A microbiological and molecular study of *Campylobacter* and related species isolated from ostriches (*Struthio camelus*).

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae (Biotechnology) in the Department of Natural Sciences, University of the Western Cape

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DECLARATION

I declare that *A microbiological and molecular study of* Campylobacter *and related species isolated from ostriches (*Struthio camelus*)* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.



Belinda Margaret Peyrot

Signed _____ Date _____

Abstract

*Campylobacter* and related Epsilonproteobacteria (*Arcobacter* and *Helicobacter*) are currently viewed as emerging pathogens and are able to cause gastroenteritis, bacteraemia, the Guillain-Barré syndrome, reactive arthritis and other diseases in humans. While poultry, cattle and sheep are known reservoirs for campylobacter, very little is known about ostriches as a vector for these organisms. What is known, however, is that these birds can and sometimes do get infected. Studies by various researchers have provided evidence that various species of animals shed Epsilonproteobacteria in their faeces. In this study, qualitative microbiological assays were performed on liver, caecum and colon samples predominantly from healthy ostriches presented for slaughter, to detect any Epsilonproteobacteria present. Samples were collected at an abattoir in the Western Cape between February and December 2010. Qualitative microbiological assays were also performed on 50 faecal samples collected on a farm. Epsilonproteobacteria were isolated from the tissue samples and characterized following the phenotypic and biochemical scheme presented in the Cape Town protocol. This protocol uses membrane filtration onto antibiotic-free Tryptose blood agar plates, and incubation at 37 ºC in a hydrogen-enhanced microaerobic atmosphere (Lastovica, 2006). The isolates were identified as *C. jejuni* subsp. *jejuni*. The multiplex PCR of Neubauer and Hess (2006) was applied to some of these isolates. A panel of isolates consisting of *C. jejuni* subsp. *jejuni*, *C. fetus* and *E. coli* was used to verify the DNA extraction procedure. The *C. fetus* and *E. coli* isolates were used as negative controls, and although DNA was successfully extracted from them, no bands were observed in the respective lanes of electrophoresed gels after application of the PCR. Another panel of cultures

including more of the ostrich *C. jejuni* subsp. *jejuni* isolates was used for continued optimization of the PCR, and amplificates of the correct size were observed for all the *C. jejuni* subsp. *jejuni* isolates tested. The banding patterns observed in the electrophoresed gels confirmed the phenotypic identification of the isolates. Attempts to extract DNA directly from *Campylobacter* isolates that had been stored frozen at -80 ºC were unsuccessful. The low percentage (4,9 %) of samples that tested positive in this study suggests that the likelihood of Epsilonproteobacteria contamination of ostrich meat products presented at this abattoir during this period was minimal. No Epsilonproteobacteria were isolated from the faecal samples. The data gathered in this study may be useful for determining the risk of campylobacter and other emerging Epsilonproteobacteria to human health from the processing and subsequent consumption of ostrich meat products in South Africa.

Keywords

Ostrich

*Campylobacter*

Microbiology

Molecular

Food safety

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**Abbreviations**

| | |
|---|---|
| ™ | trademark |
| ® | registered trademark |
| µg | microgram |
| µL | microlitre |
| µm | micrometer |
| µM | micromoles |
| AFLP | amplified fragment length polymorphism |
| AIDS | acquired immune deficiency syndrome |
| ATCC | American Type Culture Collection |
| ATL | a tissue lysis buffer (Qiagen®) |
| bp | base pairs |
| C | celcius |
| CA | California |
| CDT | cytolethal distending toxin |
| CFU | colony forming unit(s) |
| CHRO | *Campylobacter*, *Helicobacter* and related organisms |
| cm | centimeter |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| DOH | Department of Health |
| Doi | digital object identifier |
| Ed. | edition |
| e.g. | for example |
| ELISA | Enzyme-linked Immunosorbent Assay |
| et al. | *et alii* (and others) |
| g | gram |
| h | hour(s) |
| HACCP | Hazard Analysis Critical Control Point |
| HIV | human immunodeficiency virus |
| i.e. | *id est* (that is) |
| km | kilometer |
| Ltd | limited |
| mA | milliampere |
| mL | millilitres |
| mm | millimeters |
| MLST | multilocus sequence typing |
| m/v | mass per volume |
| NASBA | nucleic acid sequence-based amplification |

| | |
|---|---|
| n.d. | no date |
| OBIS | Oxoid Biochemical Identification System |
| PA | Pennsylvania |
| PCR | polymerase chain reaction |
| PCR-RFLP | PCR-restriction fragment length polymorphism |
| PFGE | pulsed field gel electrophoresis |
| Pty | proprietary |
| RAPD | random amplified polymorphic DNA |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RSA | Republic of South Africa |
| sp. | species |
| sp. nov. | *species nova* (new species) |
| spp. | several species |
| subsp. | subspecies |
| subsp. nov. | *subspecies nova* (new subspecies) |
| TE | Tris-EDTA buffer |
| UHT | ultra high temperature |
| UK | United Kingdom |

| | |
|---|---|
| USA | United States of America |
| V | volts |
| VBNC | viable but non-culturable |
| Vol | volume |

## Output emanating from thesis

**Isolation and microbiological characterization of Epsilonproteobacteria from fresh ostrich (*Struthio camelus*) tissue samples in the Western Cape, South Africa.**

(presented at the 2012 Congress of the South African Association of Veterinary Technologists, Berg-en-Dal, Kruger National Park, South Africa)

## Chapter 1: Introduction

*Campylobacter* is the most common cause of bacterial gastroenteritis in humans worldwide (Maher et al., 2003; Moore et al., 2005). The most susceptible sections of the population to Epsilonproteobacteria (*Campylobacter, Arcobacter* and *Helicobacter*) infections are young children, the elderly and immunocompromised people (Allos & Lastovica, 2011). In rare cases, intestinal *Campylobacter* infections can initiate disease in other sites in the body and result in secondary infections and sequelae like reactive arthritis, septicaemia, abortions and Crohn's Disease (Zhang, 2008). Results of data analysed by Poropatich et al. (2010) showed that 31 % of 2 502 cases of Guillain-Barré Syndrome (GBS) were attributable to previous *Campylobacter* infection. GBS is a rare but serious autoimmune disease of the nervous system in humans that can result in paralysis or even death.



It is generally accepted that chickens are the primary host of *Campylobacter jejuni*, and chicken meat products are the main source of *C. jejuni* infections in humans (Dasti et al., 2010; Moore et al., 2005). Thermophilic camplylobacters are well adapted to inhabit the intestinal tracts of birds and also frequently infect other species of poultry (Zhang, 2008). Extensive research has been conducted on campylobacters and related organisms in chickens and poultry products in various parts of the world (Amisu et al., 2003; Eifert et al., 2003; Jauk et al., 2003; Lammerding et al., 1996; Lynch et al., 2011; Neubauer & Hess, 2006; Rahimi & Tajbakhsh, 2008; Rossi et al., 2009; van Nierop et al., 2005).

*C. jejuni*, as well as other *Campylobacter* species, are also found in other food animals, milk, wild birds, water, soil and mud, beach sand and domestic pets (Bolton et al., 1999; Chai et al., 2009; Kemp et al., 2005; Lynch et al., 2011; Stuart et al., 2010; Taylor et al., 2012). It is becoming increasingly apparent that many of the emerging species of Epsilonproteobacteria are capable of causing disease in humans, but have not been accounted for because of limitations in culturing (Gaynor & Szymanski, 2012) and, in the case of *Arcobacter*, the considerable diversity in isolation methods (Collado & Figueras, 2011).

The likelihood of the presence of campylobacters in other farm animals, including ostriches, has been somewhat overlooked. Only a few publications dealing with research into campylobacters in ostriches are available in the literature (Cuomo et al., 2007; Oyarzabal et al., 1994; Perelman et al., 1992; Post et al., 1992; Stephens et al., 1998; Welsh et al., 1997). Similarly, published research on the prevalence of campylobacters and related organisms in ostrich meat is scanty (Ley et al., 2001).

Ostrich meat is promoted as a healthy red meat because of its low cholesterol and fat contents when compared with other meats commonly available to consumers (Orumbayev et al., 2012). No specific regulations currently exist in South Africa that enforce routine testing of ostrich meat products for the presence of *Campylobacter* species, although the Department of Health Directorate: Food Control has proposed guidelines for acceptable levels in ready-to-eat foods, cold meal items and raw meat (Department of Health Directorate: Food Control, n.d.).

Studies have suggested that flies could possibly serve as a vector for *Campylobacter* transmission on farms, especially during summer (Zhang, 2008). Assuming that *Campylobacter* could potentially be transmitted by flies, rodents and other small mammals or birds to extensively and semi-extensively farmed ostriches, it follows then that these birds could potentially be colonized by *Campylobacter* species, yet still appear clinically healthy at pre-slaughter veterinary inspections. If ostriches are carrying these organisms in their intestines at the point of slaughter, faecal contamination of the carcasses during slaughtering could occur. No specific procedure or steps in the slaughtering process appear to be in place to detect or eliminate the potential contamination of meat by campylobacters or related organisms.

This assumption suggests that a pertinent place to start looking for these organisms in ostriches is in the healthy, adult birds that are presented for slaughter for meat for human consumption. This study aims to address the questions: a) which campylobacters and related organisms are present in the ostriches delivered to an abattoir for slaughter, and b) what is their prevalence?

Tissue samples were collected at a local ostrich and game abattoir during slaughtering. Samples were analysed microbiologically and isolates were identified using routine biochemical analyses. Cultures were subjected to molecular analysis by PCR for confirmation of the microbiology results. A set of faeces samples was collected from a local farm, also for microbiological analysis. None of the birds that were sampled at the abattoir originated from this farm.

This report details the results of the microbiological investigations, firstly of the fresh tissues samples collected and secondly of the faeces samples tested. Following that findings of the molecular analyses performed on bacterial isolates are presented.

## Chapter 2: Literature review

Numerous publications are available in the public domain regarding research that has been carried out on various features of *Campylobacter* spp., *Helicobacter* spp. and *Arcobacter* spp.. Authors have investigated and reported various ways in which these organisms can infect humans (Gorkiewicz, 2002; Lastovica & Skirrow, 2000; Moore et al., 2005), and animals and birds (Debruyne et al., 2009a,b,c; Inglis et al., 2004; Moore et al., 2005; Oyarzabal et al., 1994; Post et al., 1992; Rossi et al., 2009; Weijtens et al., 1997), as well their environmental distribution, epizoology, prevalence and persistance (Amisu et al., 2003; Moore et al., 2005; Rosef et al., 2008; Siemer et al., 2004; Westgarth et al., 2009).

Studies have been done to determine which of these organisms can infect or colonize poultry and livestock (Hughes et al., 2009; Lammerding et al., 1996 Moore, 2001; Neubauer & Hess, 2006; Siemer et al., 2004), and how this can lead to the risk of infected meat, especially poultry, being consumed by humans (Eifert et al., 2003; Jauk et al., 2003; Lynch et al., 2011; Rahimi & Tajbakhsh, 2008;). Very little information, however, is available regarding similar attributes of Epsilonproteobacteria in ostriches.

It was decided to firstly review the literature available indicating which of the organisms in question can infect humans and to what degree. The roles that these Epsilonproteobacteria play in animal diseases and their epidemiology was then considered, paying particular attention to poultry and other avian species. The various molecular techniques that have been used in these and similar studies

were reviewed to try to determine which method would be most suitable for use in this survey.

## 2.1 Key concepts

### Epsilonproteobacteria

*Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. are found in many different environments and cause various disease conditions in humans and animals. They are members of the class Epsilobacteria (On, 2005). The class Epsilobacteria, as proposed in 2002, was subsequently found to be illegitimate and is no longer widely accepted (Euzéby, 1997 and updated). I am going to use the approved class name, Epsilonproteobacteria, to collectively refer to *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. in this report.



According to Euzéby (1997 and updated) the genera *Campylobacter* and *Arcobacter*, of the family *Campylobacteraceae*, and the genus *Helicobacter*, of the *Helicobacteraceae* family are three of the eleven genera of the order Campylobacterales. Campylobacterales is the type order of the class Epsilonproteobacteria, of the phylum Proteobacteria.

### Molecular

The following molecular techniques were used in this study: 1) extraction of DNA from bacterial cultures, 2) polymerase chain reaction (PCR) to amplify Epsilonproteobacterial DNA present in the extract, if any, and 3) agarose gel

electrophoresis to separate and visualise the amplified DNA fragments based on size.

**Microbiological**

This survey relied primarily on the isolation, cultural characteristics and biochemical reactions of the relevant Epsilonproteobacteria. This encompassed bacterial culture on agar media, sub-culturing for purification of the isolates and phenotyping by evaluating biochemical characteristics of the bacteria and using the resulting observations to identify them.



## 2.2 Epsilonproteobacteria in humans

The leading agents of acute bacterial gastroenteritis in most countries are *Campylobacter* species (Maher et. al., 2003), with *C. jejuni* and *Campylobacter coli* being the most frequently reported cause of intestinal infections worldwide (Moore et al., 2005). Both of these species, in spite of having different main reservoirs (chickens and pigs), seem to cause the same type of enteric disease (Moore et al., 2005). People with a decreased immune response (due to diseases like diabetes, cirrhosis, cancer and HIV infection), neonates and children up to the age of two years and the elderly are at higher risk of developing severe enteric infection (Lastovica, 2012, personal communication; Moore et al., 2005). The most frequently isolated *Campylobacter* from humans by far, in developed and developing countries, is *C. jejuni* subsp. *jejuni* (Lastovica, 2006). Infection with

this organism is accepted as the most common precursor event associated with the Guillain-Barré syndrome (Lastovica, 2006). *C. jejuni* subsp. *jejuni* has also been associated with other diseases such as septicaemia, meningitis and reactive arthritis (Lastovica, 2006).

Other species of *Campylobacter* that are implicated in enteric disease in humans include *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter fetus* (Moore et al., 2005). *C. upsaliensis* was one of the more recently recognized human pathogens, according to Sylvester et al. (1996). In a study of the campylobacters present in stool samples of paediatric patients with diarrhoea in Australia, 225 of the 676 patients tested were positive for *Campylobacter* spp.. In 121 of these patients, no other recognized enteric pathogens were present in their stools. *C. upsaliensis* was the sole pathogen present in ten of these, and was present together with other campylobacters in fifteen of these patients (Albert et al., 1992). In 1990 in South Africa, at the Red Cross War Memorial Children's Hospital, clinical isolates of *C. upsaliensis* comprised 22 % of all campylobacters isolated during routine monitoring of patients suffering from diarrhoea (Da Silva Tatley et al., 1992). It is a human pathogen in immunocompetent as well as immunocompromised patients, causing acute as well as chronic, recurring diarrhoea (Lastovica & Skirrow, 2000). In 2009 the results of a study to investigate the risk factors for the carriage of *C. upsaliensis* by dogs in a community in England were published (Westgarth et al., 2009). The prevalence of *C. upsaliensis* in 183 healthy pet dogs was determined to be 25,1 %, which may be of public health significance (Westgarth et al., 2009).

The disease potential of recently described *Campylobacter* species and subspecies (*Campylobacter avium, Campylobacter cuniculorum, Campylobacter peloridis* sp. nov., *C. lari* subsp. *concheus* subsp. nov., *C. lari* subsp. *lari* subsp. nov. and *Campylobacter subantarcticus* sp. nov.) is presently unknown (Debruyne et al., 2009a; Debruyne et al., 2009b; Rossi et al., 2009; Zanoni et al., 2009).

Rosef and colleagues (2008), when comparing *C. lari* isolates from water, animals and humans by genetic analyses, determined that some of them were highly similar. A total of 49 isolates were investigated, of which 18 were of human origin, 21 were from poultry and ten were from water, ducks and a pig. The conclusion was drawn that, since the virulence factors of *C. lari* are unknown and the isolates from these various sources were so similar, the transmission of *Campylobacter* should be avoided (Rosef et al., 2008).



In a study of the distribution of *Campylobacter* spp. in patients in the Red Cross Children's hospital in Cape Town between 1990 and 2005, campylobacters other than *C. jejuni* subsp. *jejuni* constituted two thirds of the *Campylobacter* and related organisms isolated from paediatric patients with enteritis and septicaemia (Lastovica, 2006). Of these, *C. fetus* (which is not considered an emerging *Campylobacter* species) presents most commonly in children and adults as meningoencephalitis (Lastovica, 2006). It is "under-isolated and under-appreciated" and the most typically infected population now is AIDS patients (Lastovica, 2006). *Campylobacter hyointestinalis* has been isolated from human stools, and may be the cause of watery diarrhoea in children, and *C. lari* can produce acute diarrhoea in immunocompetent hosts and diarrhoea and bacteraemia in immunocompromised patients (Lastovica & Skirrow, 2000). A

case report was published detailing the transmission of *C. hyointestinalis* from a pig to a human in Austria (Gorkiewicz et al., 2002). The patient, an 88-year old woman, had been suffering from constant diarrhoea, abdominal pain and sporadic vomiting for over a month (Gorkiewicz et al., 2002).

In a South African study, 187 out of 1519 *Campylobacter* isolates had an essential growth requirement for hydrogen, and were all phenotypically characterized as *C. concisus*. The isolates were mainly from paediatric diarrhoeal stool samples (Lastovica et al., 1993). Moore et al. (2006) referred to *Campylobacter hominis*, *Campylobacter lanienae*, *Campylobacter sputorum* subsp. *paraureolyticus* and *C. hyointestinalis* subsp. *lawsonii* as examples of species that had been found in the faeces of healthy domestic animals and humans (Moore et al., 2006). Discussions at the 16th International *Campylobacter*, *Helicobacter* and related organisms (CHRO) meeting in 2011, which coincided with the 30th anniversary of CHRO workshops, also presented evidence that lesser-known *Campylobacter* spp. are capable of causing disease, but have not always been accounted for due to culturing shortcomings (Gaynor & Szymanski, 2012).

Of the more than 32 described *Helicobacter* species (Euzéby, 1997 and updated), *Helicobacter pylori* is the most well-known human pathogen. *Helicobacter pullorum* has been isolated from the caeca of healthy broilers, and from the livers and intestinal contents of layers with hepatic lesions (Corry et al., 2002). It's also been isolated from humans with gastro-enteritis and chronic liver disease (Corry et al., 2002). *Helicobacter fennelliae* is often isolated together with *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei* or *C. upsaliensis* (Lastovica & Skirrow, 2000). *Helicobacter cinaedi* and *H. fennelliae* have been isolated from the stools

of homosexual men with diarrhoea or proctitis (Lastovica & Skirrow, 2000). They can also cause bacteraemia, especially in HIV positive patients (Lastovica & Skirrow, 2000). At a paediatric hospital in South Africa, 3,2 % of paediatric blood culture isolates were *H. fennelliae* and 2,3 % were *H. cinaedi* (Lastovica & Skirrow, 2000). Various studies have shown that gastroenteritis due to these two organisms has also been found in heterosexual men, women and children (Lastovica & Skirrow, 2000). It was suggested that transmission from animals is a possible source of human infections (Lastovica & Skirrow, 2000). Other helicobacters have also been implicated as human pathogens. *Helicobacter canis*, originally found in dogs, was also isolated from a 5½-year-old boy with diarrhoea (Lastovica & Skirrow, 2000). *Helicobacter westmeadii* was isolated from the blood of two AIDS patients in Australia, and *Helicobacter rappini* has been isolated from patients with diarrhoea, and the blood culture of a pneumonia patient (Lastovica & Skirrow, 2000).



Black et al. (1988) reported that *C. jejuni* in doses ranging between 800 and 2 x $10^9$ colony forming units (CFU) could cause diarrhoea in experimentally infected adults. Similar to the lower value of this range, Kothary and Babu (2001) reported an infectious dose of 500 cells of *C. jejuni* for humans. Virulence may depend on the *Campylobacter* strain involved. As an example, although all strains possess the gene for cytolethal distending toxin (CDT), they don't all express this gene due to point mutations and deletions (Lastovica, 2012, personal communication). Other factors affecting virulence include the immune status of the host as well as the number of infective organisms (Lastovica, 2012, personal communication).

The contributions of virulence-related factors like toxin production, adherence to and invasion of tissue has not clearly been understood (Misawa & Blaser, 2000).

## 2.3 Epsilonproteobacteria in animals

The venereal disease bovine genital campylobacteriosis, also known as vibriosis, is caused by *C. fetus* subsp. *venerealis* (Irons et al., 2004). It presents as reduced fertility and sometimes even abortions. *C. fetus* subsp. *fetus* is known to cause sporadic abortions in sheep and cattle (Irons et al., 2004). *C. sputorum* subsp. *bubulus* is a commensal of cattle and sheep, inhabiting the alimentary canal (Irons et al., 2004). *C. jejuni* commonly occurs in avian species, livestock and pets and is shed in their faeces (van der Walt, 2004). Although *C. jejuni* is considered part of the normal intestinal flora of healthy cattle, it has been implicated in the development of a self-limiting enteritis in calves and sheep (van der Walt, 2004). It has reportedly also caused occasional cases of mastitis and abortion in cattle, and abortion in goats and sheep (van der Walt, 2004). In their study on *Campylobacter* shed by beef cattle, Inglis et al. (2004) found evidence to suggest that *C. lanienae* may be pathogenic to cattle.



*C. jejuni* and *C. coli* are often present in the intestinal tracts of poultry and other avian species (Stephens et al., 1998; Zhang 2008). Campylobacters infect birds via the faecal-oral route, and the organisms colonize in the caecal and cloacal crypts. Sometimes the organisms can also be isolated from the small intestines, liver and other organs (Zhang, 2008). Under natural conditions, in spite of extensive colonization, *Campylobacter* infections of poultry seldom produce clinical signs (Zhang, 2008). Young chicks are partially protected from *Campylobacter* infection by maternal antibodies. Experimentally, though, various challenge studies have shown that *Campylobacter* caused gastroenteritis, weight loss and varying degrees of mortality in day-old to three-day-old chicks. Similar

observations were made in 4-day-old turkey poults and 3-week-old Japanese quails (Zhang, 2008).

Ostriches are known to suffer from *Campylobacter* infections. In 1990 in Israel, a syndrome resembling avian vibrionic hepatitis in other birds was observed in young ostriches (Perelman et al., 1992). Pure cultures of *C. jejuni* were isolated from the livers of all the sick birds examined (Perelman et al., 1992). *C. jejuni* was allegedly isolated from 3-month-old rheas and 2-week-old ostriches in 1990 at the Texas Veterinary Medical Diagnostic Laboratory in Amarillo, Texas (Post et al., 1992). Although no other etiological agents were detected, only a limited number of confirmatory tests were performed on these isolates, some of which were hippurate hydrolysis positive and some negative. Welsh et al. (1997) reviewed the infectious diseases seen in ratites between 1990 and 1996 at the Oklahoma Animal Disease Diagnostic Laboratory. Only 0,2 % of the ratite enteric samples tested for the presence of various bacteria were positive for *C. jejuni*. The culture conditions used, however, were not optimal for *Campylobacter* isolations. Laboratories that are not familiar with the isolation of *Campylobacter* may often miss strains (Lastovica, 2006), and at the Oklahoma laboratory enteric samples were placed in a *Salmonella* selective enrichment broth (tetrathionate with iodine) at 42 ºC for overnight incubation. While *C. jejuni* was isolated in this way, most other *Campylobacter* species may not have been detected (Corry et al., 2002). An outbreak of infectious hepatitis in 2-8-week-old ostrich chicks was reported from six farms in Australia between November 1995 and April 1996 (Stephens et al., 1998). This appeared to be the first definitive report of infectious hepatitis in ostriches caused by *C. coli*.

Between 2004 and 2006, 2 084 samples from wild birds in northern England were analysed in a survey to investigate the epidemiology of *Campylobacter* species (Hughes et al., 2009). The authors wanted to determine the host range and prevalence of campylobacters in a population of wild birds, and whether these wild birds were a probable source of human or livestock *Campylobacter* infections. *C. jejuni*, *C. lari* and *C. coli* collectively were present in 29 of the 2084 birds tested. The *C. jejuni* isolates were characterized by multilocus sequence typing (MLST) and the results were compared with those in the public MLST database. Livestock-associated strains of *C. jejuni* were detected in wild bird samples, yet novel wild bird strains seemed to be absent in livestock. Hughes et al. (2009) suggested that this may indicate that the direction of infection is mainly from livestock to wild birds, rather than the reverse (Hughes et al., 2009). The findings of this study are worth noting, considering the large amount of direct and indirect contact local farmed ostriches have with wild birds, which is due to the mainly extensive nature of ostrich farming practices in South Africa.



In a study to detect only the thermotolerant *Campylobacter* species in clinically health ostriches in Italy, 60 of 150 birds tested were positive for *C. jejuni* and/or *C. coli* (Cuomo et al., 2007) indicating that ostriches are indeed *Campylobacter* carriers. Findings of Oyarzabal et al. (1994) also determined that in the food animal environment ostriches were potential *Campylobacter* carriers. Emerging, newly described campylobacters have been found in production animals like pigs, sheep, cattle and poultry, and in pets and wild animals (Lastovica, 2006). In a bacterial investigation regarding the prevalence of *H. pullorum* in poultry, a previously unknown *Campylobacter* species, *C. avium*, was isolated from broiler

chicks and a turkey and subsequently described in 2009 (Rossi et al., 2009). Between 2005 and 2007 a new *Campylobacter* sp., *C. cuniculorum* was isolated from farmed rabbits in Italy (Zanoni et al., 2009). Other non-mammalian species like wild birds and shellfish have also been implicated as reservoirs for emerging campylobacters (Lastovica, 2006). This was substantiated by the recent description of the novel species *C. subantarcticus*, isolated from birds in the sub-Antarctic (Debruyne et al., 2009b). In 2007 a new species, *Campylobacter canadensis*, was described after it was isolated from whooping cranes in a Canadian zoo (Inglis et al., 2007).

Many *Helicobacter* species are found in the stomachs of cats, dogs, monkeys, pigs, rodents and other animals (Corry et al., 2002). Despite this, some authors report that the roles of these organisms in causing disease in animals are unclear (Coetzer & Tustin 2004, Lastovica & Skirrow, 2000). Some helicobacters colonize the large intestine. *H. pullorum* has been isolated from the livers and intestines of laying hens with hepatic lesions (Corry et al., 2002).

*Arcobacter* species can cause porcine, equine, bovine and ovine abortion, and gastroenteritis in sheep, cattle and primates (Lastovica & Skirrow, 2000). A recently described species, *Arcobacter thereius*, was isolated from livers and kidneys of aborted piglets, but its pathogenicity still needs to be confirmed (Collado & Figueras, 2011). Poultry species could be a natural reservoir of *Arcobacter* because they are recognized as faecal shedders of these organisms, yet *Arcobacter* has not been associated with diseases in poultry (Collado & Figueras, 2011). The presence of *Arcobacter* has also been reported in some exotic and non-domesticated animals including rheas (Collado & Figueras, 2011).

## 2.4 Epsilobacteria in food animals, water and other sources

Many foods of animal origin may be contaminated with *Campylobacter* species. Poultry has been identified as a significant source of human *C. jejuni* infections, and to a lesser extent *C. coli* and *C. lari* infections, with infection of the birds and contamination of the meat products occurring at pre-harvest and harvest levels respectively (Moore et al., 2005). Cross-contamination can also occur during slaughter and the subsequent processing steps. If broiler chicks are colonized with campylobacters, they normally remain asymptomatic carriers up to slaughter age (Moore et al., 2005). In a study to compare phenotypic and genotypic differentiation of *Campylobacter* species collected at broiler farms in Austria, Jauk et al. (2003) found 71 *C. jejuni* isolates, 53 *C. coli* isolates and 2 isolates of *C. fetus*/*C. hyointestinalis*. They were unable to phenotypically identify the latter, but the PCR-RFLP method that they used placed these isolates in a group comprising *C. fetus* and *C. hyointestinalis* (Jauk et al., 2003).



*C. jejuni* and *C. coli* are present in the gastrointestinal tract of many other food animals, with their prevalence varying between ±20 % in sheep, absent to 80 % in cattle, and their prevalence in pigs possibly being higher than both of these (Moore et al., 2005). Pigs are the primary reservoir of *C. coli*, yet the results of epidemiology studies using amplified fragment length polymorphism (AFLP) analysis indicated that poultry appears to be a plausible source of human infection, while pigs are less frequent contributors to human campylobacteriosis (Siemer et al., 2004). Moore et al. (2005) recognized that high throughputs and the resulting necessity for automated equipment during certain poultry slaughtering steps creates many opportunities for cross-contamination to occur.

During initial stages of the slaughter procedure, pig carcasses also undergo a communal scalding step. Furthermore the skin is left on the carcass. These two factors contribute significantly to the high prevalence of *Campylobacter* in pig carcasses (Moore et al., 2005). Rahimi & Tajbakhsh (2008), when studying the prevalence of *Campylobacter* species in poultry meat (raw chicken, quail, turkey and ostrich) found that 47 % of all the samples tested were positive, in spite of having used selective isolation procedures and thereby possibly under-isolating many of the emerging campylobacters that could have also been present.

*C. jejuni* is also commonly isolated from waterborne outbreaks due to drinking water contamination (Moore et al., 2005). *H. pylori* may also be a water contaminant. *C. coli* and *C. jejuni* were isolated from sewage in Sweden, and *Campylobacter* and other bacterial pathogens from rural water sources in South Africa (Moore et al., 2005). In China *C. jejuni* was identified as a human health risk because it was often a contaminant of retail chicken meat, raw milk and environmental water, and on New Zealand farms it was isolated from the faeces of dairy cows, beef cattle, sheep and ducks, and also from chicken carcasses and surface waters (Moore et al., 2005). Feedlot steers may shed more than $10^9$ cells per animal per day (Inglis et al., 2004).

Collado and Figueras (2011) concluded that *Arcobacter* strains can tolerate elevated sodium chloride concentrations, grow at low temperatures used for refrigeration, can attach to various surfaces and can resist desiccation. They have been isolated from various types of environmental waters, and some species are more prevalent in faecally contaminated water (Collado & Figueras, 2011).

The threshold of *C. jejuni* cells for human infections, according to Inglis et al. (2004), is approximately 10-100 cells, so even only one animal shedding this organism in their faeces is a potential food safety risk when slaughtered. According to Black et al. (1988) 800 CFU is the lowest infectious dose for humans, established during a volunteer study. These threshold differences could be ascribed to variations in strain colonization, pathogenic potential and host immune status. Black et al. (1988) found that dosage increases from 800 up to 2 x $10^9$ CFU affected the rates of infection, but not the development of illness (1988). According to an advisory committee for the Food Standards Agency of the United Kingdom the general infectious dose of *Campylobacter* is thought to be <500 cells (Food Standards Agency, 2005).



## 2.5 Potential reservoirs of epsilonproteobacteria for human infections

It seems evident that healthy domestic animals could potentially be important reservoirs of atypical and emerging *Campylobacter* species in humans (Moore et al., 2005). Maher et al. (2003) suggested that finding *Campylobacter* DNA in faecal specimens from patients with diarrhoea, when no other identified cause of diarrhoea was found, may suggest that *Campylobacter* species other than *C. jejuni* and *C. coli* may be the causative agent. In their study, the method for bacterial isolation was biased towards *C. jejuni* and *C. coli*, while the DNA probe assay also detected *C. concisus*, *C. curvus* and *C. gracilis*. They suggested that *C. concisus*, as a causative organism of gastro-enteritis, might be an opportunistic pathogen in young children or immunocompromised patients (Maher et al., 2003).

In 2010, Inglis et al. published the results of their study on campylobacters in bovine manure compost. They found that campylobacters could readily be isolated from bovine pen manure, and may persist for long periods in manure compost (Inglis et al., 2010). It follows, therefore, that because these cattle were healthy carriers of these organisms, their carcasses could potentially become contaminated with the bacteria during slaughter, creating a food safety risk for consumers (Lastovica & Skirrow, 2000). In a similar way, ostrich meat products could be contaminated by Epsilonproteobactera if healthy ostriches were carriers of these organisms.

Eifert et al. (2003) reported that, according to previous studies, *Arcobacter* species are present on many retail poultry carcasses and other meat products. *Arcobacter butzleri* was most common, and can cause human enteritis. Two other species, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* can also cause human and animal diseases (Eifert et al., 2003; Lastovica & Allos, 2008). Each of these three *Arcobacter* species is linked to enteritis and abortions in cattle and pigs (Coetzer & Tustin, 2004). As with the campylobacters, arcobacters have been isolated from a range of food and water sources (Amisu et al., 2003). In a Canadian study, after primary processing, 121 of 125 broiler chicken carcasses were positive for *A. butzleri* (Lammerding et al., 1996). Amisu et al. (2003), in their article about *A. butzleri* strains isolated from poultry abattoir effluent in Nigeria, referred to the arcobacters as emerging food-borne pathogens of public health importance. *A. butzleri* was isolated from drinking water plants in Germany, from a contaminated well in Idaho, and can survive up to 16 days in groundwater (Lastovica &

Skirrow, 2000). Infections of humans are mainly gastrointestinal, and disease may be more prevalent in developing countries (Amisu et al., 2003).

Some recognized sources of *Helicobacter* are humans (*H. cinaedi*, *H. fennelliae* and *H. rappini*), poultry (*H. pullorum*) and some rodents (*H. cinaedi* and *H. rappini*), and *H. rappini* can also be transmitted from sheep (Lastovica and Skirrow, 2000). According to Corry et al. (2002) whether *Helicobacter* species are transmitted to humans via food or water is unknown. *H. pylori* was reportedly detected in raw cow's milk in Japan by Fujimura et al. (2002) and these authors suggested that milk may be a vehicle of transmission in *H. pylori* infections in children. They could not confirm the survival of this organism in pasteurized milk (Fujimura et al., 2002). More recently, in Italy, results of a study to evaluate the survival of *H. pylori* in pasteurized and ultra-high temperature (UHT) treated milk showed that the organism survived for an average of nine days in pasteurized milk and twelve days in UHT milk (Quaglia et al., 2007). In 2012 *H. pylori* was isolated from sheep and buffalo milk in Iran (Rahimi & Kheirabadi). These authors also showed that *H. pylori* DNA could be detected in buffalo and camel milk.

In a report detailing the transmission of *C. hyointestinalis* from a pig to a woman, the authors suggest that *Campylobacter* species other than *C. jejuni* and *C. coli* may go undetected during regular human faecal specimen testing because of sub-optimal culture methods used (Gorkiewicz et al., 2002). The use of alternate sample preparation methods, like filtration, or PCR-based analyses was advocated (Gorkiewicz et al., 2002). There are a number of *Campylobacter* species that are uncommon human pathogens, while the status of others is not yet fully defined

(Maher et al., 2003). Moore et al. (2006) stated that healthy domestic and wild animals could be significant reservoirs of emerging campylobacters in humans. In a longitudinal study to establish chronic shedding of *Campylobacter* species in beef cattle, 100 % of the steers that were monitored over a four-month period shed campylobacters (Inglis et al., 2004). Avian species and shellfish have been implicated as reservoirs of *Campylobacter* species other than *C. jejuni* and *C. coli* (Lastovica & Skirrow, 2000). The suggestion was made that more research is needed to establish the incidence and extent of potential disease associated with these organisms (Lastovica & Skirrow, 2000). Siemer et al. (2004) investigated the epidemiology of *C. coli* isolates from humans, food and animals. They found that poultry species other than chicken may be an important source of this organism. Of the 177 isolates that they analysed, that had been collected between 1999 and 2001, they also discovered that 21 % of the human isolates and 10 % of the poultry isolates were actually *C. jejuni* (Siemer et al., 2004). This indicated the need for improved routine identification (Siemer et al., 2004).



If emerging *Campylobacter* species are present in the food chain, their potential as a health risk still needs to be determined (Lastovica, 2006). Lynch et al. (2011) investigated the source and role of fastidious *Campylobacter* species. Although the adapted methods that they used allowed the detection of some emerging and fastidious species not normally found, they were less successful in detecting *C. jejuni* in chicken meat (Lynch et al., 2011). There are currently 32 recognized and described species and 13 subspecies of *Campylobacter* (Euzeby, 1997: website accessed 20 October 2012), five of which were described in 2009 (Debruyne et.

al., 2009a; Debruyne et. al., 2009b; Debruyne et. al., 2009c; Rossi et al., 2009; Zanoni et al., 2009).

Arcobacters have been isolated from various types of environmental waters, and are highly prevalent in livestock faeces and the intestinal tracts of healthy farm animals (Collado & Figueras, 2011). *A butzleri*, *A. cryaerophilus* and *A. skirrowii* are more prevalent in water that is contaminated with faeces than water that is not (Collado & Figueras, 2011). Meat products are probably contaminated by these bacteria when faeces containing arcobacters comes into contact with slaughtered carcasses. Studies have been done on the prevalence of arcobacters in foods mainly of poultry, beef and pork origins, including raw milk (Collado and Figueras, 2011).



2.6 Avoiding the pitfalls encountered in isolation

In the past, many studies and culture methods of detecting *Campylobacter* spp. have been biased towards the common pathogens, *C. jejuni* subsp. *jejuni* and *C. coli* (Maher et al., 2003; Lastovica, 2006; Lynch et al., 2011). One of the ways in which this was done, was by increasing the growth temperature of the primary cultures to 42 ºC (Maher et al., 2003). Most *Campylobacter* spp. grow optimally at 37 ºC, with some being able to grow at 42 ºC, some at 25 ºC and some at all of these temperatures (Quinn et al., 1994). In a survey carried out by Rahimi and Tajbakhsh (2008) on poultry meat, all isolation attempts were performed at 42 ºC. Cuomo et al. (2007), in their study of ostriches in Italy also only cultured the thermotolerant *Campylobacter* strains.

*Helicobacter* spp. grow optimally at 37°C while their growth at 42 ºC is variable (Holt et al., 1994). *Arcobacter* spp. grow between 15 ºC and 37 ºC, with 25 ºC to 30 ºC being optimal for them (Eifert et al., 2003). Amisu et al. (2003) reported that most arcobacters grow at 37 ºC and only some grow at 42 ºC.

The duration of incubation of cultures must be long enough to facilitate the observation of the slower-growing epsilonproteobactera (Lastovica, 2006). After detection of fast-growing species like *C. jejuni*, additional incubation of the plates should be carried out for detection of any other suspect organisms that may be present in the sample (Lastovica, 2006). At a hospital in South Africa, plates are routinely incubated for six days before discarding (Lastovica, 2012, personal communication). It was found that up to 17 % of clinical samples tested at that facility yielded two to five species of *Campylobacter* or related bacteria on the primary isolation plate (Lastovica, 2006). Another way in which some *Campylobacter*-like organisms may go undetected is by using sub-optimal atmospheric conditions for cultures (Maher et al., 2003). Arcobacters are microaerophilic on initial isolation, but then adapt to increased oxygen levels and subsequently become aerobic (Eifert et al., 2003). Helicobacters are all microaerophilic (Corry et al., 2002) and Fox (1997) recommended added hydrogen for their growth. *Campylobacter* species are generally microaerophilic (Lastovica, 2006), but some emerging species have an essential growth requirement for hydrogen, and are extremely difficult to culture by the standard routine isolation techniques used by most laboratories.

Many commonly used selective *Campylobacter* media include antibiotics as chemical selective substances to reduce concurrent isolation of other

contaminating bacteria in samples. Antibiotic susceptibility of some *Campylobacter*-like organisms and the routine use of these antibiotics in selective media also limits the range of Epsilonproteobacteria that can be isolated (Lastovica, 2006; Maher et al., 2003; Moore et al., 2005). The use of media containing antibiotics can be avoided by filtering samples onto antibiotic-free blood agar plates (Lastovica, 2006) thereby utilizing the natural motility of the organisms to remove undesirable bacteria before incubation. Various passive membrane filtration techniques have been used effectively to reduce levels of contaminating bacteria in samples (Moore et al., 2005). Cellulose acetate or mixed cellulose ester filters are commonly used, and cellulose nitrate filters have been shown to restrict penetration of competing bacteria when the filter pore size is 0,65 µm (Moore et al., 2005). Lynch et al. (2010) reported that during their investigation the best recovery of *Campylobacter* cells was achieved using mixed cellulose ester membranes with 0,65 µm pore size, as outlined in the Cape Town protocol (Lastovica, 2006).



To avoid the pitfalls that could result in failure to isolate certain strains, the following isolation criteria were used in this study. Samples were filtered and cultured on Tryptose blood agar plates (Oxoid™ CM0233, containing 10 % unlysed horse/sheep blood). The culture plates were incubated at 37 ºC for a total of six days in a hydrogen-enriched microaerobic atmosphere (anaerobic jars with Oxoid™ BR38 gas-generating pouches and without catalyst) as described in the Cape Town protocol (Lastovica, 2006). Corry et al. (2002) also describe the need to use membrane filtration and non-selective media, and incubating plates at 37 ºC to facilitate isolation of arcobacters and some fastidious campylobacters.

2.7 Isolation and phenotypical identification

The Cape Town protocol (Lastovica, 2006) has been used exclusively for the isolation of Epsilonproteobacteria from clinical specimens containing a high bacterial load (Lynch et al., 2010). Subsequently this method was adapted to facilitate the isolation of a wide range of *Campylobacter* species from meat, where these bacteria may be damaged or present in low numbers. In 2010 Lynch et al. reported how they had developed a universal protocol for the detection of twenty species and subspecies of *Campylobacter* by the experimental inoculation of meat and subsequent target organism recovery. They adapted the Cape Town protocol by including an initial enrichment step, and added a centrifugation step to reduce filter blockage by particles in the sample (Lynch et al., 2010). This was the first report of the use of a single cultural procedure to isolate a wide variety of *Campylobacter* species and recover them from spiked meat samples. When cultures had been incubated, the authors reported good results when performing the KOH test together with the use of the OBIS Campy *Campylobacter* kit (Oxoid™, Basingstoke, UK) for presumptive identification of suspect colonies. This finding is consistent with the good results obtained by Hoosain and Lastovica (2009) on evaluation of the OBIS Campy kit for the presumptive characterisation of *Campylobacter* and *Arcobacter*. Isolates were confirmed either by the phenotypical tests listed in the Cape Town protocol, or by PCR (Lynch, et al., 2010). When Lynch et al. (2011) later applied this adapted cultural protocol to investigate the source and role of fastidious species in meat and poultry, they concluded that this method allowed the isolation of a wider range of *Campylobacter* species than normally reported. They again used the biochemical

test matrix presented in the Cape Town protocol to phenotypically identify their isolates. They also used a *Campylobacter* latex test kit (Microgen Bioproducts Ltd, Camberley, UK) to distinguish thermophilic from non-thermophilic species (Lynch et al., 2011). Three latex agglutination tests for the rapid identification of *Campylobacter* spp. were evaluated by Miller et al. (2008). The Microgen *Campylobacter* kit mentioned above was recommended for testing campylobacters isolated from humans and food samples (Miller et al., 2008).

One of the common ways of differentiating *C. jejuni* from *C. coli* in clinical laboratories is by means of the hippurate hydrolysis test (Holt, 1994). *C. coli* is hippurate negative, while *C. jejuni* is usually hippurate positive, although atypical hippurate negative isolates of *C. jejuni* have been reported (Jauk et al., 2003). Initial *C. jejuni* isolates cultured from ratite samples were found to be hippurate negative, although subsequent ratite cases mentioned in that report yielded hippurate positive isolates (Post et al., 1992).



Moore (2001) described an optimised method to isolate thermophilic *Campylobacter* species from porcine liver samples. Various selective agars were used, and a simple direct swabbing technique of the samples was found to be economical (Moore, 2001). Homogenization of the liver tissue should be avoided, as intracellular inhibitory substances may be released that could impede the growth and multiplication of campylobacters (Moore, 2001).

## 2.8 Molecular techniques

As a result of the limitations of culture-based methods for the detection of campylobacters, alternative detection methods were developed (Moore et al., 2005). Genotypic analyses and distinction was an obvious alternative (Jauk et al., 2003). Differentiation of campylobacters is carried out using PCR, pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) and PCR restriction fragment length polymorphism (PCR-RFLP) (Jauk et al., 2003; Silva et al., 2011). For the development of a rapid, two-step identification scheme for campylobacters, arcobacters and helicobacters, one set of primers was directed at 26 species (Marshall et al., 1999). All of these organisms could be identified to genus level with just one restriction enzyme, *Dde*I (Marshall et al., 1999), which also gave unique banding patterns for the identification of 11 organisms to species level. Latex agglutination tests using polyclonal antibodies provide faster confirmation of species than conventional phenotypic tests (Moore et al., 2005). The use of a commercial enzyme-linked immunosorbent assay, ProSpecT Microplate assay, also provided faster results when compared to conventional culture and may be advantageous when early diagnosis is critical for patient therapy (Tolcin et al., 2000 in Moore et al. 2005).

The first PCR applied to detect *C. jejuni* and *C. coli* in stool samples and among various other *Campylobacter* isolates was described in 1992 by Oyofo et al. (Moore et al., 2005), but this assay could not distinguish between the two species (Oyofo et al., 1992). The report showed the potential of PCR methods to detect low numbers of the target cells, although its use was still restricted to research laboratories because of complicated sample preparation steps and gel

electrophoresis for end-point determination (Moore et al., 2005). Macrorestriction of whole-cell DNA fragments of *Campylobacter* spp. isolates, and subsequent pulsed-field gel electrophoresis, were used effectively to determine significant epidemiological information concerning an outbreak of infectious hepatitis in ostriches in Australia (Stephens et al., 1998). After developing a panel of PCR-ELISA assays to detect and distinguish between seven *Campylobacter* spp., Lawson et al. in 1999 noted that PCR was "more expensive and labour-intensive than culture" (cited by Moore et al., 2006).

A comparison was carried out on the phenotypic and genotypic differentiation of Epsilonproteobacteria that are known to colonize birds, and had been isolated from faecal samples from poultry and various animal species on broiler farms in Austria (Jauk et al., 2003). The authors aimed to develop a genotypic identification scheme based on PCR-RFLP and a PCR assay based on the hippuricase gene (Jauk et al., 2003). Primers were designed to amplify the 16S rRNA gene of *C. jejuni* subsp. *jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari*, *A. butzleri* and *H. pullorum* isolates (Jauk et al., 2003). The hippuricase gene PCR assay gave the added advantage of detecting the *C. jejuni* strains that were phenotypically hippuricase-negative. Overall, when the phenotypic differentiation results were compared with those of the molecular characterization, they corresponded in 91,5 % of the cases (Jauk et al., 2003). An advantage of the multiplex PCR method of Persson and Olsen (2005) to identify *C. coli* and *C. jejuni* directly in stool samples was the detection of viable as well as non-viable organisms present in the sample.

The first PCR assay developed for the detection of *Campylobacter* species in foods was able to show a limit of detection of 25 CFU per gram of tissue when applied to artificially inoculated samples of chicken skin (Moore et al., 2006). A more sensitive limit of detection of one CFU per 25 gram of food is, however, required of a PCR assay for it to replace conventional culture methods (Moore et al., 2006). This level of sensitivity appears to be attainable owing to the development of real-time PCR methods potentially capable of detecting 1 CFU in under two hours (Debretsion et al., 2007 in Silva et al., 2011).

Inefficient sample preparation steps in subsequent reports of PCR assays for campylobacters revealed limitations in the methods when attempting to separate low numbers of target cells from the sample matrix, even though some methods claimed a detection limit of one cell per reaction (Moore et al., 2006). Persson and Olsen (2005) reported that their multiplex PCR for *C. coli* and *C.jejuni* identification gave a sensitivity limit of 10 to 100 cells per PCR performed on pure cultures. The sensitivity limit when DNA was extracted directly from spiked stool samples was $10^5$ cells per mL sample (Persson & Olsen, 2005).

Real-time PCR methods have been used to develop quantitative PCR assays to detect campylobacters in foods, milk and environmental water (Moore et al., 2006). When applied to raw meat rinse fluids though, the limit of detection was forfeited by PCR inhibitors and the low numbers of cells present (Sails et al., as cited by Moore et al., 2006). If nucleic acid based detection methods could permit direct detection of target organisms without an enrichment culture step, detection time would be reduced to hours instead of days (Moore et al., 2006). Conventional PCR assays cannot distinguish between viable and non-viable cells which does

limit the use of such results in food microbiology (Moore et al., 2006). There is uncertainty around the relationship between the presence of DNA and viability of cells (Astorga & Alonso, 2010). Assays to detect viable cells using messenger RNA as the target for reverse transcriptase PCR have been investigated (Moore et al., 2006). Nucleic acid sequence-based amplification (NASBA) has been used to establish the viability of *Campylobacter* (Astorga & Alonso, 2010). During this procedure single-stranded RNA sequences are amplified. Real-time NASBA was reportedly suitable for determining the viability of targeted nucleic acid as 16S rRNA genes (Astorga & Alonso, 2010). The use of ethidium monoazide was applied successfully to amplify DNA only from *Campylobacter* cells with intact cell membranes in a study on the persistence of these organisms in bovine manure compost (Inglis et al., 2010).



Maher et al. (2003) assessed a DNA Probe-based PCR assay for the direct detection of *Campylobacter* species in clinical faecal samples. They reported inconsistencies in the amplification of *Campylobacter* DNA from some of the samples. They ascribed this to the likely presence of PCR inhibitors in the samples and concluded that the assay should be modified to include an internal standard control (Maher et al., 2003). This control should then be amplified together with samples, thus revealing any PCR inhibitors in the sample matrix and whether or not samples should be diluted for repetition of the PCR (Maher et al., 2003). A multiplex PCR was developed to identify only the most relevant pathogens associated with poultry and poultry products (Neubauer & Hess, 2006). No species identification, however, is possible with this method (Neubauer & Hess, 2006).

In a study to detect *Campylobacter* species in faecal samples from steers, samples were tested using only molecular techniques. The use of an internal control though indicated adequate removal of PCR inhibitors from the faeces, and the analyses proved to be accurate, sensitive and rapid (Inglis et al., 2004). In 2009 several species-specific PCRs for various *Campylobacter* species were used to confirm that the novel *C. avium*, which was initially incorrectly identified as *C. jejuni*, was this new species (Rossi et al., 2009).

Persson and Olsen (2005) acknowledged the difficulties in routine *C. jejuni* and *C. coli* differentiation, and developed a multiplex PCR to identify these two organisms. They applied this method to DNA that was directly extracted from faecal samples, as well as to pure cultures. Although the sensitivity of direct culturing of these species was greater than that of direct DNA extraction, applying the PCR to DNA directly extracted from samples gave the added advantages of the detection of non-viable organisms, a reduced testing time, and the correct identification of hippurate-negative strains of *C. jejuni* (Persson & Olsen, 2005). In a PCR-RFLP- based method developed to identify avian Epsilonproteobacteria, a PCR assay based only on the hippuricase gene was included to distinguish *C. jejuni* from *C. coli* (Jauk et al., 2003). This assay allowed the correct identification of seven hippurate-negative *C. jejuni* strains (Jauk et al., 2003). Subsequently Astorga and Alonso (2010) reported that this gene is no longer used in *C. jejuni* identification since the discovery of hippurate negative strains. In an epidemiological study of *C. coli* isolates from Denmark, twelve percent of the isolates were found to be *C. jejuni* by molecular techniques, that were previously misidentified as *C. coli* by phenotypical analyses (Siemer et al., 2004). Marshall et

al. (1999) promoted the rapid identification of epsiloproteobacteria by PCR-RFLP as a means to bypass the often subjective phenotypical classification methods. Even just the hippuricase PCR - part of their identification scheme alone could be used as a diagnostic tool when simple differentiation of *C. jejuni* and *C. coli* is required, thereby also eliminating the effects of atypical strain characteristics (Marshall et al., 1999).

## 2.9 The need for research

Various sources have stated that there is a need for more research and epidemiological studies on the occurrence of campylobacters and related organisms (Maher et al., 2003) especially relating to their possible transmission to humans via foods of animal origin (Corry et al., 2002; Lastovica & Skirrow, 2000, Rosef et al., 2008). The roles of the emerging *Campylobacter* spp. in particular in disease processes are not fully realized, and improvements to isolation and identification protocols for these organisms are essential to obtain the necessary epidemiological and other information (Lastovica, 2006). For example the habitat of *C. lari* is not fully understood, and data regarding how long it can survive in the environment is still lacking (Rosef et al., 2008). It has been isolated from the faeces of wild birds, dogs, horse intestine, pigs, poultry, shellfish and from natural water sources (Debruyne et al., 2009a; Hughes et al., 2009; Rosef et al., 2008).

When investigating the occurrence of fastidious *Campylobacter* species in fresh meat and poultry, Lynch et al. (2011) recorded some significant results. *C. concisus*, that had not previously been associated with food, was isolated from

poultry products. *C. mucosalis* , which is normally associated with pigs, was detected in 3 % of the *Campylobacter*-positive poultry samples tested. *C. lari* and *C. fetus* were also detected in chicken meat, which was a rare finding (Lynch et al., 2011). The authors recovered a wider range of emerging *Campylobacter* species that are of clinical significance (Lynch et al., 2011). The method that they used also permitted detection of *C. coli* more frequently than *C. jejuni*, which is usually the most prevalent species in poultry. They suggested that the detection rates of these species may be an indication of the methods applied, instead of the actual occurrence in food products (Lynch et al., 2011).

There seems to be an increasing rise in antibiotic resistance of *Campylobacter* strains from both clinical specimens and food animals (Zhang, 2008). In a study on the prevalence of *Salmonella*, *Campylobacter* and *Escherichia coli* on ostrich carcasses in the United States, the authors concluded that more antibacterial susceptibility tests on ostrich bacterial isolates are needed for this industry (Ley et al., 2001). Increases in antibiotic resistance in clinical isolates from paediatric diarrhoeic stools in Cape Town between between 1998 and 2005 were reported by Lastovica (Moore et al., 2006). Some of these isolates even showed multiple resistance (Lastovica et al., 2012, unpublished data). Various presentations at the 16th CHRO workshop in 2011 evidenced increases in antibiotic resistance to *C. jejuni* and *H. pylori* (Gaynor & Szymanski, 2012).

During the ostrich slaughtering process in abattoirs, particularly during the evisceration steps, there is a significant risk of faecal contamination of the carcass. Strict guidelines are in place in many abattoirs in South Africa to adhere to good Hazard Analysis and Critical Control Points (HACCP) principles and risk

management systems. This approach during harvest to control and reduce faecal contamination is recommended by Moore et al. (2006). Ostrich meat products (processed and un-processed) are tested before consumption for the presence of various potentially pathogenic bacteria and spoilage organisms. These tests usually include the enumeration of *E. coli*, Enterobacteriaceae and/or coliforms, *Staphylococcus aureus*, *Pseudomonas* spp. as well as total heterotrophic plate counts. The presence or absence of *Salmonella* spp., *Clostridium perfringens*, and *Listeria* spp. is also monitored. The European Union's regulation 2073/2005 on microbiological criteria for foodstuffs (2005), clause 22, states that "sampling of the production and processing environment can be a useful tool to identify and prevent the presence of pathogenic microorganisms in foodstuffs". Testing for the presence of *Campylobacter* and related organisms in food is not routine practice in South Africa.



Considering the high incidence of HIV/AIDS infections currently in the South African population and the indicators that AIDS patients are vulnerable to infections by *Campylobacter* and related species (Lastovica, 2006; Lastovica & Skirrow, 2000), it appears prudent to apply the suggestion made by the European Union in commission regulation no. 2073/2005. Clause 26 states that the set microbiological criteria should be revised or supplemented if developments in the field of food safety, such as possible outputs from risk assessments and changes in the population of vulnerable consumers, deem it appropriate (2005).

## Chapter 3: Isolation and characterization of Epsilonproteobacteria from fresh ostrich tissue samples

### 3.1. Abstract

*Campylobacter* and related Epsilonproteobacteria (*Arcobacter* and *Helicobacter*) are currently viewed as emerging pathogens and are able to cause gastroenteritis, bacteraemia, the Guillain-Barré syndrome, reactive arthritis and other diseases in humans. These bacteria can cause a variety of diseases in animals, including venereal disease, reduced fertility, abortions, gastroenteritis and mastitis in cattle, sporadic abortions in sheep and goats, and equine and porcine abortions. While poultry, cattle and sheep are known reservoirs for campylobacter, very little is known about ostriches as a vector for these organisms. What is known, however, is that these birds can and sometimes do get infected. In this study, qualitative microbiological assays were performed on liver, caecum and colon samples predominantly from healthy ostriches presented for slaughter, to detect any Epsilonproteobacteria present. Samples were collected at an abattoir in the Western Cape between February and December 2010. One three-to-four-month old bird was also tested. Bacterial isolates were characterized following the phenotypic and biochemical scheme presented in the Cape Town protocol. This protocol uses membrane filtration onto antibiotic-free Tryptose blood agar plates, and incubation at 37 ºC in a hydrogen-enhanced microaerobic atmosphere. Most isolates were identified as *C. jejuni* subsp. *jejuni*. Beef, pork, chicken and other poultry meat products, including ostrich meat, have previously been surveyed overseas for the presence of Epsilonproteobacteria. The low percentage (4,9 %) of samples that tested positive in this study suggests that the likelihood of

Epsilonproteobacteria contamination of ostrich meat products presented at this abattoir during this period was minimal. Various possible reasons for this low prevalence were explored. The data gathered in this study may be useful for determining the risk of campylobacter and other emerging Epsilonproteobacteria to human health from the processing and subsequent consumption of ostrich meat products in South Africa.

## 3.2. Introduction

Thermophilic campylobacters (*C. jejuni, C. coli, C. lari* and *C. upsaliensis*) occur as part of the normal intestinal flora in a variety of animals and birds (Astorga & Alonso, 2010). Studies have been carried out on these and other Epsilonproteobacteria to determine which of them can colonize livestock and poultry destined for human consumption (Hughes et al., 2009; Lammerding et al., 1996; Moore, 2001; Neubauer & Hess, 2006; Siemer et al., 2004). As faecal contamination of meat may occur during slaughtering, humans can become infected with campylobacters after consuming undercooked, tainted meat (Astorga & Alonso, 2010; Rahimi & Tajbakhsh, 2008). The source of fastidious *Campylobacter* species and the role that fresh food may play in their transmission to humans also still needs to be established (Lynch et al., 2011).



*C. jejuni* and *C. coli* are often present in the intestinal tracts of poultry and other avian species (Stephens et al., 1998; Zhang, 2008). Birds are infected by campylobacters via the faecal-oral route, and the organisms colonize in the caecal and cloacal crypts. Sometimes the organisms can also be isolated from the small intestines, liver and other organs (Zhang, 2008). Some helicobacters can colonize

the large intestine. *H. pullorum* has been isolated from the livers and intestines of laying hens with hepatic lesions (Corry et al., 2002). *Arcobacter* has regularly been isolated from the intestines and faeces of various farm animals (Collado & Figueras, 2011).

Ostriches are known to suffer from *Campylobacter* infections. In 1990 in Israel, a syndrome resembling avian vibrionic hepatitis in other birds was observed in young ostriches (Perelman et al., 1992). Pure cultures of *C. jejuni* were isolated from the livers of all the birds showing symptoms (Perelman et al., 1992). *C. jejuni* was allegedly isolated from 3-month-old rheas and 2-week-old ostriches in 1990 in Texas, USA (Post et al., 1992). Although no other etiological agents were detected, only a limited number of confirmatory tests were performed on these isolates, some of which were hippurate hydrolysis positive and some negative. Welsh et al. (1997) reviewed the infectious diseases seen in ratites between 1990 and 1996 at the Oklahoma Animal Disease Diagnostic Laboratory. Only 0,2 % of the ratite enteric samples tested for the presence of various bacteria were positive for *C. jejuni*. The culture conditions used, however, were not optimal for *Campylobacter* isolations. An outbreak of infectious hepatitis in two-to-eight-week-old ostrich chicks was reported from six farms in Australia between November 1995 and April 1996 (Stephens et al., 1998). This appeared to be the first definitive report of infectious hepatitis in ostriches caused by *C. coli*.

In a study to detect only the thermotolerant *Campylobacter* species in clinically health ostriches in Italy, 60 of 150 birds tested were positive for *C. jejuni* and/or

*C. coli* (Cuomo et al., 2007) indicating that ostriches are indeed *Campylobacter* carriers. Findings of Oyarzabal et al. (1994) also determined that in the food animal environment they were potential *Campylobacter* carriers. Emerging, newly described campylobacters have been found in production animals such as pigs, sheep, cattle and poultry, and in pets and wild animals (Lastovica, 2006). In a bacterial investigation regarding the prevalence of *H. pullorum* in poultry, a previously unknown *Campylobacter* species, *C. avium*, was isolated from broiler chicks and a turkey and subsequently described in 2009 (Rossi et al., 2009). Arcobacters have frequently been isolated from the intestines of farm animals including poultry, and they are reportedly present in various other species including rheas (Collado & Figueras, 2011).



Relatively little is known about ostriches as a vector for all of these organisms. To establish what the prevalence of Epsilonproteobacteria in healthy adult ostriches is, 344 tissue samples were collected at a local abattoir from adult birds presented for slaughter.

One ostrich chick (3 to 4 months old) was submitted to the Provincial Veterinary Laboratory, Department of Agriculture: Western Cape for post mortem examination, and was included in this survey. Findings of the examination indicated gastric stasis, and a sample of the caecum was collected for Epsilonproteobacteria screening.

## 3.3. Materials and methods

Fresh ostrich tissue samples (344) were collected at a local abattoir (Swartland Ostriches and Game Abattoir, Malmesbury, Western Cape) from adult birds presented for slaughter during an eleven month period between February and December 2010. The abattoir is situated 61 km away from the laboratory where the samples were processed and analyses were performed. Ostriches presented for slaughter at this abattoir undergo an on-site veterinary inspection directly before they are slaughtered to confirm that they are healthy. Batches of samples, usually from ten birds, were collected per sampling day. Initially colon, caecum and liver samples were collected from each bird (from Farms A to H). Later (from Farms I to P) only caecum and colon samples were collected. Samples were collected during the evisceration step of the slaughter process. They were placed in a cool box containing frozen ice-packs and transported directly to the laboratory. Samples were processed within 6 hours of arrival at the laboratory.



All samples collected were analysed by conventional microbiological culture. For a positive growth control, colonies of *C. jejuni* (ATCC 33560) and an *Arcobacter* sp. were inoculated separately into 4 mL volumes of Brain Heart Infusion (Oxoid™ CM1135, Oxoid Ltd, Basingstoke, Hampshire, England), and incubated overnight in 5 % $CO_2$. A positive control sample was freshly prepared each morning before departure to the abattoir. The control sample for each day consisted of a test tube containing ±1 g of colon content (previously verified negative for Epsilonproteobacteria) inoculated with 100 – 500 µL of each of the overnight broth cultures. The tube was mixed well by vortexing and was tightly sealed (double layer). This control sample accompanied the samples collected on

that day for the duration of the sample collection, processing and incubation steps and was processed simultaneously.

Liver samples constituted a single piece of liver of about 200 g with no visible signs of perforation. Intestinal samples constituted a length of about 15 cm of the appropriate region of the intestine. Samples were collected individually in sterile 125 mL and 250 mL polypropylene or polystyrene sample jars during evisceration. They were placed in a cooler box containing frozen ice packs and transported to the laboratory immediately for processing. Samples were processed within 6 hours of arrival at the laboratory.

Sample processing, bacterial isolation, phenotypical and biochemical characterization procedures were carried out at the Provincial Veterinary Laboratory, Department of Agriculture: Western Cape. This laboratory is situated in Stellenbosch. Sample processing started immediately on arrival at the laboratory. Liver samples were seared with a stainless steel spatula heated over a flame, before an incision was made through the surface of the organ to a depth of at least 2 cm. A sterile, dry, cotton-tipped swab or a sterile, disposable, 10µL plastic loop was inserted as deep as possible into the incision to collect deep tissue material, and plated onto Tryptose Blood Agar Base (Oxoid™ CM0233) blood agar plates. The plates were prepared with 10 % unlysed whole sheep or horse blood, and pre-reduced in 5 % $CO_2$ overnight before use.



Each intestinal sample was processed by making a small incision into the intestine wall, avoiding either “open” end thereby minimising exposure to air. A sterile cotton –tipped swab was carefully inserted into the incision to collect intestinal

content, concentrating on the mucosal wall by applying pressure from the inside. The swab was removed and placed into 4 mL sterile saline. The saline suspension was mixed by vortexing for 5-10 seconds, and filtered through a 0,65 µm membrane filter with 47 mm diameter ( mixed cellulose ester filters: Schleicher and Schuell, Dassel, Germany, or cellulose nitrate filters: Sartorius Stedim Biotech, Göttingen, Germany) onto pre-reduced Tryptose Blood Agar plates as above. Filtration was carried out by applying the membrane filter to the agar plate, and flooding it 3 times with volumes of between 20 and 100uL, waiting 4-5 minutes after each flooding, giving a total inoculum of between 180 and 220uL and total time of filtration of about 15 minutes. The membrane was then removed, taking care that any residual fluid on the membrane did not run over the edge of it and contaminate the plate. After inoculation, the plates were immediately sealed in anaerobic jars, each containing an Oxoid™ BR38 microaerophilic gas-generating pouch without a catalyst. The jars were incubated at 37 ºC.



One colon sample and one caecum sample from each batch collected was also plated onto blood agar and streaked for single colonies. This was a crude evaluation of the total microbial flora present in the intestinal samples at the time of sampling.

One ostrich chick (3 to 4 months old) was submitted to the Provincial Veterinary Laboratory, Department of Agriculture: Western Cape for post mortem examination, and was included in this survey. Findings of the examination indicated gastric stasis, and a sample of the caecum was collected for Epsilonproteobacteria screening.

All the agar plates were examined every 48-72 hours. All suspicious colonies were subjected to Gram's staining to identify any potential Epsilonproteobacteria. Colonies of typical Gram negative curved rods, often observed lying in an "S", "V" or "gull-wing" formation, were sub-cultured using sterile culture loops to obtain pure cultures. Original agar plates were re-incubated under the same conditions, and inspected again after another 4 days. Where more than one suspect isolate was isolated from the same sample, each was ascribed a unique identification symbol to ensure traceability.

Biochemical tests were performed on each of these isolates and they were stored on Microbank™ beads at -80 ºC where possible. All biochemical characteristics were determined together with selected reference strains. Applying the Cape Town protocol (Lastovica, 2006) phenotypical characteristics of each isolate were determined by the following observations and biochemical tests: growth in an increased hydrogen atmosphere, growth in 5 % $CO_2$, growth in ambient air, growth on MacConkey's agar, indoxyl acetate hydrolysis, hippurate hydrolysis, presence of L-Alanine aminopeptidase, motility, catalase and oxidase. In addition, each isolate was tested for sensitivity to Naladixic acid (30 µg) and Cephalothin (30 µg) using the Kirby-Bauer disk-diffusion method.



## 3.4. Results and discussion

Phenotypical characteristics of all the isolates were consistent with those of *C. jejuni* subsp. *jejuni*. They grew in an increased hydrogen atmosphere and in 5 % $CO_2$, but not in ambient air. They also grew on MacConkey's agar. They were

catalase positive, oxidase positive and motile, displaying typical corkscrew-like motility. The indoxyl acetate hydrolysis test was positive, hippurate was hydrolysed and they all tested positive for the presence of L-Alanine aminopeptidase. All isolates tested were resistant to Cephalothin and sensitive to Nalidixic acid.

### 3.4.1 Liver samples

No Epsilonproteobacteria were isolated from any of the 71 liver samples that were investigated from eight farms (see Table 3.1). Eleven samples were collected from birds from Farm B. Five samples each were collected from Farms D and E, as only a small number of birds from these farms were presented for slaughter on the day of sample collection.

**Table 3.1 Number of liver samples tested from each farm**

| Farm identification | Number of liver samples |
|---|---|
| **A** | 10 |
| **B** | 11 |
| **C** | 10 |
| **D** | 5 |
| **E** | 5 |
| **F** | 10 |
| **G** | 10 |
| **H** | 10 |
| *Total number of farms = 8* | ***Total number of samples = 71*** |

### 3.4.2 Caecum samples

135 caecum samples from sixteen farms yielded six Epsilonproteobacteria, four of which were found to be *C. jejuni* subsp. *jejuni* after biochemical analyses (see Table 3.2). The identification of two isolates (numbers 1 and 2) was not confirmed, because they could not be recovered from the frozen microbeads. From one farm (Farm B) three out of the ten samples tested yielded *C. jejuni* subsp. *jejuni*. On two farms, one isolate each from ten samples were found. On one farm (Farm O), one out of five samples was positive.

In total, six positive samples out of 135 indicates a recovery rate of 4,4 %.

**Table 3.2 Results of Epsilonproteobacteria isolations from caecum samples**

| **Farm identification (and sampling month)** | **Number of samples tested** | **Number of positive samples** | **Assigned isolate number** |
|---|---|---|---|
| **A** (February 2010) | 9 | 0 | - |
| **B** (February 2010) | 10 | 3 | **1,2,3** |
| **C** (February 2010) | 9 | 0 | - |
| **D** (March 2010) | 5 | 0 | - |
| **E** (March 2010) | 5 | 0 | - |
| **F** (March 2010) | 10 | 0 | - |
| **G** (March 2010) | 10 | 0 | - |
| **H** (March 2010) | 10 | 1 | **8** |
| **I** (November 2010) | 10 | 0 | - |
| **J** (November 2010) | 10 | 0 | - |
| **K** (November 2010) | 10 | 0 | - |
| **L** (November 2010) | 10 | 0 | - |
| **M** (November 2010) | 7 | 0 | - |
| **N** (December 2010) | 10 | 1 | **13** |
| **O** (December 2010) | 5 | 1 | **15** |
| **P** (December 2010) | 5 | 0 | - |
| *Total number of farms = 16* | ***Total number of samples = 135*** | ***Total number of positive samples = 6*** | |



*C. jejuni* subsp. *jejuni* was also isolated from the caecum of the three-to-four-month old ostrich chick that was submitted for post mortem.

### 3.4.3 Colon samples

Most Epsilonproteobacteria isolates were recovered from the colon samples. 138 colon samples were taken from 136 birds from sixteen farms. Duplicate samples from one bird from each of Farms A and B accounts for the extra two samples analysed. Of all of these samples, ten yielded Epsilonproteobacteria (see Table 3.3), which is a 7,2 % recovery rate.

**Table 3.3 Results of isolation of Epsilonproteobacteria from colon samples**

| Farm identification (and sampling month) | Total number of samples tested | Number of positive samples | Assigned isolate number |
|---|---|---|---|
| **A (February 2010)** | 11 | 0 | - |
| **B (February 2010)** | 11 | 2 | **4,5** |
| **C (February 2010)** | 9 | 0 | - |
| **D (March 2010)** | 5 | 0 | - |
| **E (March 2010)** | 5 | 0 | - |
| **F (March 2010)** | 10 | 0 | - |
| **G (March 2010)** | 10 | 2 | **6,7** |
| **H (March 2010)** | 10 | 1 | **9** |
| **I (November 2010)** | 10 | 0 | - |
| **J (November 2010)** | 10 | 2 | **11,12** |
| **K (November 2010)** | 10 | 0 | - |
| **L (November 2010)** | 10 | 0 | - |
| **M (November 2010)** | 7 | 0 | - |
| **N (December 2010)** | 10 | 1 | **14** |

| | | | |
|---|---|---|---|
| **O (December 2010)** | 5 | 2 | **16, 17** |
| **P (December 2010)** | 5 | (1) | **-** |
| ***Total no. of farms = 16*** | ***Total no. of samples = 138*** | ***Total no. positive samples = 10 (11)**** | |

***** Only one suspect Epsilonproteobacteria colony was isolated from Farm P, and it could not be confirmed later.

One sample from Farm P yielded only one suspect Epsilonproteobacteria colony. It was stored on microbeads at -80 ºC until further testing could be carried out. Unfortunately no bacterial growth could later be recovered from the stored bacteria.

### 3.4.4 Analysis of results of caecum and colons sampled from adult birds

**Table 3.4 Comparisons of caecum and colon *Campylobacter* isolation rates**

| **Organ(s)** | **Number of positive birds/ total number tested (Farm identification)** |
|---|---|
| Both caecum and colon positive | 1/10 (**B**); 1/10 (**H**); 1/10 (**N**); 1/5 (**O**) |
| Only caecum positive | None |
| Only colon positive | 2/10 (**G**); 2/10 (**J**); 1/5 (**O**) |

Collectively, the results of isolation attempts from the intestinal samples reflect 136 birds tested. Of these birds, four yielded isolates from both the colon and caecum samples (see Table 3.4). Positive results from only the colon are observed from five birds. No birds gave positive isolates from the caecum only. The total number of samples from which *C. jejuni* subsp. *jejuni* was isolated from all the intestinal samples collected at the abattoir is 16 (5,9 %).



### 3.4.5 Other results

From the evaluation of the total aerobic and facultative microbial flora in the representative intestine samples, some interesting observations were made (unpublished data). The birds from most farms appeared to have a healthy, diverse microbial population. Some intestinal samples, though, contained only Gram positive bacteria, suggesting that the birds had been treated with an antibiotic substance targeting Gram negative bacteria some time prior to slaughtering. Intestinal samples from Farm A revealed the presence of some type of broad

spectrum antibiotic residue in the birds' intestinal content. No bacteria were cultured from the samples at all, indicating the complete absence of normal intestinal flora. No indication of this was observed in any of the other samples. Lastly, the birds from one farm were infested with tapeworms.

## 3.5. Conclusions

The consumption of contaminated offal, especially liver, may be a source of *Campylobacter* spp. in humans, and scientists have searched for a specific and sensitive isolation procedure to screen large numbers of them (Moore, 2001). According to Moore (2001) the swabbing method that was used to process the liver tissue is better than any procedure that includes mechanical disruption of the tissue. With this in mind, it appears that the method used to isolate Epsilonproteobacteria from liver tissue was indeed suitable for this study. One flaw, though, was the absence of a positive control for the liver culturing process, and any kind of evaluation of the method sensitivity. This could be addressed in future studies.



No Epsilonproteobacteria was cultured from the 71 liver samples tested from eight farms. Of these farms, and of the same birds sampled, birds from three farms yielded isolates from the intestines sampled (Farms B, G and H), indicating that those birds were carriers of the organisms at the time of slaughter.

All the *C. jejuni* subsp. *jejuni* isolates from the caecum and colon samples may be considered to be part of the birds' normal intestinal flora, because no clinical signs of disease were evident during the pre-slaughter veterinary inspection. Poultry and

other avian species are recognized carriers of *C. jejuni* and *C. coli* (Stephens et al., 1998; Zhang, 2008), and *Arcobacter* (Collado & Figueras, 2011).

There appears to be no significant difference between the number of positive samples identified from those collected in February and March 2010, and the November and December 2010 samples. In spite of this, a larger population size would need to be evaluated to substantiate this observation.

In a study conducted in the United States (Ohio and Indiana) in slaughterhouses, 3 % of the large intestine samples collected from ostrich carcasses were positive for thermotolerant *Campylobacter* species (Ley et al., 2001). In the South African study documented in this report, the 273 intestinal samples collected represent 136 birds tested. Twelve of these birds (8,8 %) were carrying *C. jejuni* subsp. *jejuni* in either the colon or both the colon and caecum.



None of the emerging or fastidious Epsilonproteobacteria were isolated during this survey. Although precautions were taken to ensure that the viability of these organisms, if present in the samples, was maintained, stricter control measures to evaluate this are necessary. Perhaps the use of a fastidious Epsilonproteobacterium instead of, or in addition to, the robust *C. jejuni* subsp. *jejuni* and the *Arcobacter* sp. used as positive control organisms would give greater confidence in the isolation techniques used.

In a study to determine bacterial contamination of chicken carcasses in Gauteng, South Africa, 32,3 % of the retail chicken carcasses tested were contaminated with campylobacter (van Nierop et al., 2005). Since these organisms were detected in the slaughter-age ostriches tested, it may be advantageous to conduct a

study to evaluate the level of *Campylobacter* contamination of ostrich meat products as well.

## Chapter 4: Isolation of Epsilonproteobacteria from ostrich faecal samples

### 4.1 Abstract

While poultry, cattle and sheep are known reservoirs for campylobacter, very little is known about ostriches as a vector for these organisms. What is known, however, is that these birds can and sometimes do get infected. Studies by various authors have provided evidence that various species of animals shed Epsilonproteobacteria in their faeces. In this study, qualitative microbiological assays were performed on 50 faecal samples collected on one farm. This farm was not associated with any of the farms from which samples were collected for analyses in Chapter 3, and was selected for ease of access. Again the Cape Town protocol was employed with passive membrane filtration onto antibiotic-free Tryptose blood agar plates, and incubation at 37 ºC in a hydrogen-enhanced microaerobic atmosphere. No Epsilonproteobacteria were isolated. Possible reasons for the absence of these organisms in these samples were considered.



### 4.2 Introduction

Studies have been done to determine the prevalence of Epsilonproteobacteria in faecal samples of various species (Jauk et al., 2003). Significant numbers of feedlot cattle have been shown to chronically shed campylobacters in their faeces, which persist for long periods (Inglis et al., 2004). In studies done on wild bird populations, the prevalence of *Campylobacter* species in faeces has differed. This could be due to factors such as different life stages (e.g. breeding or migration), or variations in sampling procedures or isolation methods. All these factors need to

be taken into account when evaluating the prevalences of these organisms in wild birds (Hughes et al., 2009).

Poultry may shed arcobacters in their faeces, but do not appear to develop any disease from these bacteria, making them possible natural reservoirs of *Arcobacter* spp. (Collado & Figueras, 2011). *A. cryaerophilus* has been isolated from porcine, bovine and equine faeces (Lastovica & Skirrow, 2000).

In a brief investigation in June 2010 to evaluate the incidence of Epsiloproteobacteria in ostrich faeces, five groups of ten fresh faecal samples each were collected where 7 to 9-month-old birds were being kept in camps on a farm. One group of ten samples was taken from each camp. Between six and seven birds were in each camp. The birds were not receiving any medication at the time of sample collection. The camps were "dry" (i.e. only a few weeds were growing within the perimeters). The ostriches were receiving a balanced ration that was prepared by the owner, and had constant access to fresh water. No mortalities had occurred in the month immediately prior to sampling and the birds appeared to be in a good condition. The farm on which the ostriches were being raised is in the Stellenbosch district of the Western Cape, which has a Mediterranean climate. Sampling was done in winter, when the days are usually cool and sometimes rainy and the average daytime temperature is about 16 ºC.



Samples were only taken of freshly excreted faeces, and were analysed individually.

## 4.3 Materials and methods

The samples were collected individually in sterile 40 mL polypropylene or polystyrene sample jars, placed on ice and transported directly to the laboratory for processing. In the laboratory a swab was taken from the centre of each sample and each swab was suspended in ±3 mL sterile saline. Columbia blood agar plates (pre-reduced in 5 % $CO_2$) were prepared for each sample by inverting them and placing a 0,65 µm membrane filter (either mixed cellulose ester or cellulose nitrate) in the centre of each plate on the surface of the agar. The swab suspension was mixed by vortexing thoroughly and 240µL of each sample was inoculated onto the centre of a membrane. The inoculum was divided into three smaller volumes (100µL, 100µL and 40µL) allowing 4 minutes between each inoculation. This prevented the fluids from overflowing the edges of the membranes. After a total time of 15 minutes, the membranes were carefully removed. The plates were immediately sealed in anaerobic jars, each containing an Oxoid™ BR38 gas-generating sachet without catalyst. The jars were incubated at 37 ºC for a total of six days. The plates were removed from the jars for inspection after 48H and again after 6 days' incubation. All suspicious colonies were subjected to Gram's staining to identify any potential Epsilonproteobacteria.



Control isolates (*C. jejuni* ATCC 33560 and an *Arcobacter* sp.) were used as described above to prepare a positive control which accompanied the samples throughout this process.

## 4.4 Results and discussion

No Epsilonproteobacteria were isolated from the fifty ostrich faeces samples that were tested. Various reasons for this result were considered. Firstly, it has been determined that some Epsiloproteobacteria are very susceptible to adverse environmental conditions (Corry et al., 2002). Despite this, a study by Inglis et al. (2010) indicated that campylobacters could easily be isolated from pens housing feedlot cattle. Campylobacters excreted in cattle faeces also remained viable in bovine manure compost for extended periods (Inglis et al., 2010). During the present study, all possible precautions were taken to minimise any potential exposure that organisms contained in the faecal samples may have had to ambient air and dessication. Sample processing commenced as soon as possible after the samples were collected.



Secondly, the efficacy of the sample filtration method was considered. The permeability of the membrane filters used was evaluated by counting the number of colony forming units that passed through the filters after a suspension of known bacterial concentration was inoculated onto the filters. In this test, when a *C. jejuni* suspension of > 2,48 x $10^8$ CFU/ mL was filtered, only 8 CFU/ mL were recovered. A suspension of *Arcobacter* sp. of similar concentration yielded 1,5 x $10^2$ CFU/ mL. It is evident that a large percentage of the organisms either became non-viable during this step, or were retained on top of the membrane and did not pass through the pores at all to form colonies on the agar surface. Some *C. jejuni* strains are known to show autoagglutination (Misawa & Blaser, 2000). Such strains would likely not pass through the filter (Lastovica, 2013, personal

communication). If Epsiloproteobacteria had been present in the faeces samples in low concentrations, the method used for their isolation may not have been sufficiently sensitive to detect the low numbers.

## 4.5 Conclusion

No Epsilonproteobacteria could be detected in the fifty ostrich faecal samples that were tested. Any horizontal transmission of these organisms could not be investigated. Although some groups of birds were kept in adjacent camps where they had contact with each other, the likelihood that this setup could facilitate cross-contamination between groups could also not be explored. Likewise, the likelihood that wild birds could be a source of Epsilonproteobacteria infection could not be assessed.



In a study of *Campylobacter* species in bovine manure compost, a two-step centrifugation method was used to facilitate isolation of the organisms from manure samples (Inglis et al., 2010). The first step removed large substrate particular matter, and the second step concentrated the *Campylobacter* cells into a pellet. These principles could perhaps be applied to improve the sensitivity of the direct filtration method used in this study.

Some Epsilonproteobacteria are sensitive to adverse environmental conditions, and in dry, warm or aerobic circumstances their survival is reduced (Corry et al., 2002; Persson and Olsen, 2005). *Campylobacter* spp. have been isolated from faecal samples maintained in Cary-Blair medium at 4 ºC for 3 weeks (Oyarzabal et al., 1994). Lastovica (2012, personal communication) observed that after

human faecal samples were seeded with *C. jejuni* subsp. *jejuni* and *Arcobacter* spp., and stored at 4ºC, the campylobacter could still be recovered after filtration after several weeks, and arcobacters could be recovered more than seven weeks later.

The samples were collected in June, which is winter in South Africa. This could also have contributed to the absence of Epsilonproteobacteria in the birds tested. Future studies could include sample collections during summer months to assess this.

# CHAPTER 5 Molecular characterisation of ostrich Epsilonproteobacteria isolates

## 5.1 Abstract

As a result of the scarcity of information regarding the prevalence of Epsilonproteobacteria in ostriches (*Struthio camelus*), genetic data about these isolates is also limited. When *C. jejuni* subsp. *jejuni* strains were isolated from ostriches (as detailed in Chapter 3 above) the multiplex PCR of Neubauer and Hess (2006) could be applied to these isolates. As *C. jejuni* subsp. *jejuni* was the only bacterium isolated and identified by phenotypical testing, one ostrich isolate was randomly selected for inclusion in a panel of isolates used to verify the DNA extraction procedure. This panel consisted of *C. jejuni* subsp. *jejuni* isolates, one *C. fetus* and one *E. coli*. The *C. fetus* and *E. coli* isolates were used as negative controls, and although DNA was successfully extracted from them, no bands were observed in the respective lanes of electrophoresed gels after application of the PCR. Another panel of isolates including more ostrich *C. jejuni* subsp. *jejuni* isolates was used for continued optimization of the PCR, and amplificates of the correct size were observed for all the *C. jejuni* subsp. *jejuni* isolates tested. The banding patterns observed in the electrophoresed gels confirmed the phenotypic identification of the isolates. Attempts to extract DNA directly from *Campylobacter* isolates that had been stored frozen at -80 ºC were unsuccessful.



## 5.2 Introduction

The Epsilonproteobacteria isolated during this study were characterized using the multiplex polymerase chain reaction (PCR) developed by Neubauer and Hess

(2006). These authors used the method to detect and differentiate significant pathogenic food-borne *Campylobacter*, *Arcobacter* and *Helicobacter* species in poultry. Although a molecular identification procedure for these organisms has also been described by Marshall et al. (1999), it comprises a two-step scheme (PCR amplification of the target fragment as well as restriction enzyme digestion), while the method described by Neubauer and Hess and used in this study is a single reaction followed by fragment separation in agarose gel (2006). In keeping with this method, after isolation and phenotypical identification of the isolates collected from the ostrich samples, DNA was extracted from the cultures and subjected to the multiplex PCR. The PCR products were visualized in agarose gel after electrophoresis using an ultraviolet transilluminator.



To avoid re-culturing stored bacterial isolates, attempts were made to extract DNA directly from stored frozen cultures for use in the multiplex PCR. Hughes et al. (2009) reported the preparation of *Campylobacter* spp. DNA extracts from cultures stored at -80 ºC in Microbank™ tubes (Pro-Lab Diagnostics, Ontario, Canada). One bead removed from a tube was boiled in 0,5 mL of sterile distilled water for 20 minutes, and the boilate was stored at 4 ºC for up to 2 weeks (Hughes et al., 2009). In another attempt, three beads from each isolate were removed into 100 µL of TE buffer in a microcentrifuge tube. The tubes were incubated at 56ºC for one hour, with vortexing every 20 minutes. DNA from the fluids from each of these methods attempted was then also extracted and used in the multiplex PCR of Neubauer and Hess (2006).

## 5.3 Materials and methods

### 5.3.1 Initial DNA extraction procedure comparison

Three methods of extracting DNA from the *C. jejuni* subsp. *jejuni* isolates were investigated. Two test isolates (ostrich isolate no. 3 and a chicken isolate), two positive extraction control cultures (*C. jejuni* subsp. *jejuni* biotypes 1 and 2) and two negative controls (*E. coli* and *C. fetus*) were selected for investigation. The isolates were plated onto blood agar plates (Oxoid™ Tryptose blood agar CM0233 plates or Columbia blood agar CM331 plates, with 10 % whole unlysed sheep blood).



After incubation for two days, a loopful of each culture was transferred into a microcentrifuge tube containing 100 µL of TE buffer. The tubes were vortexed and incubated for 2,5 H at room temperature. The contents of each tube were transferred to 0,2 mL PCR tubes, and heated at 95 ºC in a thermal cycler (Px2 Thermal Cycler, Thermo Electron Corporation) for five minutes. The tube contents were then transferred to new microcentrifuge tubes and stored frozen at -20 ºC until DNA extraction could be performed. This protocol was referred to as Method Q.

DNA was also extracted from the above cultures using the Qiagen® DNeasy Blood and Tissue kit (Qiagen Inc., Valentia, CA) according to the manufacturer's instructions. The Spin-Column protocol for animal tissues (without the pretreatment protocol for Gram negative bacteria) was used. Loopfuls of the

bacterial growth from the plates were transferred to 1,8 mL microcentrifuge tubes for the lysis step. Lysis time was two to three hours. This method was called Method E.

A combination of Methods Q and E was also carried out. 50µL of each of the extracts from Method Q were added to 180µL of ATL buffer. Samples were lysed for two hours. The rest of the manufacturer's instructions for the same protocol as above, were followed and this was called Method QE.

As an initial screening to detect any DNA present in the extracts prepared by the above three methods, the extracts were electrophoresed. Agarose gel (1,5 % m/v) containing ethidium bromide (5 µL of 1 % m/v per 120 mL gel) was prepared. A 100 base-pair DNA ladder (Fermentas O'GeneRuler™) was used to indicate fraction sizes. Loading dye (Thermo Scientific® 6 x Orange DNA loading dye; Thermo Scientific, Pittsburgh, PA) was used, and gels were electrophoresed using 110V and 240mA for 60minutes.

Gel images were captured using a ChemiDoc™ XRS gel documentation system (Bio-Rad Laboratories Pty Ltd, Parklands, Johannesburg, RSA).

### 5.3.2 PCR on gradient block

To optimize the annealing temperature of the PCR suggested by Neubauer and Hess (2006), the use of a gradient block in the thermocycler allowed a comparison of three different annealing temperatures. The extracts prepared using Method E above were selected as the optimal template to use in the PCR reaction. Promega PCR Master Mix M7502 (Promega Corporation, Madison, WI) was used to amplify the DNA templates in 25µl reaction volumes. Each of the three forward and one reverse primers used by Neubauer and Hess (2006) were included at a final concentration of 0,4 µM (see Table 5.1).

**Table 5.1 Oligonucleotide sequences used in this study (Neubauer & Hess, 2006)**



| Name | Sequence | Fragment size (bp) |
|---|---|---|
| **REVERS** | 5′-gtg gag tac aag acc cgg gaa-3′ | |
| **HELIP2** | 5′-cca agg gct atg acg ggt gta tcc-3′ | 1107 (*Helicobacter pullorum* accession no. AY631956) |
| **CAMPCJL1** | 5′-act cct ttt ctt agg gaa gaa ttc-3′ | 946 (*C. jejuni* accession no. Y19244) |
| **ARCOB1** | 5′-tgt agg cgg att gat aag ttt gaa-3′ | 822 (*Arcobacter butzleri* accession no. L14626) |

The annealing temperature recommended in the method is 65 ºC, so for this step temperatures of 62 ºC, 64,5 ºC and 66,5 ºC were compared (see Table 5.2).

**Table 5.2 PCR cycling conditions used to compare annealing temperatures**

| **Step** | | Recommended conditions | Adjustments attempted |
|---|---|---|---|
| **Initial denaturation** | | 94 ºC, 5 minutes | 94 ºC, 2 minutes |
| **Denaturation** | *30 cycles* | 94 ºC, 2 minutes | 94 ºC, 1 minute |
| **Annealing** | | 65 ºC, 1 minute | 62 ºC/ 64,5 ºC/ 66,5 ºC, 1 minute |
| **Extension** | | 72 ºC, 1 minute | 72 ºC, 1 minute |
| **Final extension** | | 72 ºC, 10 minutes | 72 ºC, 10 minutes |



A second round of adjustments of the annealing temperatures was attempted (see Table 5.3) when it became evident that the lowest annealing temperature gave the best results. This time six ostrich *C. jejuni* subsp. *jejuni* isolates, one positive control culture (*C. jejuni* subsp. *jejuni* biotype 2) and one negative control (*C. fetus*) were selected for use. Template DNA was prepared using Method E above. The same master mix and primers were used. Annealing temperatures selected were 60,2 ºC and 62,1 ºC.

**Table 5.3 PCR cycling conditions used for the second round of annealing temperature adjustments**

| **Step** | | Recommended conditions | Second adjustments attempted |
|---|---|---|---|
| **Initial denaturation** | | 94 ºC, 5 minutes | 94 ºC, 2 minutes |
| **Denaturation** | *30 cycles* | 94 ºC, 2 minutes | 94 ºC, 1 minute |
| **Annealing** | | 65 ºC, 1 minute | 60,2 ºC/ 62,1 ºC |
| **Extension** | | 72 ºC, 1 minute | 72 ºC, 1 minute |
| **Final extension** | | 72 ºC, 10 minutes | 72 ºC, 10 minutes |

A third round of annealing temperature adjustments was carried out using the same reaction mixtures as prepared for the second round of adjustments described above. The adjustments to the annealing temperature are detailed in Table 5.4. This time annealing temperatures of 56,4 ºC, 58,7 ºC and 60,9 ºC were compared.

**Table 5.4 PCR cycling conditions used for the third round of annealing temperature adjustments**

| **Step** | | Recommended conditions | Third adjustments attempted |
|---|---|---|---|
| **Initial denaturation** | | 94 ºC, 5 minutes | 94 ºC, 2 minutes |
| **Denaturation** | *30 cycles* | 94 ºC, 2 minutes | 94 ºC, 1 minute |
| **Annealing** | | 65 ºC, 1 minute | 56,4 ºC/ 58,7 ºC/ 60,9 ºC |
| **Extension** | | 72 ºC, 1 minute | 72 ºC, 1 minute |
| **Final extension** | | 72 ºC, 10 minutes | 72 ºC, 10 minutes |



### 5.3.3 Recovering DNA from frozen culture beads

Two methods for recovering DNA from cultures on microbeads stored at -80 ºC were compared. For the first method (called method H), three microbeads of culture were placed in 500µL of sterile water in a microcentrifuge tube. The tubes were placed in a boiling waterbath for 20minutes. The second method (called method V) comprised placing three microbeads into 100 µL TE buffer in a microcentrifuge tube. The tubes were incubated at 56 ºC for one hour, with vortexing every 20 minutes.

Isolates selected for methods H and V included two positive extraction controls (*C. jejuni* subsp. *jejuni* biotypes 1 and 2), three negative controls (*C. upsaliensis*, *C. fetus* and *C. concisus*), one ostrich *C. jejuni* subsp. *jejuni* isolate (Isolate no. 3)

and one chicken *C. jejuni* subsp. *jejuni* isolate. Extraction of the samples from both methods was carried out using method E above (Qiagen® DNeasy Blood and Tissue kit). Lysis time was one hour.

PCR reaction mixtures for the samples comparing these two methods were again prepared using the Promega PCR Master Mix and the same primers as above. Specifications of thermocycling were the same as previously used, but an annealing temperature of 61,9 ºC was selected. Agarose gel (1,5 % m/v) containing ethylene bromide was prepared for electrophoresis. Gels were electrophoresed using 110V and 240mA for 60minutes. A 100 base-pair DNA ladder (Fermentas O'GeneRuler™) was used to indicate fraction sizes. Loading dye (Thermo Scientific 6 x Orange DNA loading Dye) was used to visually trace the loaded samples. Gel images were captured using the same ChemiDoc™ system.

## 5.4 Results and discussion

### 5.4.1 DNA extraction

Molecular methods of detecting and identifying Epsilonproteobacteria from bacterial cultures include a DNA extraction step. This could involve culturing the unknown bacterium and suspending an amount of cultured cells in water before boiling (Marshall et al., 1999). Alternatively, DNA can be extracted by standard methods like the phenol-chloroform method, or using commercially available extraction kits (e.g. Promega, Qiagen®) as was done by Neubauer and Hess (2006). The comparison during this study of two other methods (not detailed here) together with the one used by these authors, confirmed that the Qiagen® extraction kit (called "Method E" in this study) that they had used was effective under the existing conditions (See Figures 5.1a and 5.1b).



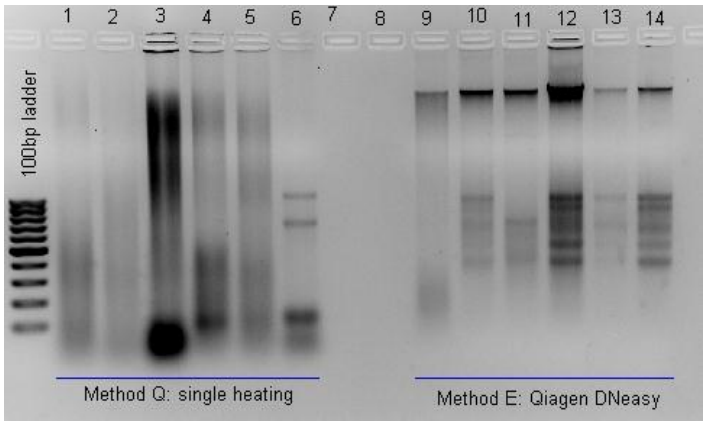


**Figure 5.1a: Comparison of DNA extraction methods.**

Lanes 1 and 14: *C. jejuni* subsp. *jejuni* (chicken strain); lanes 2 and 13: *C. jejuni* subsp. *jejuni* 2 (positive extraction control); lanes 3 and 12: *C. jejuni* subsp. *jejuni* 1 (positive extraction control); lanes 4 and 11: *C. fetus* (negative extraction control); lanes 5 and 10: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 3); lanes 6 and 9: *E. coli* (negative control).

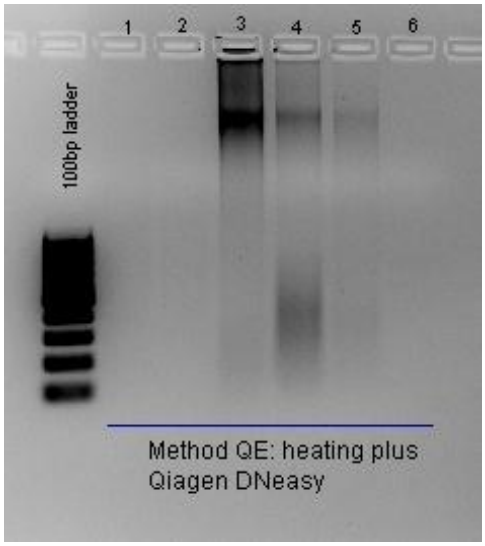


**Figure 5.1b: Comparison of DNA extraction methods (continued).**

Lane 1: *C. jejuni* subsp. *jejuni* (chicken strain); lane 2: *C. jejuni* subsp. *jejuni* 2 (positive extraction control); lane 3: *C. jejuni* subsp. *jejuni* 1 (positive extraction control); lane 4: *C. fetus* (negative extraction control); lane 5: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 3); lane 6: *E. coli* (negative control).



### 5.4.2 Annealing temperatures for PCR

When the PCR method with primers designed by Neubauer and Hess (2006) were used to identify *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. it was unsuccessful. It seemed likely that the annealing temperature was not optimal (Basardien, L., 2011, personal communication). This prompted three comparisons of various annealing temperatures. Results from the first temperature comparison (see Fig 5.2) indicated that annealing at 62 ºC gave bands of DNA of the correct fragment size, although non-specific amplificates of a smaller fragment were also observed. This comparison was performed in duplicate.

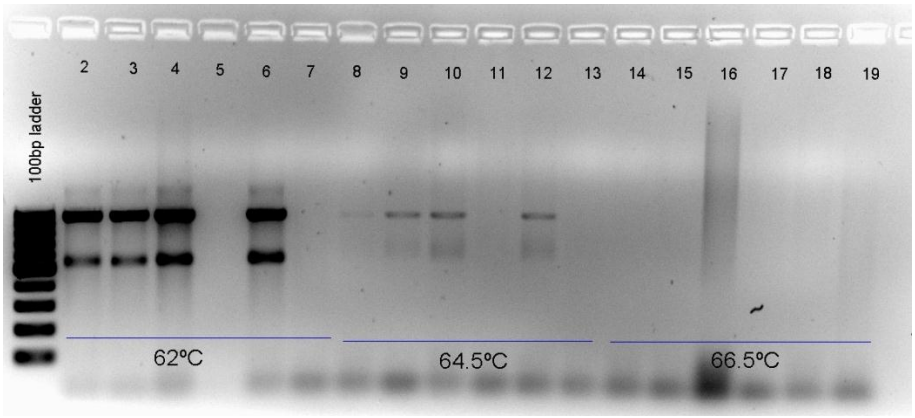


**Figure 5.2: First comparison of three annealing temperatures.**

Lanes 2, 8 and 14: *C. jejuni* subsp. *jejuni* (chicken strain); lanes 3,9 and 15: *C. jejuni* subsp. *jejuni* 2 (positive control); lanes 4,10 and 16: *C. jejuni* subsp. *jejuni* 1 (positive control); lanes 5,11 and 17: *C. fetus* (negative control); lanes 6,12 and 18: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 3); lanes 7,13 and 19: *E. coli* (negative control).

Eight cultures were selected for the second round of annealing temperature adjustments, including six ostrich *C. jejuni* subsp. *jejuni* isolates. The two control isolates (*C. jejuni* subsp. *jejuni* 2 and *C. fetus*) used were the same as those used in the first comparison. The banding patterns of 7 of the 8 cultures tested were similar and the higher annealing temperature gave additional non-specific bands (see Fig 5.3). The "No template control" was prepared by substituting extracted DNA with sterile, DNA- and RNA-free water when preparing the mastermixes.

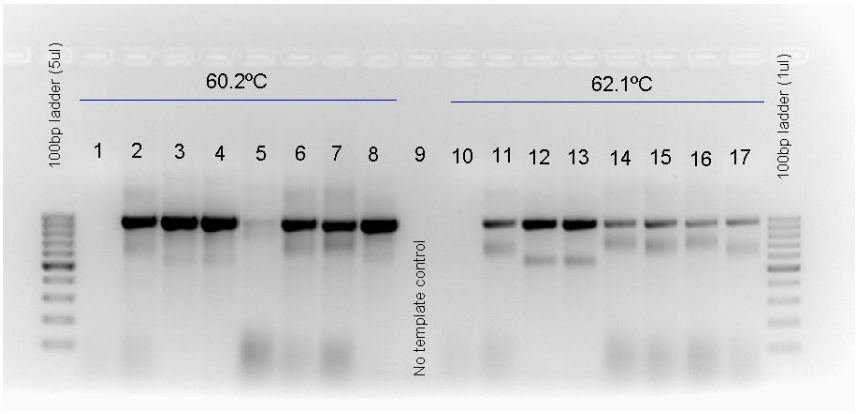


**Figure 5.3: Second comparison of two annealing temperatures.**

Lanes 1 and 10: *C. fetus* (negative control); lanes 2 and 11: *C. jejuni* subsp. *jejuni* 2 (positive control); lanes 3 and 12: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 6); lanes 4 and 13: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 7); lane 5: no DNA loaded into well; lanes 6 and 15: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – small colony form); lanes 7 and 16: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – pooled small and large colony forms); lanes 8 and 17: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 9); lane 14: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – large colony form) .



It appeared as though the bands of the correct fragment size were slightly stronger when the lower annealing temperature was applied, so it was decided to adjust it even lower still and compared temperatures of 60,9 ºC, 58,7 ºC and 56,4 ºC (see Fig 5.4a and 5.4b) in a third comparison exercise.

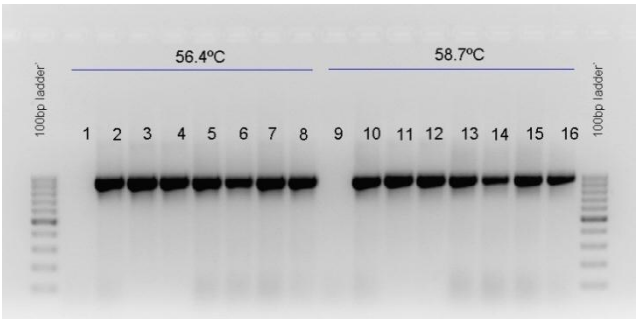


Figure 5.4a: **Third comparison of three annealing temperatures.**

Lanes 1 and 9: *C. fetus* (negative control); lanes 2 and 10: *C. jejuni* subsp. *jejuni* 2 (positive control); lanes 3 and 11: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 6); lanes 4 and 12: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 7); lanes 5 and 13: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – large colony form); lanes 6 and 14: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – small colony form); lanes 7 and 15: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – pooled small and large colony forms); lanes 8 and 16: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 9)

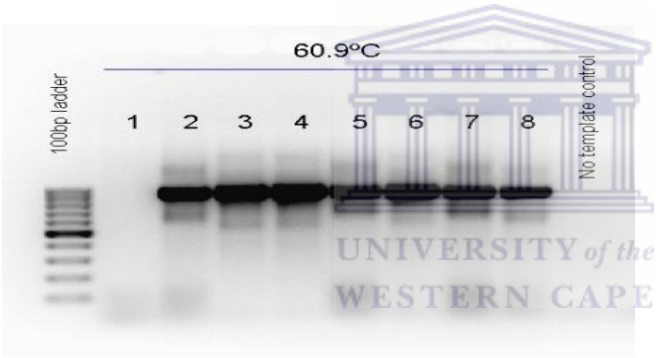


Figure 5.4b: **Third comparison of three annealing temperatures (continued).**

Lane 1: *C. fetus* (negative control); lane 2: *C. jejuni* subsp. *jejuni* 2 (positive control); lane 3: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 6); lane 4: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 7); lane 5: . *jejuni* subsp. *jejuni* (ostrich isolate no. 8 – large colony form); lane 6: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – small colony form); lane 7: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 8 – pooled small and large colony forms); lane 8: *C. jejuni* subsp. *jejuni* (ostrich isolate no. 9)

### 5.4.3 DNA from culture beads

Some authors have described methods of extracting bacterial DNA from frozen microbeads used for the storage of bacterial isolates (Miller et al., 2007; Smuts & Lastovica, 2011). In this study two attempts were made to recover DNA directly from cultures frozen on microbeads at -80 ºC for analysis by the PCR described by Neubauer and Hess (2006).

Unfortunately both of the attempted methods failed. No visible amplificates were observed on electrophoresed gels from any of the panel of samples tested, which included positive and negative controls. One reason for this could be that the PCR is not sufficiently sensitive to detect the amount of DNA that may have been present on each bead. Another possibility is that the DNA had degraded during storage. The cells may also have been resistant to the heating steps applied.



Considering the results of comparing various annealing temperatures (see 5.4.2), it may also be possible that other parameters of the PCR assay are not optimal.

## 5.5 Conclusions

The PCR of Neubauer and Hess (2006), when applied to fresh *Campylobacter* cultures, gave the expected results. Attempts to apply the same assay to frozen bacteria were not successful. There is no data on long term storage of campylobacters in the Microbank™ system (Lastovica, 2013, personal communication). Bacterial viability and possibly also the condition of the bacterial DNA could deteriorate with time. Future studies would require a more

comprehensive evaluation of various methods available to extract DNA from the particular storage substrate used (microbeads). If an extraction method providing adequate sensitivity for this PCR could be identified, it would be useful in confirming the phenotypic identification results of the stored cultures.

Another possible future investigation could comprise using different PCR assays. The restrictions inherent in the PCR used here to detect and identify the relatively small group of Epsilonproteobacteria that are most commonly associated with food products, precludes the possibility of discovering other strains that may be present in ostriches by molecular means.

# CHAPTER 6 Conclusions and recommendations

The most significant findings of this survey are the incidences of viable *C. jejuni* subsp. *jejuni* in the various tissues of healthy ostriches at the point of slaughter. Ten samples out of 138 colon samples tested (7,2 %) yielded the bacterium. Of the 135 caecum samples analysed, six tested positive for the presence of *C. jejuni* subsp. *jejuni* (4,4 %). No Epsilonproteobacteria were isolated from 71 livers tested although the isolation method used appeared appropriate for the sample type (Moore, 2001).

Collectively, 16 of 273 intestinal samples (5,9 %) yielded *C. jejuni*. This prevalence is similar to results obtained overseas. In a study conducted in the United States (Ohio and Indiana) in slaughterhouses, 3 % of the large intestine samples collected from ostrich carcasses were positive for thermotolerant *Campylobacter* species (Ley et al., 2001). These authors also reported detecting campylobacters on about 10 % of the ostrich carcasses tested (Ley et al., 2001). This prevalence is slightly lower than that found in poultry products by Lynch et al. (2011), who identified seven different *Campylobacter* species



All the faecal samples analysed tested negative for the presence of Epsilonproteobacteria. *C. jejuni* and other Epsilonproteobacteria can enter a viable but non-culturable (VBNC) state, which is a survival strategy in response to environmental stress (Astorga & Alonso, 2010). Speculations exist about how they transform back to a culturable state, if indeed they do, and if VBNC is the same as being non-viable (Silva et al., 2011). During this survey, all attempts were made to ensure that samples were protected from unfavourable conditions to

protect the viability of Epsilonproteobacteria. It would have been interesting to evaluate faecal samples from the same birds as were presented for slaughter, or at least collected on the same farms. Thermophilic campylobacters occur as part of the normal intestinal flora in a variety of animals and birds (Astorga & Alonso, 2010). Consequently, if this applies to ostriches as well, one would expect to encounter them in faecal samples, yet none were isolated. In chickens also, although campylobacter is common, there are flocks from which not a single *C. jejuni* has been isolated (Lastovica, 2012, personal communication). A greater distribution of samples from more than just one farm is a recommendation for future studies.

Blood from various animal species (usually defibrinated or lysed) is usually included in solid media used for culturing Epsilonproteobacteria (Corry et al., 2002). Lysed blood neutralizes the toxic effect of compounds formed in growth media due to the effects of light and oxygen (Astorga & Alonso, 2010). Persson and Olsen (2005) used 5 % defibrinated horse blood in their media. At the Red Cross War Memorial Children's Hospital in Cape Town, all Tryptose blood agar plates used for primary isolation or maintenance of Epsilonproteobacteria are prepared using horse blood (Lastovica, 2012, personal communication). Other compounds like charcoal and haematin added to media can serve the same purpose, and when used together with lysed blood in the same media, may further increase the oxygen-quenching abilities of the media (Astorga & Alonso, 2010). During this survey, horse blood was used for primary cultures and sheep blood was used for sub-culturing. Sheep blood was readily available at short notice and could be used to prepare plates as the need for sub-culturing arose.

*C. jejuni* and *H. pylori*, like numerous other pathogenic and non-pathogenic bacteria, can enter a VBNC state, which is a survival strategy in response to environmental stress (Astorga & Alonso, 2010; Oliver, 2005). Speculations exist about how they transform back to a culturable state, if indeed they do, and if VBNC is the same as being non-viable (Silva et al., 2011). Oliver (2005) concluded that resuscitation of VBNC cells can occur, and some species even retain their virulence. He also listed some environmental stresses that could induce a VBNC state in bacteria: starvation, temperature stress, osmotic pressure and oxygen concentration (Oliver, 2000 cited in Oliver, 2005). The interaction of infectious bacteria including *Campylobacter*, *Helicobacter* and related organisms (CHRO) with resident microbial communities was discussed in a summary of recent advances in CHRO research (Gaynor & Szymanski, 2012). Their genomic diversity allows both campylobacters and helicobacters the flexibility of activating some of their proteins to allow phenotypic adaptions according to given selection pressures (Gaynor & Szymanski, 2012). Of the environmental stresses listed by Oliver above, oxygen concentration seems to be the only factor that could reasonably have influenced the attempts to isolate Epsilonproteobacteria during this study. Whether this was indeed the case during this survey is doubtful, because all samples were fresh and were processed without delay after collection. Thermophilic campylobacters occur as part of the normal intestinal flora in a variety of animals and birds (Astorga & Alonso, 2010). The prevalence of Epsilonproteobacteria reflected by the results of this survey appears to be an accurate representation of these bacteria in the slaughter-age ostriches that were

tested. To eliminate speculation, though, it may be advisable to replace the *C. jejuni* culture that was used as a control organism with (or use it in conjunction with) an Epsilonproteobacteria species that is more susceptible to unfavourable conditions.

Strategies to combat the spread of campylobacters in poultry flocks, apart from standard biosecurity principles, include limiting contact that the birds have with the environment, because campylobacters are common in wild animals and birds. Extensive ostrich production practices in South Africa preclude this possibility. Segregating positive and negative flocks of ostriches, a practice that has been attempted with success in poultry flocks (Silva et al., 2011) may also be unpractical in ostrich farming.



If the intestines of a carcass are ruptured during evisceration, subsequent contamination of succeeding carcasses on the abattoir slaughter line is probable. If there is no critical control point to counteract this contamination during meat processing, it seems likely that ostrich meat products in South Africa may indeed contain campylobacters. As faecal contamination of meat may occur during slaughtering, humans can become infected with campylobacters after consuming undercooked tainted meat (Astorga & Alonso, 2010). Rahimi and Tajbakhsh (2008) reported a prevalence of 11,7 % of thermotolerant campylobacters in ostrich meat samples investigated in Iran.

Freezing did not completely eliminate these organisms from chicken carcasses (Silva et al., 2011). The most effective way of eliminating campylobacters from

ostrich meat products rests on two practices in food preparation areas. Firstly, meat must be cooked properly at the correct temperatures for the correct times. Campylobacters are easily inactivated by heat treatments (Silva et al., 2011) so this should be easy to achieve. At 51 ºC, 90 % of *C. jejuni* present in ground chicken meat were destroyed in 9 minutes, while the same result was noted by heating at 57 ºC for 47 seconds (Silva et al., 2011). Secondly, cross-contamination of cooked meat from raw meat, food preparation surfaces and incompletely sanitized utensils should be strictly avoided.

In their summary of recent advances in CHRO research Gaynor and Szymanski noted an increasing appreciation for Epsilonproteobacteria other than *C. jejuni* and *H. pylori* (2012).

Molecular analysis of the *C. jejuni* subsp. *jejuni* isolates from this survey confirmed the phenotypical identifications performed, insomuch as the multiplex PCR used (Neubauer & Hess, 2006) allows. Bands of the correct fragment size were produced when a lower annealing temperature was used than was specified in the method. There appears to be scope for further optimization of this PCR, which could be a subject for further study.

A major disappointment encountered was failure to extract DNA from bacterial cultures frozen on microbeads that was detectable by this PCR. Further investigation is needed to establish possible reasons and solutions for this. Consideration could also be given to extending the scope of this survey to include more samples.

CHAPTER 7 **References**

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