) isopropanol (Merck, South Africa) was added to precipitate the nucleic acids. The microcentrifuge tubes were shaken back and forth until the stringy white DNA precipitate became visible and then centrifuged. The liquid mixture in the tube was carefully poured off. The DNA was then washed with 200 μl 70% ice cold ethanol (Kimix, South Africa) and subsequently centrifuged. The ethanol was then carefully poured off and the DNA pellet was air-dried by placing it in a class two Bio-Flow laminar flow for 1 h or until all residual ethanol had evaporated. The pellet was dissolved in 100 μl TE buffer in a refrigerator overnight.

The concentration of the DNA ($\text{ng}\cdot\mu\text{l}^{-1}$) was determined using the NanoDrop[®] ND-1000 (Thermo Scientific, USA) and all DNA samples were diluted to $\sim 50 \text{ ng}\cdot\mu\text{l}^{-1}$ with TE buffer for use in the AFLP protocol.

6.3.3 AFLP analysis

AFLP analysis were performed on the *Campylobacter* species ($n = 22$) according to adaptations of the AFLP microbial fingerprinting method of Perkin-Elmer (PE) Applied Biosystems (Duim *et al.* 1999), and the fluorescent AFLP (F-AFLP) genotyping technique of Messens *et al.* (2009). Briefly, 500 ng of genomic DNA was simultaneously digested in a 30 μl reaction volume, according to manufacturers instructions, with one microlitre FastDigest *Hind*III (Thermo Scientific, USA) and one microlitre FastDigest *Hha*I (Thermo Scientific, USA) in the presence of two microlitres of $10 \times$ FastDigest Buffer (Thermo Scientific, USA). The suspension was mixed gently, briefly spun down and then incubated in a water bath set at 37°C for 10 min. Subsequent inactivation of the enzymes was not required and five microlitres of the restriction digestion products were electrophoresed in a 1.5% Tris-Acetic Acid-EDTA (TAE) - agarose gel (Lonza, Whitehead Scientific, South Africa) at $4\text{V}\cdot\text{cm}^{-1}$ for 3 to 4 h to check that the desired size products were obtained. The desired smear of product in the 100 – 1500 bp region should clearly be visible (results not shown).

Double stranded oligonucleotide adaptors designed by Duim and Savelkoul (2003) were synthesized by Inqaba Biotech (South Africa). The sequences of the adaptors are listed in Table 6.1. The adaptors were synthesized as single stranded oligonucleotides and annealing of the adaptors were therefore required prior to the ligation step. The adaptors were annealed by combining equal volumes of the forward and reverse strands of Adaptor *Hha*I and Adaptor *Hind*III in a microcentrifuge tube and heating it for 5 min at 100°C in a water bath. The adaptors were then allowed to slowly cool to room temperature by leaving it on the work bench for 10 min. The

ligation step was performed in a 50 µl reaction volume by adding five microlitres of 10 × T4 DNA Ligase Buffer (Thermo Scientific, USA), five microlitres of 50% PEG 4000 solution (Thermo Scientific, USA), 0.5 µl of 20 µM Adaptor *HhaI* and 0.5 µl of 2 µM of Adaptor *HindIII* and 2 U of T4 DNA Ligase, to the microcentrifuge tube containing the remaining 25 µl restriction digestion product. The suspension was mixed thoroughly, briefly spun down and incubated in a water bath at 22°C for 1 h. The enzyme was subsequently heat inactivated by incubating the mixture at 65°C for 10 min. The ligation products were diluted two-fold.

Table 6.1 Sequences of adaptors used to ligate to the restriction fragments generated (adapted from Duim and Savelkoul 2003)

Adaptor	Forward strand	Reverse strand
<i>HhaI</i>	5'-GACGATGAGTCCTGATCG-3'	5'-ATCAGGACTCATCG-3'
<i>HindIII</i>	5'-CTCGTAGACTGCGTACC-3'	5'-AGCTGGTACGCAGTC-3'

The pre-selective PCR was performed in 25 µl reaction volumes containing 2.5 µl of 10 × PCR Buffer (Bioline, UK), 1.25 µl of 50 mM MgCl₂ (Bioline, UK), 0.5 µl of 10 mM dNTPs (KapaBiosystems, South Africa), 1.25 U BioTaq (Bioline, UK), 2.2 µl of 0.1 µM pre-selective adaptor-specific *HhaI* primer (IDT, Whitehead Scientific, South Africa), 2.2 µl of 0.44 µM pre-selective adaptor-specific *HindIII* primer (IDT, Whitehead Scientific, South Africa) and 5 µl of the ligation product that was used as the DNA template. Amplification was performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Life Technologies, USA) using an initial denaturation step at 94°C for 2 min, followed by 25 cycles consisting of denaturation at 94°C for 20 s, annealing at 56°C for 30 s and extension at 72°C for 2 min. The primers used were designed by Duim and Savelkoul (2003) and the sequences thereof are listed in Table 6.2.

The selective PCRs were carried out in 20 µl reaction volumes containing 2 µl of 10 × PCR Buffer, 1 µl of 50 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 1.25 U BioTaq, 3 µl of 0.15 µM selective adaptor-

specific *HhaI* primer (IDT, Whitehead Scientific, South Africa), 3 µl of 0.75 µM FAM-labelled selective adaptor-specific *HindIII* primer (IDT, Whitehead Scientific, South Africa) and 3 µl of the undiluted pre-selective PCR product that was used as the DNA template. Amplification was performed in a GeneAmp PCR System 2700 thermocycler using an initial denaturation step at 94°C for 2 min, followed by 20 cycles consisting of denaturation at 94°C for 20 s, annealing at 66°C for 45s, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The primers used were designed by Duim and Savelkoul (2003) and the sequences thereof are listed in Table 6.2.

Table 6.2 Sequences of the primers used in both the pre-selective and selective PCR amplification of the AFLP technique (adapted from Duim and Savelkoul 2003)

Primer	Pre-selective PCR	Selective PCR
<i>HhaI</i> primer	5'-GATGAGTCCTGATCGC-3'	5'-GATGAGTCCTGATCGCA-3'
<i>HindIII</i> primer	5'-GACTGCGTACCAGCTT-3'	5'-56-FAM-GACTGCGTACCAGCTTA-3'

The selective PCR products were diluted 1:1(vol/vol) in TE buffer (Appendix B) and 1 µl of the diluted product was mixed with 2 µl of a loading buffer mix containing 1.25 µl deionized formamide, 0.25 µl blue dextran/50mM EDTA loading solution and 0.5 µl of GeneScan-500 (ROX) (GS-500 (ROX)) internal lane size standard in a 0.2 ml microcentrifuge tube. The tubes were heated in the thermocycler at 95°C for 3 min to denature the DNA and immediately removed and snap-cooled on ice to prevent re-annealing of DNA strands until loaded. The sample (2 µl) was loaded onto a 5% denaturing Long Ranger gel using 1 × Tris-Borate-EDTA (TBE) (Appendix B) running buffer. The electrophoresis conditions were 2500 V for 4 h.

Data generated during electrophoresis were collected by GeneScan software (Applied Biosystems, Life Technologies, USA). At the completion the electrophoresis, the gel lanes were tracked and extracted using the Gel Processor (Applied Biosystems, Life Technologies, USA) and GeneScan software and the densitometric curves generated were imported into the GelCompar II version 5.1

software (Applied Maths, Belgium). Gel lanes were normalized by use of the reference positions of the GS-500 (ROX) internal lane size standards.

DNA fragments ranging in size from 50 to 500 bp were used for comparison. The AFLP curves were calculated with the Pearson product-moment correlation co-efficient of similarity. Cluster analysis was subsequently carried out by the UPGMA clustering method (Duim *et al.* 1999; Duim *et al.* 2000; Duim *et al.*; 2001; Duim and Savelkoul 2003).

6.4 RESULTS

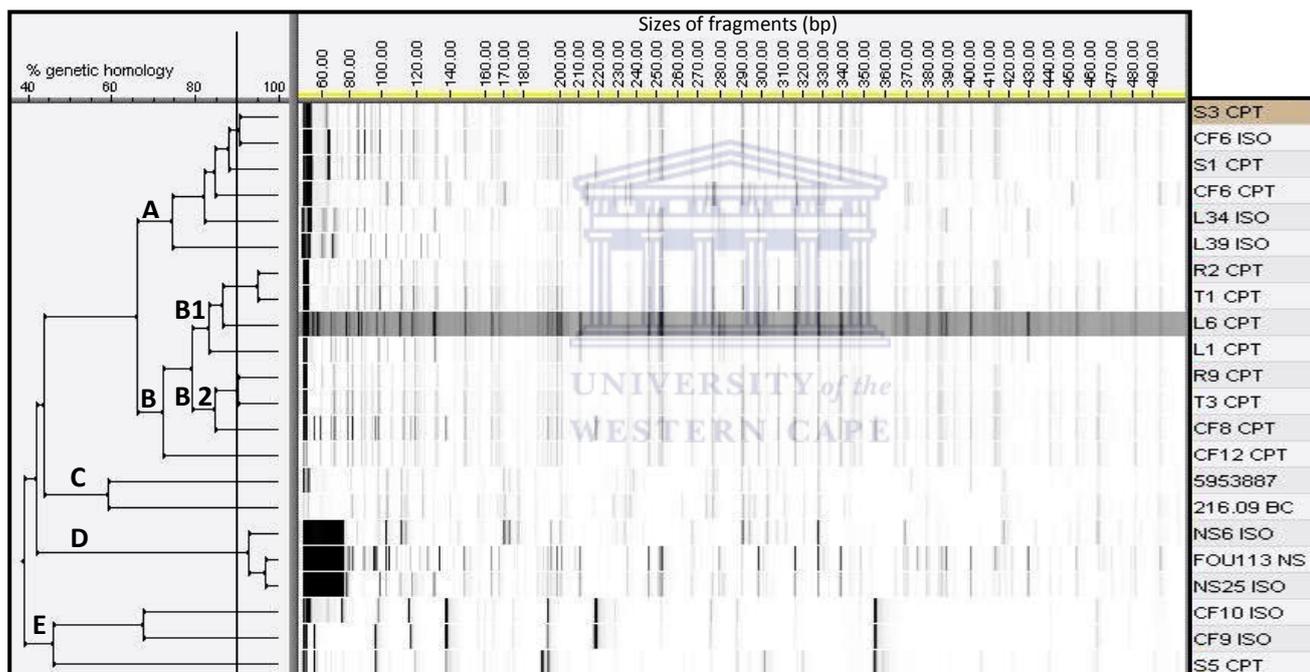


Fig. 6.1 Dendrogram derived from AFLP patterns of 22 *Campylobacter* species of free range and commercial chicken origin

6.5 DISCUSSION

The *Campylobacter* strains (n = 22) used in this study were randomly selected from a pool of isolates obtained from a separate study in which *Campylobacter* was isolated from free range and commercial chicken and identified and subtyped using biochemical tests (Chapter 3). The aim of applying the AFLP technique to the selected strains was to determine the relatedness between the strains that were isolated from chicken bred in different regions of the Western Cape based on its genetic composition.

After polyacrylamide gel electrophoresis, a pattern of 20-200 bands were obtained for the isolates (Figure 6.1). Initially, according to the biotyping results obtained from the separate study (Chapter 3), it showed that 7 *C. coli* strains were included in this study for AFLP analysis. However, the high discriminatory power of the AFLP technique showed that four of the isolates believed to have been *C. coli*, were indeed *C. jejuni*. This is further testimony that phenotypic subtyping of *Campylobacter* is not a reliable tool and that the AFLP technique provides higher discriminatory power for the subtyping of strains. This reclassification of strains brings the total number of *C. jejuni* isolates analysed by the AFLP technique to 17. The *C. coli* isolates are represented in cluster E of the dendrogram (Figure 6.1).

Five genotypes were obtained after UPGMA cluster analysis (clusters A-E) (Figure 6.1). The scale represents percentages of similarity as determined with the Pearson coefficient. The cut-off for similarity was set to 90% and therefore isolates that shared a similarity percentage of 90% or more were considered to be identical (Duim *et al.* 2001; Zweifel *et al.* 2008; Messens *et al.* 2009). Groups of isolates sharing a similarity of 90% and more were grouped to form a phenon (Olive and Bean 1999; Zweifel *et al.* 2008). Isolate reference numbers are included on the far right of Figure 6.1.

Cluster C contains the two clinical *C. jejuni* isolates that were included in the study to determine the differences in the genetic make-up of clinical *C. jejuni* strains in comparison to *C. jejuni* strains from chicken origin. The two clinical strains are clustered together; however they share a genetic similarity of < 60%. The *C. jejuni* strain 216.09 BC was a blood culture isolated from a hospitalized patient (Groote Schuur Hospital) in 2009, while the *C. jejuni* strain 5953887 was isolated from the stools of a hospitalized patient (Groote Schuur Hospital) in 2012. This could account for the two strains not being related (80% or more). These clinical strains share less than 45% similarity with *C. jejuni* strains of chicken origin. This implies that the source of the clinical *C. jejuni* isolates cannot be linked to any of the *C. jejuni* strains of chicken origin and that the hospitalized patients did not acquire their infection(s) from the chicken(s) of which the *C. jejuni* strains were isolated.

Cluster A (Figure 6.1) is comprised of *C. jejuni* strains of both commercial (S3CPT, CF6ISO, S1CPT, CF6CPT) and free range (L34ISO and L39ISO) origin. The *C. jejuni* strains S3CPT and CF6ISO share a ~91% similarity and are therefore considered to be identical. These two strains originate from chicken bred on two different commercial farms in the Stellenbosch area of the Western Cape province of South Africa. These two farms supply the same distributor of retail chicken in the Western Cape. A common source that is supplied to both the farms such as the feed or the hatchery could be implicated as the original source of the *Campylobacter* infection. The *C. jejuni* strains S1CPT and CF6CPT displays similarity (~87% and ~84% respectively) to the S3CPT and CF6ISO phenon. Strain CF6CPT was characterized as *C. jejuni* subsp. *jejuni* biotype 2 using the biochemical characterization scheme. A distinct banding pattern is obtained for this strain from other *C. jejuni* strains of which most were characterized as *C. jejuni* subsp. *jejuni* biotype 1. CF6ISO and CF6CPT were isolated from the same chicken leg (Chapter 3) from a batch of one farm while S3CPT and S1CPT were from the same batch of samples of another farm. It can therefore be deduced that the original source(s) of contamination or the farm environment(s) are abundant with different strains of *Campylobacter*. However, it should be noted that the chickens on these farms

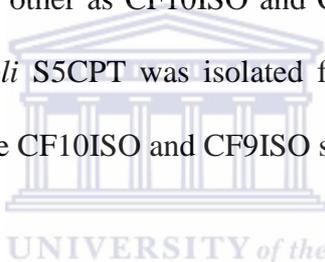
are kept in cages as it is commercial farms. Therefore the *Campylobacter* strains are introduced to the chicken through sources such as the workers entering the chicken houses or the drinking water and feed brought into the chicken house (Humphrey *et al.* 2007; Horrocks *et al.* 2009).

The free range *C. jejuni* strain, L34ISO shows 82% similarity to the commercial *C. jejuni* strain CF6CPT and the strain L39ISO displays a 75% similarity to L34ISO. This difference in the somewhat similar free range *C. jejuni* strains suggest that the free range farm environment is most likely to also be abundant in different strains of *Campylobacter* as campylobacters are considered to be ubiquitous in the environment (Allos and Lastovica 2011).

The second genotype in the dendrogram (cluster B) (Figure 6.1) contains two phenons; the *C. jejuni* strains R2CPT and T1CPT which share a 95% similarity and the *C. jejuni* strains R9CPT and T3CPT which share a similarity of 90%. The two former strains are identical to each other while the two latter strains are identical to each other. R2CPT and T1CPT are from different farms while R9CPT is from the same farm as R2CPT and T3CPT is from the same farm as T1CPT. Therefore, it could also be concluded that a common source supplied to the two farms are the original source of *Campylobacter* infection. This cluster also comprises of the *C. jejuni* strains of free range chicken origin (L6CPT, L1CPT). This could also imply that a vehicle of transmission exists in the environment such as flies or wild birds (Zweifel *et al.* 2008) as the location that these strains originate from are in different regions of the Western Cape. Further investigation and analysis of the sources of the chicken feed or the chick supplier is required. CF8CPT is similar (85%) to the R9CPT and T3CPT phenon while CF12CPT is ~72% similar to clusters B1 and B2. CF8CPT and CF12CPT are from the same batch of chicken samples as the CF6ISO and CF6CPT strains and are from the same farm. The heterogeneity between the isolates indicates that there are multiple sources of *Campylobacter* infection on the farm.

The *C. jejuni* strains NS6ISO, FOU113NS and NS25ISO are of free range chicken origin and have all originated from the same free range farm. They are grouped together in cluster D of the dendrogram (Figure 6.1). The strains FOU113NS and NS25ISO are grouped in a phenon and has a ~96% degree of similarity. These strains were isolated 6 months apart; strain FOU113NS was isolated in cooler month of May 2011 while the NS25ISO strain was isolated in the warmer month of November 2011. This could represent a strain that is dominant or persistent on the farm. Strain NS6ISO is ~91% similar to the phenon described above. NS6ISO and NS25ISO were isolated from different batches of chicken samples almost two months apart. This too, could represent a dominant strain in the environment as a similarity of 90% or more is considered to be identical strains.

The strains CF10ISO, CF9ISO and S5CPT are *C. coli* isolates of commercial chicken farming origin. These strains are grouped together in cluster E (Figure 6.1). The *C. coli* strains show a greater degree of heterogeneity to each other as CF10ISO and CF9ISO, of one batch of samples, have a ~68% genetic similarity. *C. coli* S5CPT was isolated from a different batch of chicken samples and has a ~43% similarity to the CF10ISO and CF9ISO strains.



The AFLP fingerprints of the 22 strains included in the study were vastly heterogeneous. The main reasons for the heterogeneity of the fingerprints obtained amongst the strains is due to the mutations in the restriction sites as well as in sequences adjacent to the restriction sites as well as insertions and deletions within the amplified fragments (Savelkoul *et al.* 1999). Several studies have identified a high genetic diversity amongst both human and animal *C. jejuni* strains (Aeschbacher and Piffaretti 1989; Duim *et al.* 2000). The heterogeneity of the *C. jejuni* strains in this study could be a reflection of this normal genetic diversity. Inconsistency in the PCR could account for the minor differences obtained in the AFLP fingerprints of genetically identical (90% or more similarity) strains.

6.6 CONCLUSION

The AFLP technique applied in this study indicates that it is a powerful and reliable method for demonstrating whole genome polymorphisms among strains of *C. jejuni* and *C. coli* from chickens in the Western Cape, South Africa. It was clearly indicated that strains that cluster at and above 90% similarity can be considered to be genetically or epidemiologically related while similarity of 80% or more could still be significant for identifying epidemiologically related strains. The strains of free range chicken origin were found to be more heterogeneous than those of commercial chicken farming origin. Overall, the fingerprint data obtained had indicated that all the strains had shown a high genetic diversity. No similarity was found between the genotypes of the strains from chicken and those of human infections. The high discriminatory power of the technique has the ability to track the source of *Campylobacter* infections. Future studies on the genetic diversity of campylobacters should include a larger number of clinical strains as well as more strains isolated within and between the supplier farms. This could lead to a better conclusion as to which strains contribute to the disease burden in the Western Cape or on a larger scale, in South Africa.

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6.7 REFERENCES

Aeschbacher, M. and Piffaretti, J.C. (1989) Population genetics of human and animal enteric *Campylobacter* strains. *Infect Immunol* **57**, 1432-1437.

Allos, B.M. and Lastovica, A.J. (2011) *Campylobacter* Infections. In *Tropical Infectious Diseases: Principles, Pathogens and Practise* ed. Guerrant, R.L., Walker, D.H. and Weller, P.F. pp.145-149. Philadelphia: Elsevier.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J. (1999) High-Resolution Genotyping of *Campylobacter* Strains Isolated from Poultry and Humans with Amplified Fragment Length Polymorphism Fingerprinting. *Appl Environ Microbiol* **65**, 2369-2375.

Duim, B., Ang, C.W., van Belkum, A., Rigter, A., van Leeuwen, N.W.J., Endtz, H.P. and Wagenaar, J.A. (2000) Amplified Fragment Length Polymorphism Analysis of *Campylobacter jejuni* Strains Isolated from Chickens and from Patients with Gastroenteritis or Guillain-Barré or Miller Fisher Syndrome. *Appl Environ Microbiol* **66**, 3917-3923.

Duim, B., Vandamme, P.A.R., Rigter, A., Laevens, S., Dijkstra, J.R. and Wagenaar, J.A. (2001) Differentiation of *Campylobacter* species by AFLP fingerprinting. *Microbiol* **147**, 2729 – 2737.

Duim, B and Savelkoul, P. (2003) Typing of bacteria using Amplified Fragment Length Polymorphism (AFLP) Analysis. In *Experimental Approaches for Assessing Genetic Diversity Among Microbial Pathogens* ed. Van Belkum, A., Duim, B. and Hays, J. pp.81-91. Wageningen, The Netherlands: Ponsen and Looijen BV.

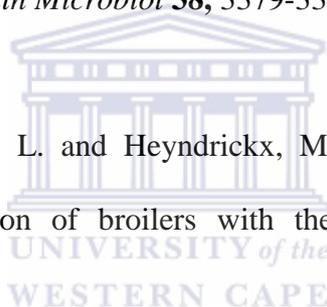
Horrocks, S.M., Anderson, R.C., Nisbet, D.J. and Ricke, S.C. (2009) Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* **15**, 18-25.

Humphrey, T., O'Brien, S. and Madsen, M. (2007) Campylobacters as zoonotic pathogens: A food production perspective. *Int J Food Microbiol* **117**, 237-257.

Kokotovic, B. and On, S.L.W. (1999) High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. *FEMS Microbiol Lett* **173**, 77-84.

Lindstedt, B.A., Heir, E., Vardund, T., Melby, K.K. and Kapperud, G. (2000) Comparative Fingerprinting Analysis of *Campylobacter jejuni* subsp. *jejuni* Strains by Amplified-Fragment Length Polymorphism Genotyping. *J Clin Microbiol* **38**, 3379-3387.

Messens, W., Herman, L., De Zutter, L. and Heyndrickx, M. (2009) Multiple typing for the epidemiological study of contamination of broilers with thermotolerant *Campylobacter*. *Vet Microbiol* **138**, 120-131.



Meudt, H.M. and Clarke, A.C. (2007) Almost Forgotten or Latest Practise? AFLP applications, analyses and advances. *Trends Plant Sci* **12**, 106-117.

Newell, D.G., Frost, J.A., Duim, B., Wagenaar, J.A., Madden, R.H., van der Plas, P. and On, S.L.W. (2000) New Developments in the Subtyping of *Campylobacter* Species. In *Campylobacter* ed. Nachamkin, I. and Blaser, M. J. pp. 27-44. Washington, D.C.: ASM Press.

Olive, D.M. and Bean, P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* **37**, 1661-1669.

On, S.L.W. and Harrington, C.S. (2000) Identification of taxonomic and epidemiological relationships among *Campylobacter* species by numerical analysis of AFLP profiles. *FEMS Microbiol Lett* **193**, 161-169.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. and Lenstra, J.A. (1999) Amplified Fragment Length Polymorphism Analysis: the State of an Art. *J Clin Microbiol* **37**, 3083-3091.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijjers, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407-4414.

Wilson, K. (1994) Preparation of genomic DNA from bacteria. In Current protocols in molecular biology ed. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. pp.2.4.1-2.4.2. New York: Greene Publishing and Wiley-Interscience.

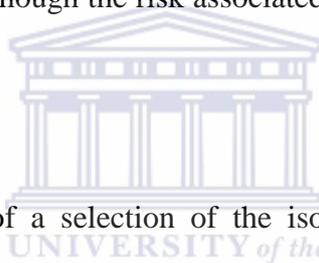
Zweifel, C., Scheu, K.D., Keel, M., Renggli, F. and Stephan, R. (2008) Occurrence and genotypes of *Campylobacter* in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. *Int J Food Microbiol* **125**, 182-187.

Chapter 7 – Conclusion

Campylobacter is the leading bacterial agent of human gastroenteritis and is responsible for 400 to 500 million reported cases globally. The illness is usually self-limiting but individuals with a high risk profile such as young children, the elderly and immunocompromised individuals often experience extra-intestinal infections such as meningitis and endocarditis or post-infection complications such as Guillain-Barré Syndrome and Reactive Arthritis.

Chicken is considered as the main reservoir of *Campylobacter* species and is reported to be prevalent in 35% to 85% of poultry flocks globally. These figures suggest that chicken can be considered as the main vehicle of transmission of campylobacteriosis to humans. The sporadic nature of the illness suggest that the majority of campylobacteriosis cases originate from the domestic kitchen where raw chicken is mishandled and working surfaces and ready-to-eat (RTE) foods become cross-contaminated with campylobacters. The present study surveying the prevalence of *Campylobacter* in South African chicken showed that 27% of retail chicken (n = 84) and 73% of chicken sampled directly from the abattoir (n = 182) but also intended for human consumption are contaminated with *Campylobacter* species. Furthermore, 79% of free range chicken (n = 118) and 56% of commercial chicken (n = 64) were contaminated with *Campylobacter*. The Cape Town protocol was optimized by including the enrichment broth used in the ISO10272-1:2006 method for the recovery of and enrichment of damaged or stressed *Campylobacter* cells from food samples. Therefore, the growth of emerging *Campylobacter* species that may have been present were not recovered as Bolton broth suppresses the growth of these campylobacters. The total number of *Campylobacter* species isolated in this study was 156 of which 102 isolates were *C. jejuni* and 51 were *C. coli*. The speciation of 3 *Campylobacter* isolates could not be determined. There was no major difference (3%) between the two isolation techniques but results obtained using the Cape Town Protocol was in less time (48 h) than the ISO10272-1:2006 method.

The survival of four randomly selected strains in chicken meat at refrigeration and freezer temperatures was determined. The study was designed to simulate refrigeration and freezing of chicken in the domestic kitchen. All the strains in duplicate were able to survive the eight day period at refrigeration temperature. The survival of the strains at freezer temperatures was more variable. The freezing of the artificially contaminated chicken showed no immediate decline upon visual examination as was the case in several other similar studies across the world. A *Campylobacter* strain of free range chicken origin, *C. jejuni* subsp. *jejuni* biotype 1 NS24a, lost its culturability on the eighth day of the survival period. The rest of the strains were of commercial chicken origin. *C. coli* CF10a survived for 25 days while *C. coli* CF9a and *C. jejuni* S1b were able to survive the 30 day sampling period and still maintained its viability after the 30 day period. A decline in number for the survival period was noted upon visual examination at each sampling time. These results indicate that extra caution should be taken when handling fresh raw chicken that is stored in the refrigerator and freezer, although the risk associated with frozen chicken declines over time.



The antibiotic susceptibility patterns of a selection of the isolates (n = 122) were performed. Susceptibility to the antibiotics nalidixic acid, tetracycline, erythromycin and ciprofloxacin were tested using the disk diffusion method and results interpreted using the NHLS guidelines. The percentages of resistant *C. coli* strains (n = 33) of free range chicken were 18, 39, 61 and 85% respectively while *C. jejuni* strains (n = 56) isolated from free range chicken were resistant to the above-mentioned antibiotics by 4, 11, 50 and 64% respectively. *C. coli* isolates (n = 4) of commercial chicken origin were 100, 50, 100 and 25% resistant to the antibiotics respectively while *C. jejuni* strains (n = 29) showed 100, 86, 97 and 79% resistance to the antibiotics respectively. The resistance to the respective antibiotics are very high and indicates that humans with severe campylobacteriosis cases requiring antibiotic therapy will not respond to the antibiotics when it is administered.

The genetic relatedness of a selection of isolates (n = 22) were determined using the AFLP technique. A genetic similarity of 90% or more was observed for five phenons of *C. jejuni* strains from different farms and even between free range and commercial farms. All *C. coli* and *C. jejuni* strains displayed a high genetic diversity with heterogeneous AFLP fingerprints, indicating that there were various sources of *Campylobacter* infections of the chickens, even within the same farm. A few strains isolated from chicken bred on different farms were found to be identical and this indicates that a source common to both farms such as the feed or the hatchery could be implicated as the source of a diversity of *Campylobacter* strains. The AFLP technique is also useful for the subtyping of bacteria and four strains incorrectly characterized as *C. coli* by biochemical tests were genotyped as *C. jejuni* strains with the high discriminatory power of the AFLP technique.

This study has shown that *Campylobacter* species is prevalent in South African chicken and the environment at high rates. This poses a high risk of acquiring campylobacteriosis to South Africans as undercooked chicken as well as other foods cross-contaminated with *Campylobacter* is likely to be consumed. The South African population therefore need to be informed of the serious risks associated with the consumption of undercooked or mishandled chicken and need to use good hygienic practises to reduce or eliminate the risk of acquiring the illness.

Appendix A

1. Luria Bertani (LB) broth (150ml)

1.5 g Tryptone Powder (Pancreatic Digest of Casein)

0.75 g Yeast Extract

0.75 g NaCl (58.44 g/mol)

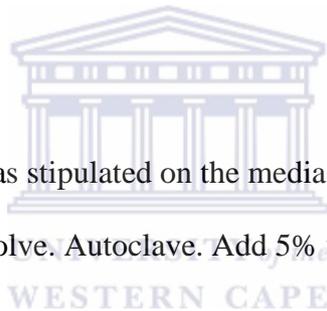
Weigh out each constituent and add to a clean 250ml Schott bottle. Add 150ml distilled water Mix by shaking. Autoclave.

2. Tryptose Blood Agar

Weigh out the required amount of agar as stipulated on the media bottle. Add distilled water to the required volume. Shake to dissolve. Autoclave. Add 10% unlysed horse blood and prepare plates.

3. Columbia Blood Agar

Weigh out the required amount of agar as stipulated on the media bottle. Add distilled water to the required volume. Boil the media to dissolve. Autoclave. Add 5% unlysed horse blood and prepare plates.



4. Mueller Hinton Agar

Weigh out the required amount of agar as stipulated on the media bottle. Add distilled water to the required volume. Boil the media to dissolve. Autoclave. Add 5% unlysed horse blood and prepare plates.

Appendix B

1. 10X TAE Buffer (1 litre)

48.4 g Tris

11.4 ml Glacial acetic acid

3.7 g EDTA

Weigh and measure out each constituent. Dissolve in 1 litre distilled water. It is not necessary to have the pH of the solution done. The solution does not need to be autoclaved.

2. 10X TBE Buffer (500ml)

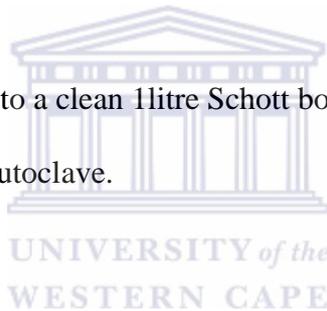
0.9 M Tris

0.89 M Boric Acid

25mM EDTA

Weigh out each constituent and add to a clean 1litre Schott bottle. Add 500ml distilled water.

Heat to dissolve while stirring and autoclave.



3. 1X TE Buffer (500ml)

10mM Tris – HCl

1mM EDTA

Weigh out each constituent and add to a clean 1litre Schott bottle. Add 500ml distilled water.

Mix by shaking. Autoclave.

4. 1X PBS pH 7.2 (1 litre)

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂PO₄

Add 800ml of distilled water to a clean 1 litre Schott bottle and add the required weight of each constituent. Adjust the solution to pH 7.2. Adjust to the final volume of 1litre and autoclave.

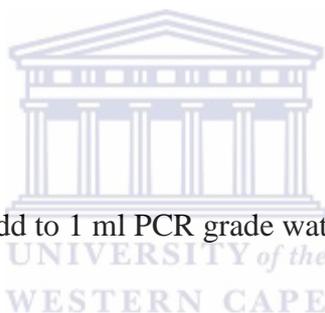
5. 10% Sodium dodecyl sulphate (SDS) (400ml)

40 g SDS

Weigh out the SDS and add to a clean 1 litre bottle. Add 400ml distilled water. Dissolve by placing on a hot plate with stirring. DO NOT AUTOCLAVE.

6. 20mg/ml proteinase K

Weigh out 20mg proteinase K and add to 1 ml PCR grade water. Filter sterilize. Store in small single-use aliquots at -20 °C.



7. 5M NaCl (200ml)

Weight out 58.44 g NaCl and add to a clean 500ml bottle. Add 200ml distilled. Dissolve by heating and stirring. Autoclave.

8. Hexadecyltrimethyl ammonium bromide (CTAB) / NaCl Solution (100ml)

4.1 g NaCl

10 g CTAB Dissolve the NaCl in 80ml (PCR grade) water. Slowly add the CTAB while heating and stirring. If necessary, heat to 65 °C to dissolve. Adjust to a final volume of 100ml with (PCR grade) water. Do not autoclave.

8. 24:1 Chloroform/Isoamyl alcohol (50ml)

Measure out 48ml chloroform and 2ml isoamyl alcohol and add each constituent to a clean 50ml Greiner tube. Invert gently to mix. Cover with foil.

9. 25:24:1 Phenol/ Chloroform / Isoamyl alcohol (50ml)

Measure out 25ml phenol, 24ml chloroform and 1ml isoamyl alcohol and add each constituent to a clean 50ml Greiner tube. Invert to mix. Cover the tube with foil.

10. 70% Ethanol (500ml)

Add 150 ml distilled water to 350 ml 99.9% Ethanol. Mix by inverting

