Molecular detection and study of *Campylobacter* and related microorganisms

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor Philosophiae in the Department of Biotechnology, University of the Western Cape.

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November 2010

DECLARATION

I declare that "Molecular detection and study of *Campylobacter* and related microorganisms" is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Nisreen Hoosain November 2010

Signed:…….

ACKNOWLEDGEMENTS

First and foremost, I wish to thank Allah for giving me the strength, wisdom and guidance during my PhD and allowing me to obtain my PhD degree.

I would like to thank my supervisor, Prof. P.A. Gouws, and my co-supervisor, Prof. A.J. Lastovica for their support, guidance, encouragement and advice.

A special thanks to my colleagues in the laboratory as well as all staff in the Biotechnology Department at the University of the Western Cape. Thank you for your friendship, advice and making my stay an enjoyable one.

To my beloved parents, brother, sister in-law, niece, nephew and other family members: thank you for your prayers, love, understanding, encouragement and support through the years. It is greatly appreciated, I am truly blessed!

In addition, I would like to extend my sincere gratitude to my financial assistants: Department of Biotechnology at the University of the Western Cape, the National Research Foundation (NRF) of South Africa, Muslim Hands of South Africa and the South African National Zakah Fund (SANZAF).

CONTENTS

Chapter 1:

INTRODUCTION

Chapter 2 (Literature Review)

Identification of *Campylobacter* and related microorganisms in chicken and *in vitro* testing of strains

Chapter 3

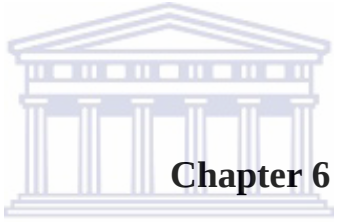
Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples

Chapter 4

Detection and isolation of *Campylobacteraceae* in retail and "Free-range" organic chicken

Chapter 5

In vitro Survival test of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures



Chapter 6

Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

Chapter 7: CONCLUSION AND FUTURE PROSPECTS

LIST OF FIGURES

CHAPTER 3:

Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples

CHAPTER 4:

Detection and isolation of *Campylobacteraceae* in retail and "Free-range" organic chicken

Chapter 6

Inhibitory effect of selected culinary products vs. antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

LIST OF TABLES

CHAPTER 4:

Detection and isolation of *Campylobacteraceae* in retail and "Free-range" organic chicken

CHAPTER 5:

In vitro Survival test of *Campylobacteraceae* and *Helicobacter* species in "free-range" organic chicken blood at various temperatures

Chapter 6

Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

LIST OF ABBREVIATIONS

AEB	*Arcobacter* enrichment broth
AFLP	Amplified fragment length polymorphism
AK	Amikacin
ARCOB1	forward *Arcobacter* primer
Aryl Sulf.	Aryl Sulfatase
blastn	Blast nucleotide search tool
Ca^{2+}	Calcium
CAMPCJL1	Forward *Campylobacter* primer
Cat.	Catalase
CIP	Ciprofloxacin
CN	Gentamicin
CO_2	Carbon dioxide
CTAB	Hexadecyltrimethyl ammonium bromide
DA	Clindamycin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetate
EtBr	Ethidium bromide
EtOH	Ethanol
gDNA	Genomic Deoxyribonucleic acid
H_2	Hydrogen
H_2S	Hydrogen sulphide

HELIP2	Forward *Helicobacter* primer
Hipp.	Hippurate
IAC	Internal amplification control
Ind.	Indoxyl acetate
IS	Intermediate Sensitivity
KF	Cephalothin
LMP	Low melting point
MEGA	Molecular evolutionary genetics analysis
MgCl	Magnesium chloride
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
N.R.	Nitrate Reductase
N/T	Not tested
NA	Nalidixic Acid
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
No.	number
O_2	Oxygen
Ox	Oxacillin
Oxid.	Oxidase
P	Penicillin G
PBS	Phosphate buffer saline

PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase chain reaction- restriction fragment length polymorphism
PFGE	Pulsed-field gel electrophoresis
Pyraz.	Pyrazinamide
R	Resistant
RFLP	Restriction fragment length polymorphism
rRNA	ribosomal Ribonucleic acid
RT	Room temperature
S	Sensitive
SDS	Sodium dodecyl sulphate
SXT	Sulphamethoxazole/Trimethoprim
TBA	Tryptose blood agar
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEC	Teicoplanin
TSI	Triple sugar iron
U.K.	United Kingdom
U.S.A.	United States of America
YT	Yeast Tryptone

LIST OF UNITS

%	percent
µg ml^{-1}	microgram per milliliter
µg	microgram
µg disc^{-1}	microgram per disc
µl	microliter
µm	micrometer
bp	base pairs
cfu ml^{-1}	colony forming units per milliliter
g	centrifugal field
h	hour(s)
mg ml^{-1}	milligrams per milliliter
min	minute(s)
ml	milliliter(s)
mmol l^{-1}	millimoles per liter
mol l^{-1}	moles per liter
mm	millimeter(s)
ng µl^{-1}	nanograms per microliter
ng ml^{-1}	nanograms per milliliter
nm	nanometers
ºC	Degrees Celsius
rev. min^{-1}	Revolutions per minute
U	Units

V	Volts
v/v	volume per volume
w/v	weight per volume
µmol l^{-1}	micromoles per liter

LIST OF PUBLICATIONS/CONFERENCE CONTRIBUTIONS EMANATING FROM THE WORK REPORTED IN THIS THESIS

A publication from each of the studies in the thesis is anticipated

PCR inhibitor removal poster presentation

Improved method for the efficient removal of PCR inhibitors and detection of *Arcobacter* DNA in chicken

Nisreen Hoosain and Pieter Gouws

Food Microbiology Research Group, Department of Biotechnology, Faculty of Science, University of the Western Cape, Bellville, Cape Town, Republic of South Africa

The poster was presented at one international scientific meeting where I was awarded first prize for the scientific poster presentation competition:

DIFSC 2009

4th Dubai International Food Safety Conference

Dubai

24-26 February 2009

Detection poster presentation

Detection poster presentation

Detection of three *Arcobacter* species from retail chicken and fresh blood samples by multiplex PCR after using an improved method for the removal of PCR inhibitors

Nisreen Hoosain, Albert Joseph Lastovica and Pieter Gouws

Department of Biotechnology, Faculty of Science, University of the Western Cape, Bellville, Cape Town, Republic of South Africa

The poster was presented at one international scientific meeting:

CHRO 2009

15th International Workshop on *Campylobacter*, *Helicobacter* and related organisms

Toki Messe, Niigata, Japan

2-5 September 2009

Spice poster presentation

Inhibitory effect of some spices, herbs, lemon juice, onion, honey and antibiograms against the growth of *Campylobacteraceae* and *Helicobacter* species

Nisreen Hoosain, Albert Lastovica and Pieter Gouws

Food Microbiology Research Group, Department of Biotechnology, Faculty of Science, University of the Western Cape, Bellville, Cape Town, Republic of South Africa

The poster was presented at one international scientific meeting and at the Departmental Open Day:

DIFSC 2010

5th Dubai International Food Safety Conference

Dubai

22-24 February 2010

U.W.C. DEPARTMENTAL OPEN DAY

First Annual Department of Biotechnology Open Day

Department of Biotechnolgy

University of the Western Cape

September 2010

ABSTRACT

Molecular detection and study of *Campylobacter* and related microorganisms

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PhD degree Thesis, Department of Biotechnology, University of the Western Cape

Species of *Campylobacter*, *Arcobacter* and *Helicobacter* have been associated with various diseases in humans and animals; and chickens have been identified as a reservoir of these microorganisms. Two published techniques and a new technique, developed in this dissertation, were evaluated to test its efficiency in removing PCR inhibitors from chicken samples. All of the techniques were based on agarose/DNA slants and were evaluated using multiplex PCR and an Internal Amplification Control. The new technique was found to be most effective and consequently used further in the study.

Three *Arcobacter* species were detected in novel sites of the chicken: *A. butzleri* in "mala" contents and blood; *A. cryaerophilus* in caeca; and *Arcobacter* sp. strain R-28314 in liver. One *A. butzleri* and two *C. jejuni* subsp. *jejuni* strains, identified by multiplex PCR and confirmed by sequencing of the 16S rRNA gene, were also isolated from novel sites: from chicken liver and chicken blood, respectively. All strains were isolated by filtration on Tryptose Blood Agar plates, pre-treated with *Arcobacter* Enrichment Broth. Amplified Fragment Length Polymorphism analysis displayed similar profiles for the *C. jejuni* subsp. *jejuni* biotype II reference strain and *C. jejuni* subsp. *jejuni* isolates. Amplified Fragment

Length Polymorphism profiles also displayed diversity among *A. butzleri*, *A. cryaerophilus* and *C. jejuni* subsp. *jejuni* strains.

A novel study was done to evaluate the survival of *Campylobacter*, *Arcobacter* and *Helicobacter* strains in chicken blood at -20, 4, 37 and 42ºC as well as at ambient room temperature (±22ºC). It was found that all strains could survive at all temperatures, albeit at different duration times. Most notably, an *A. butzleri* strain was able to survive at 4^{o}C for up to 297 days.

With increasing resistance and infection rates of *Campylobacter*, *Arcobacter* and *Helicobacter* alternative methods are being sought for treatment. Using a well-diffusion technique, several culinary products and antibiotics were tested against the growth of *Campylobacter*, *Arcobacter* and *Helicobacter* strains. Garlic, ginger, local honey, cinnamon, allspice and cloves displayed potent inhibitory effects against all strains. No antibiotic tested could demonstrate a similar effect. Through regular consumption of these culinary products, it may be possible to prevent and treat these infections in humans.

Keywords

Arcobacter, blood, *Campylobacter*, chicken, culinary products versus antibiotics, *Helicobacter*, identification, isolation, inhibition, survival

12 November 2010

INTRODUCTION

INTRODUCTION

Species of *Campylobacter*, *Arcobacter* and *Helicobacter* have been implicated in food related illnesses and are currently recognized as foodborne pathogens (Mansfield and Forsythe 2000; Ceelen *et al.* 2005; Engberg 2006). These microorganisms have been identified in chicken worldwide with high prevalence rates in some parts of the world (Friedman *et al.* 2000; Corry and Atabay 2001).

OUTLINE OF THESIS

This project is titled "Molecular detection and study of *Campylobacter* and related microorganisms". It is a broad topic which allows the incorporation of the following studies which would be explored in some detail below: 1) Evaluation of a new method to efficiently remove PCR inhibitors from poultry; 2) Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken; 3) *In vitro* survival testing of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures; and 4) Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species.

THE IMPORTANCE OF STUDIES INCLUDED

The first study is important as PCR inhibitors need to be removed before successful PCR can be performed. If these are not removed "false negative" results could

occur. Various PCR clean up strategies have been used but success seems to be sample dependant. The second study is important to determine whether *Campylobacter* and related microorganisms are present in retail "battery" and "free range"/organic chicken products and blood, with the latter being a novel part of the study. The third study is novel and important as it sheds light on the survival ability of various strains of different species of *Campylobacter*, *Arcobacter* and *Helicobacter* in chicken blood incubated at different temperatures. The last study is important as it focuses on determining the inhibitory effect of safer forms of spices and herbs (aqueous solutions thereof) on strains of *Campylobacter*, *Arcobacter* and *Helicobacter*, as opposed to using solvents such as ethanol; methanol; and chloroform to produce the spice or herb extracts.

REASON FOR CHOSEN STUDIES

A literature search suggested lack of identification of *Campylobacter*, *Arcobacter* and/or *Helicobacter* in retail chicken products and "free-range"/organic chicken and chicken blood in South African based studies. On that basis, it was decided to determine whether *Campylobacter*, *Arcobacter* and/or *Helicobacter* were present in retail chicken and "free-range"/organic chicken products; as well as in chicken and ostrich blood in Cape Town, South Africa. It was decided to incorporate the use of both PCR and culturing due to drawbacks inherent in both techniques. Some problems in culturing are that difficulties can arise due to the presence of contaminant microorganisms in the sample that can grow at a much faster rate thus masking the presence of target microorganisms (Pentimalli *et al.* 2009); or

misidentification of isolates by phenotypic and biochemical testing (Mateo *et al.* 2005). However, successful isolation of a strain can prove to be of great value, particularly if a new species is suspected or tests such as antibiotic testing are required. PCR targets DNA, therefore it cannot be used to determine if viable cells of a microorganism is present in a sample. One major problem of PCR is that it is sensitive to PCR inhibitors present in a sample (Rådström *et al.* 2004). The use of an Internal Amplification Control in PCR is imperative to avoid "false-negative" results and to illustrate that optimal PCR conditions are present in every tube (Lübeck *et al.* 2003; Josefsen *et al.* 2004). After the removal of PCR inhibitors, PCR and sequencing analysis can successfully be used in tandem to confirm target microorganisms in a sample. Typing methods such as amplified fragment length polymorphism fingerprinting can be used to determine genetic relatedness or diversity among target strains (Siemer *et al.* 2004).

A more thorough literature search indicated that some studies have been done to determine the survival capacity of species of *Campylobacter*, *Arcobacter* and *Helicobacter* in food, water and media (Valdés-Dapena and Adám 1983; Wesley 1997; Gomes and De Martinis 2004; Saumya and Cottrell 2004). It appears that the research is mainly based on *C. jejuni*, *A. butzleri*, *H. pylori* and some on *C. coli*. We detected no study based on the survival of several strains of different species of *Campylobacter*; *Arcobacter*; and *Helicobacter* in chicken blood incubated at various temperatures.

Although, there has been an increasing number of studies dealing with the antimicrobial effect of plant matter on strains of *Campylobacter*, *Arcobacter* and

Helicobacter they have mainly been focused on different extracts of herbs and spices, different types of teas and they only seem to focus on one of the genera described above (Smith-Palmer *et al.* 1998; Stamatis *et al.* 2003; Cervenka *et al.* 2006). Our study, however, was done to determine the effect various raw and aqueous culinary products have on several strains of different species of *Campylobacter*, *Arcobacter* and *Helicobacter* and comparing the results to the effect of selected antibiotics. All of these studies described above are outlined below.

AIMS OF CHAPTERS

The primary aim of the first study: "Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples" was to evaluate the new method, developed to remove PCR inhibitors from chicken products and poultry blood, against two published methods (Moreira 1998; Monteiro *et al.* 2001) using a competitive internal amplification control. The primary aim of the second study: "Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken" was to detect and/or isolate strains of *Campylobacter* and related microorganisms in retail chicken products and poultry blood. The primary aim of the third study: "*In vitro* survival testing of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures" was to determine how long strains of different species of *Campylobacter*, *Arcobacter* and *Helicobacter* could survive in chicken blood incubated at different temperatures. The primary aim of the fourth study: "Inhibitory effect of selected culinary products

versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species" was to determine the effect safer forms (heated aqueous solutions) of household spices, herbs and other culinary products (obtained from retail stores and spice shops) would have on strains of *Campylobacter*, *Arcobacter* and *Helicobacter* and compare it to the effect of selected antibiotics.

FRAMEWORK OF CHAPTERS

The thesis follows the format of the Journal of Applied Microbiology. The thesis includes the following studies: 1) Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples; 2) Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken; 3) *In vitro* survival testing of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures; and 4) Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species.

RESEARCH DESIGN OF STUDIES

In the first study, a competitive internal amplification control (IAC) would be constructed, based on the Denis *et al.* (1999) strategy, by incorporating the target fragment into a vector. A positive clone, confirmed by sequencing, would be used to determine its minimum inhibitory concentration and used as the competitive IAC to confirm the removal of PCR inhibitors in the retail chicken products and blood

samples. Thereafter, the IAC would be used to evaluate the new and published methods using spiked and unspiked chicken products and blood.

In the second study, culturing would be performed in an effort to isolate viable strains of *Campylobacter* and related microorganisms present in a sample. The IAC would be used in the multiplex PCR to detect presumptive strains present in a sample; while 16S rRNA sequencing would be used to confirm the presence of target microorganisms. Thereafter, PCR positive samples and isolates would be subjected to AFLP to determine genetic relatedness or diversity among strains with reference to reference strains.

In the third study, test strains would be cultured and confirmed to be a pure culture using phenotypic and biochemical tests. Thereafter, strains would be inoculated in chicken blood, in duplicate, and incubated at -20, 4, 37 and 42ºC as well as at ambient room temperature (±22ºC). Blood inoculums would be tested twice or thrice a week by filtration and streaking onto nonselective tryptose blood agar (TBA) plates, incubated overnight at 37ºC and confirming the survival of strains using Gram staining and other biochemical tests.

In the fourth study, test strains would be cultured and confirmed to be a pure culture using phenotypic and biochemical tests. Thereafter, standard suspensions of strains would be prepared; aliquots spread onto nonselective TBA plates and allowed to dry in the laminar low. During this time tested culinary samples would be prepared. This would be followed by making a maximum of four wells into the inoculated plates and dispensing the tested samples into the wells. For antibiotic testing, strains would be heavily streaked onto nonselective TBA plates followed by

placing a maximum of four tested antibiotics on the surfaces of streaked TBA plates. All plates would be incubated overnight at 37ºC and the results recorded in the morning.

CONCLUSIONS

The chapters of the thesis have been placed in the following order: Chapter 1: Introduction followed by Chapter 2: Literature review: "Identification of *Campylobacter* and related microorganisms in chicken and *in vitro* testing of strains". The review was constructed around three main focal points. The first part of the review focuses on culturing, PCR and typing techniques used for *Campylobacter*, *Arcobacter* and *Helicobacter*; the second part on the survival of these microorganisms in various food sources; and the final part on the treatment options used in *in vitro* and *in vivo* studies. Chapter 3: "Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples". This chapter would primarily be used to demonstrate which method best suites the removal of PCR inhibitors from poultry samples using the competitive IAC. Chapter 4: "*In vitro* survival testing of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures". This chapter is a novel study discussing the survival capacity of strains of *Campylobacter*, *Arcobacter* and *Helicobacter* in chicken blood stored at different temperatures. Chapter 5: "Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species". In this chapter, the inhibitory effect results of safer culinary products tested in this study against strains of

Campylobacter, *Arcobacter* and *Helicobacter* would be compared to the results of methanol, ethanol, chloroform and other solvent extracts of spices and herbs used in published studies. The data of the tested antibiotics against the growth of *Campylobacter*, *Arcobacter* and *Helicobacter* strains would be compared to published data. In addition, a note would be made on the inhibitory effects of the tested culinary products versus that of selected antibiotics.

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LITERATURE REVIEW

Identification of *Campylobacter* and related microorganisms in chicken and *in vitro* testing of strains

SUMMARY

Species of *Campylobacter*, *Arcobacter* and *Helicobacter* have routinely been isolated from a range of food sources, including poultry, beef and milk; and are associated with foodborne illnesses. These microorganisms have also been isolated from fresh, salt and ground water and pets such as cats, dogs and monkeys. The first part of the review focuses on culturing, PCR and typing techniques used for *Campylobacter*, *Arcobacter* and *Helicobacter*; the second part on the survival of these microorganisms in various food sources; and the final part on the treatment options used in *in vitro* and *in vivo* studies.

INTRODUCTION

Campylobacter, *Arcobacter* and *Helicobacter* are Gram-negative and spiral or rod-shaped (Lastovica 2006). *Campylobacter* and *Helicobacter* are microaerophilic microorganisms, requiring O_2 levels in the range of 2-10% for growth as their cells are damaged by normal atmospheric levels of 20% (Prescott *et al.* 1996). *Arcobacter* species are aerotolerant microorganisms capable of growing at normal atmospheric conditions and at lower temperatures than *Campylobacter* (Vandamme *et al.* 1991). *Campylobacter* and *Arcobacter* are collectively placed within the *Campylobacteraceae* family due to similar phylogenetic and phenotypic

characteristics (Vandamme and De Ley 1991). The genera of *Campylobacter* and *Arcobacter*, together with *Helicobacter* and *Wolinella* form a distinct group within the epsilon sub-division of *Proteobacteria* (Vandamme *et al.* 1991).

HISTORY

Campylobacter were the first microorganisms identified from the epsilon sub-division of *Proteobacteria*. It appears that humans have been suffering from *Campylobacter* infections for many years prior to the classification of the genus. Symptoms of *Campylobacter* infections were first described by Theodor Escherich as early as 1886 in the colons of infant patients who died as a result of infections he named "cholera infantum" (reviewed by Engberg 2006). He also identified these spiral-shaped microorganisms in stool specimens from neonates and kittens experiencing diarrhea, in the same year, but was unable to culture them on solid media (reviewed by Engberg 2006). *Campylobacter* was first isolated but misidentified as "*Vibrio*" species by McFaydean and Stockman in 1909 from the placentas and aborted fetuses of cattle (Todd 2002; Engberg 2006). In 1919, Smith and Taylor proposed the name *Vibrio fetus* for microorganisms isolated from "*Vibrio*"-related abortion specimens of cattle. Closely related microorganisms were later isolated from cattle and pigs and described as *V. jejuni* and *V. coli*, respectively (Jones *et al.* 1931; Doyle 1944). *V. fetus* and *V. jejuni* were first isolated from human blood cultures in the 1950s (King 1962 reviewed in Tauxe *et al.* 1988). The genus of *Campylobacter* was first proposed in 1963 by Sebald and Véron. In 1970, Bokkenheuser *et al.* identified 10 cases where *V. fetus* that had been isolated from

blood, cerebrospinal fluid, liver and abscess specimens of patients. Most of the patients with these infections were known to be suffering from underlying chronic illnesses and as a result *V. fetus* was considered an opportunistic pathogen (Wyatt *et al.* 1977; Schmidt *et al.* 1980; Kanj *et al.* 2001). The DNA composition of *V. fetus* was very different to other *Vibrio* species and on that basis Sebald and Véron proposed a new genus, *Campylobacter*, in 1972. Shortly thereafter, Dekeyser *et al.* (1972) and Butzler *et al.* (1973) isolated *C. jejuni* in Belgium from diarrhoeal stool samples. Ten years after their initial proposition, Véron and Chatelain classified *Campylobacter* into four distinct species: *C. coli*; *C. fetus*; *C. jejuni* and *C. sputorum* based on a comprehensive taxonomy study on microaerophilic *Vibrio*-like microorganisms (Véron and Chatelain 1973). The development of new selective culture media (Skirrow 1977; Blaser *et al.* 1979) allowed many routine laboratories to isolate *Campylobacter* from stool samples. This paved the way for many studies to determine the clinical role of *Campylobacter* in humans and animals. *Campylobacter* is recognized as a common cause of diarrhoael illnesses and gastroenteritis, worldwide (Friedman *et al.* 2000; Frost 2001; Engberg 2006). There are currently 22 validated species and eight defined subspecies of *Campylobacter* (Paulin and On 2010).

Aerotolerant *Campylobacter*-like microorganisms were first isolated from the aborted fetuses of cows and pigs dating back as early as the late 1970s (Ellis *et al.* 1977; Ellis *et al.* 1978). The name *Campylobacter cryaerophila* was only proposed in 1985 on the basis of it being aerotolerant and able to grow at 25^{o}C (Neill *et al.* 1985). In 1980 free-living nitrogen-fixing *Campylobacter* species were

found to be associated with the roots of *Spartina alterniflora* Loisel, a salt marsh plant, but was only formerly named as *C. nitrofigilis* in 1983 (McClung *et al.* 1980; McClung *et al.* 1983). Kiehlbauch *et al.* (1991a) were the first to isolate *C. butzleri* from humans and animals suffering from diarrheal-related illnesses. In addition, this study also identified two distinct groups of *C. cryaerophilia*, IA and IB, on the basis of extensive DNA homology studies. However, a more recent study proposed the loss of the groups IA and IB based on data obtained from amplified fragment length polymorphism (AFLP) and gene sequencing of the heat shock protein HSP60 (Debruyne *et al.* 2010). By analyzing 101 strains of *Arcobacter*, including 59 of *A. cryaerophilus*, they noted that *A. cryaerophilus* had a complex and diverse taxonomic structure among its strains. They also identified multiple cores of strains within *A. cryaerophilus* sharing intermediate levels of DNA-DNA hybridization but low levels of DNA-DNA hybridization with other species of *Arcobacter*. In addition, it was found that the type strain was genetically different from most of the *A. cryaerophilus* strains tested and thus seen as inappropriate for comparison purposes (Debruyne *et al.* 2010). In 1991, the genus *Arcobacter* was proposed to include four aerotolerant microorganisms: *A. cryaerophilus*, *A. nitrofigilis*, *A. butzleri* and *A. skirrowii* (Vandamme *et al.* 1991; Vandamme *et al.* 1992a). There are currently 10 described species of *Arcobacter*, namely, *A. butzleri*, *A. skirrowii, A. cryaerophilus, A. thereius*, *A. cibarius*, *A. mytili*, *A. nitrofigilis*, *A. halophilus*, *A. marinus* and *A. trophiarum* surviving in diverse ecological niches including warm-blooded animals, oil fields and aquatic environments (Wesley and Miller 2010). Seven of these species: *A. butzleri*, *A. skirrowii, A. cryaerophilus, A. thereius*, *A.*

cibarius, *A. mytili* and *A. trophiarum* have been implicated in human illness and/or isolated from food or food animals (reviewed by Wesley and Miller 2010).

In 1982, spiral-shaped microorganisms were cultivated from human gastric biopsy specimens for the first time and named *Campylobacter pyloridis* (Marshall and Warren 1984). However, subsequent 16S rRNA gene sequence analysis revealed that there were enough differences between known *Campylobacter* species and *C. pyloridis* to exclude it from the *Campylobacter* genus (Romaniuk *et al.* 1987) and placed it into a new related genus, *Helicobacter* (Goodwin *et al.* 1989). Although this bacterium was only cultured in 1982; Rappin documented, in his thesis in 1881, that he observed spiral-shaped microorganisms in gastric biopsy specimens of dogs (Rappin 1881 reviewed in Solnick and Schauer 2001). Manifestations of these microorganisms were later documented by two independent scientists, Bizzozero and Salomon in 1892 and 1896, respectively. The cultivation of *H. pylori* and recognition of its disease potential has sparked a renewed interest in microorganisms associated with the gastrointestinal and hepatobiliary tracts of humans and animals (Solnick and Schauer 2001). In the process many novel species of *Helicobacter* have been identified. The *Helicobacter* genus has expanded at an explosive rate since 1982, currently consisting of over 50 species as well as numerous candidate or unclassified species isolated from various vertebrate hosts (Solnick and Vandamme 2001 reviewed in O'Toole *et al.* 2010).

The taxonomic diversity of the epsilon division of the *Proteobacteria* group is matched by the diverse ecological niches in which the microorganisms survive, as well as the broad spectrum of disease it is associated with (On 2001).

BRIEF DISEASE SPECTRUM

Campylobacter jejuni, the most widely studied *Campylobacter* species, is recognized as one of the major causes of gastroenteritis and diarrhoea in humans and animals (Griffiths and Park 1990; Allos 2001; Lastovica 2006). It has also been associated with septicemia, meningitis, the Guillain-Barré syndrome and reactive arthritis (Lastovica 2006). Some *Arcobacter* species have been associated with enteritis, bacteraemia and chronic diarrhoea (Vandamme *et al.* 1992a; Vandamme *et al.* 1992b; Lastovica and Skirrow 2000; Wybo *et al.* 2004). There are over 50 species of *Helicobacter* (Solnick and Vandamme 2001 reviewed in O'Toole *et al.* 2010), most of which have been identified in human and animal digestive tracts and thought to be associated with human and animal disease of gastric, enteric or systemic origin (Totten *et al.* 1985; Parsonnet *et al.* 1994; Fox *et al.* 1998; De Groote *et al.* 1999; Tee *et al.* 2001; Gueneau and Loiseaux-De Goër 2002). Species of *Campylobacteraceae* and *Helicobacter* have been identified as potential foodborne and waterborne pathogens as well as zoonotic agents (Newell and Fearnley 2003; Fox 2002; Solnick 2003; Diergaardt *et al.* 2004; Houf *et al.* 2005; Ho *et al.* 2006).

This review primarily focuses on chicken as a source of infection. *Campylobacter* is known to form part of the normal enteric flora of poultry and may be transmitted to humans through the consumption and handling of contaminated food (Stern *et al.* 2003; Gallay *et al.* 2007). The handling and consumption of undercooked poultry meat and cross contamination of other foods such as salads by raw chicken are reported to be the primary sources of human *Campylobacter*

infections in industrialized countries such as the U.S.A (Friedman *et al.* 2000). Some *Arcobacter* infections are thought to be transmitted in a similar manner (Corry and Atabay 2001; Phillips 2001) as these microorganisms have mainly been recovered from poultry meat (Atabay and Corry 1997; Atabay *et al.* 1998a; Wesley and Baetz 1999; Houf *et al.* 2005). A species of *Helicobacter* (*H. pullorum*) with similar virulence properties has been associated with similar diseases in poultry and humans and thus considered a foodborne pathogen (Burnens *et al.* 1994; Stanley *et al.* 1994; Burnens *et al.* 1996; Steinbrueckner *et al.* 1997; Young *et al.* 2002; Ceelen *et al.* 2005; Ceelen *et al.* 2006; Zanoni *et al.* 2007).

ISOLATION

Different techniques have been developed over the years in an attempt to isolate *Campylobacter*, *Arcobacter* and/or *Helicobacter* from chickens but standardized methods have yet to be established as results vary from study to study. In 1984, Steele and McDermott developed a novel filtration technique allowing *C. jejuni* to be isolated from faecal samples. The pore size of the cellulose triacetate membrane filters was found to prevent certain microorganisms from passing through and as a result was found to be as effective as antibiotic media (Steele and McDermott 1984). An enrichment step is generally required when analyzing food samples (Humphrey *et al.* 2007). The type of enrichment broth used in isolation protocols has a direct effect on the rate of recovery of the microorganism (Baylis *et al.* 2000), while the sampling method has an effect on the isolation of the microorganism (Jørgensen *et al.* 2002).

H. pullorum was isolated for the first time using selective medium containing sheep blood and a cocktail of antibiotics (Stanley *et al.* 1994; Burnens *et al.* 1996). The purpose of adding antibiotic supplements to the agar is to restrict the growth of contaminant or unwanted microorganisms which may be present in large numbers in a sample (Wesley *et al.* 1983). Various enrichment methods were developed over the years in an attempt to successfully isolate *Campylobacter* and related microorganisms from chicken. These included the use of several media supplemented with antibiotics (Wempe *et al.* 1983; Wesley *et al.* 1983; Atabay and Corry 1998; González *et al.* 2000; Houf *et al.* 2001; Meeyam *et al.* 2004; Rahim *et al.* 2010); incubation of plates with an oxyrase enzyme under normal atmospheric conditions (Wonglumson *et al.* 2001); or the inclusion of blood, as a supplement (Williams *et al.* 2009). In 1999, Johnson and Murano developed solid chromogenic media consisting of a basal nutrient mix with 0.05% thioglycolic acid; 0.05% sodium pyruvate; and 5% sheep's blood (pH 6.9+/-0.2). The media demonstrated superior growth characteristics for *A. butzleri*, *A. cryaerophilus* and *A. nitrofigilis* through the formation of deep red zones around the respective colonies. Although the use of blood, as a supplement, is generally not essential in isolating *Campylobacter* and related microorganisms, it may depend on the sample and enrichment method used (Williams *et al.* 2009).

The use of antibiotics in selective media should be treated with caution as *H. pullorum*, for example, has been shown to be sensitive to polymyxin B (Atabay *et al.* 1998b). As a result, *H. pullorum* is unable to grow on selective media such as Blaser-Wang, Campy-BAP, Exeter, Skirrow and Preston and modified cefoperazone

charcoal deoxycholate agar media (Corry *et al.* 1995; Atabay *et al.* 1998b). Due to the sensitivity of some strains to antibiotics it is best to attempt to isolate microorganisms on nonselective blood agar. This is in agreement with the Atabay *et al.* (1998b) study where all of the *H. pullorum* strains were isolated on nonselective blood agar using the basic filtration technique of Steele and McDermott.

Different techniques are often used to isolate the same microorganisms from different samples. In the Atabay *et al.* (1998b) study, *H. pullorum* strains were isolated from carcass washings using the direct filtration technique without enrichment; however *H. pullorum* strains isolated from the caeca were obtained through direct filtration and after enrichment. As the carcass washing samples were diluted by the enrichment broth, it may suggest that *H. pullorum* were too few to pass through the filter. At least 900 colony forming units (cfu) are required for one colony to be detected by filtration (personal communication Prof. A. J. Lastovica). A recent study has shown that *Campylobacter* species were best isolated from commercial broiler meat using a 1:4 enrichment ratio with 25 g of sample (Oyarzabal and Liu 2010). The caeca generally aids in the absorption of water and proteins as well as the decomposition of fiber by various microorganisms (Anonymous 2010). As a result, an enrichment step would be ideal to separate microorganisms form other inhibitory components such as proteins in the caeca. In 2006 and 2007 two independent research groups used the same technique to isolate *H. pullorum* from similar samples with different results (Ceelen *et al.* 2006; Zanoni *et al.* 2007). In their studies, homogeneous caecal contents samples were diluted in a horse-serum mixture to allow an even distribution of the inoculum onto the

membrane filters and to reduce swarming of *Campylobacter* spp. on the agar. Samples were evenly spread onto the membrane filters which adhered to the agar surfaces and allowed to filter at 37^{o}C under microaerobic conditions for 1h. Thereafter, filtrates were streaked and plates were reincubated under the same conditions. Zanoni *et al.* (2007) speculated that the higher prevalence obtained in their study could have been due to the use of a bigger pore size membrane filter but could not prove this. Although this is a valid point it may simply be that *H. pullorum* were present in greater numbers in the Zanoni *et al.* (2007) study; or that the microorganisms were present in most but not all of the chickens tested by Ceelen *et al.* (2006). Another possibility is that *H. pullorum* could have agglutinated on the membrane filter resulting in a lower filtration rate. Under certain conditions, *Campylobacter* can agglutinate which could influence the filtration rate (personal communication Prof. A.J. Lastovica). This suggests a similar phenomenon in the Ceelen *et al.* (2006) study upon isolation of *H. pullorum*. Furthermore, geographical variation of the strains; differences in environmental conditions; as well as filtration time could also have played a key role in the isolation process.

Some research groups isolated *Campylobacter* and related microorganisms with great success using only the enrichment and membrane filtration techniques (Lammerding *et al.* 1996; Gude *et al.* 2005); while others followed this up with streaking (Wesley *et al.* 1983; Ceelen *et al.* 2006; Zanoni *et al.* 2007; Kabeya *et al.* 2004) or spreading (Steele and McDermott 1984) the filtrates across the agar surfaces. In some cases samples such as cloacal swabs or blood could directly be spread or squirted onto nonselective tryptose blood agar (Lastovica 2006). The

spreading technique should be used with caution when testing certain samples such as blood as it may result in the shearing of cells of target microorganisms (personal communication Prof. A.J. Lastovica). Conventional standardized isolation procedures are often time consuming with limited results (Kiehlbauch *et al.* 1991b; Corry *et al.* 1995; Mateo *et al.* 2005). The polymerase chain reaction (PCR) is specific, sensitive, reliable and rapid and it is widely used to detect target microorganisms in various samples (Hill 1996). Although live and dead microorganisms are likely to be present in some samples such as environmental and food; only live microorganisms can be detected through culture. PCR targets the DNA of microorganisms and as a result it can detect live and dead microorganisms (Humphrey *et al.* 2007). However in some cases particularly when a new species is speculated, it is essential to isolate the microorganism to do further analysis. Thus it is advisable to use PCR concurrently with isolation procedures to correctly determine the species of microorganism present in a sample.

DETECTION

PCR detection methods are directed to target regions of a microorganisms' genome, amplified and visualized after electrophoresis (Humphrey *et al.* 2007).

Genus-specific and species-specific PCR

In genus specific PCR the highly conserved region of the 16S rRNA region is often the target for amplification; while specific loci target sites are used in species specific PCR (Humphrey *et al.* 2007). Mateo *et al.* (2005) were able to detect the

presence of *C. jejuni* and *C. coli* with and without enrichment, based on the amplification of genes encoding an outer membrane protein (*mapA*) (Stucki *et al.* 1995) and putative virulence factor (*ceuE*) (Gonzalez *et al.* 1997), respectively. A range of other target sites, including the genes: benzylglycine amidohydrolase (hippuricase)/ (*hip*) (Linton *et al.* 1997; Bang *et al.* 2002); flagellin (*flaA*) (Oyofo *et al.* 1992; Alm *et al.* 1993); *gyrA* (Adelbaqi *et al.* 2007; Brightwell *et al.* 2007) have been used to identify strains of *Campylobacter* and *Arcobacter*. An Austrian research group used variable regions of the 16S rDNA of *Campylobacter* as target sites to identify diversity among different species (Gorkiewicz *et al.* 2003). Although Gorkiewicz *et al.* (2003) could successfully identify most *Campylobacter* species with this technique; a lack of discrimination among *C. jejuni*, *C. coli* and some *C. lari* strains was noted making the identification of some species more challenging or impossible. Furthermore, this technique is very time consuming and can be problematic as it is dependant on correctly cloned target fragments which has to follow a specific order; PCR of clones; sequence analysis; sequence alignments; and the construction of phylogenetic trees. The variable 23S rDNA region has also been shown to be good targets for species differentiation among *Campylobacter* (Eyers *et al.* 1993) and *Arcobacter* (Bastyns *et al.* 1995; Kabeya *et al.* 2003) strains.

As the numbers of viable *Campylobacter* and related microorganisms are often too low to allow successful isolation and/or detection in food samples, an enrichment step is often required. Although techniques vary from study to study, many demonstrate increasing success in the isolation and/or detection of *Campylobacter*, *Arcobacter* and *Helicobacter* in poultry by combining PCR with

culture enrichment (González *et al.* 2000; Bolton *et al.* 2002; Vytrasová *et al.* 2003; Paulsen *et al.* 2005; González *et al.* 2008; Katzav *et al.* 2008; Pentimalli *et al.* 2009).

Multiplex PCR

Co-infection of species of *Campylobacter* and *Arcobacter*, in a single poultry sample, has been reported (Bolton *et al.* 2002; Pentimalli *et al.* 2009). The discrimination between two or more species by PCR previously required two or more individual DNA amplifications and agarose gel electrophoresis per sample (Denis *et al.* 1999). As a result multiplex PCR was developed to allow simultaneous detection of different species of *Campylobacter* (Harmon *et al.* 1997; Denis *et al.* 1999; Al Amri *et al.* 2007) or *Arcobacter* (Houf *et al.* 2000; Kabeya *et al.* 2003) in poultry. Denis *et al.* (1999) used genus-specific 16S rRNA, *mapA* gene and *ceuE* gene primer sets to confirm isolation of *Campylobacter* species, *C. jejuni* and *C. coli*, respectively, from chicken faecal samples after enrichment and streaking on selective media. The reliability of the assay was demonstrated against a total of 294 *Campylobacter* isolates of which 220 were confirmed as *C. jejuni* and 74 as *C. coli*. The presence of a *Campylobacter* species was identified by a 857 bp fragment; while the 589 and 462 bp fragments indicated the presence *C. jejuni* and *C. coli*, respectively. They found that their multiplex PCR assay was 100% efficient in identifying *Campylobacter* species as opposed to 34% by biochemical testing. In another study Pentimalli *et al.* (2009) designed three primer sets for the detection of *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*; while the *A. butzleri* specific primer

set was designed by flanking a 203 bp DNA fragment in the 16S rRNA gene. The primer sets were evaluated against 18 h enriched cultures of retail chicken meat. They found that *Arcobacter* was present in 85.7% of the 42 fresh retail samples tested. Furthermore, they detected *A. butzleri* in 50% of the samples; noted that 35.7% of the samples were coinfected with *A. butzleri* and *A. cryaerophilus*; while *A. skirrowii* and *A. cibarius* were not detected in any sample. *Helicobacter pullorum* is the only species of *Helicobacter* that has been identified in naturally contaminated poultry produce therefore there is no need as yet to develop multiplex PCR for the detection of *Helicobacter* in poultry. Multiplex PCR was further developed to simultaneously detect *Campylobacter jejuni* and *Arcobacter butzleri* in a range of spiked dairy, raw and ready-to-eat products (Winters and Slavik 2000). Amplification of a 159 bp or 1223 bp product indicated the presence of *C. jejuni* or *A. butzleri*, respectively; while both bands were amplified when both microorganisms were present (Winters and Slavik 2000). Their PCR corresponded to results obtained by standard microbiological techniques and thus reduced the detection time for these microorgainsms. In 2006, Neubauer and Hess further developed a multiplex PCR, based on the 16S rRNA gene, to identify different species of *Campylobacter*, *Arcobacter* and *Helicobacter*. The multiplex primer set consisted of three individual primers specific for *Campylobacter*, *Arcobacter* and *Helicobacter* and a common reverse primer. After growing strains, previously isolated from various sources, on blood agar plates they extracted DNA using a kit and performed multiplex PCR on all DNA samples. They were able to differentiate

between the presence of *Campylobacter*, *Arcobacter* and *Helicobacter* by amplification of a 946, 822 or 1107 bp fragment, respectively.

Real-time PCR

The detection of conventional PCR products may often be a daunting task to inexperienced technical staff due to inexperience in preventing cross contamination of amplified DNA (Humphrey *et al.* 2007). This resulted in the development of several realtime (RT) PCR assays (Lund *et al.* 2004). This technique allows the multiplication of DNA fragments to be followed through the development of a rising curve (Sails *et al.* 2003). Ridley *et al.* (2008) developed fluorescently labeled PCR oligonucleotide probes for a realtime PCR assay targeting a highly diverse DNA segment within the *flaA* short variable region to detect *C. jejuni* in broiler flocks and their environment after enrichment. This method allowed them to detect a strain of *C. jejuni* from the environment which was later found to colonize a broiler flock. Using the multiplex realtime PCR approach other groups were able to ascertain the prevalence of *Campylobacter* and *Helicobacter* in poultry and other food products (González *et al.* 2008; Bonjoch *et al.* 2010; Mayr *et al.* 2010; Schnider *et al.* 2010). Rudi *et al.* (2004) made use of paramagnetic beads to quantitatively detect, by realtime PCR, the presence of *C. jejuni* in chicken caeca and faeces without enrichment. Their data indicated that the levels of *C. jejuni* were similar within but varied between flocks. The authors suggested that it may indicate different stages of colonization with *C. jejuni* or indicate that some flocks were colonized with highly virulent strains of *C. jejuni*. However, this could not be

determined in their study as additional techniques such as pulsed field gel electrophoresis or amplified fragment length polymorphism fingerprinting was not performed on any of the strains. The use of fluorescently labeled probes or paramagnetic beads may be very time consuming and costly therefore other groups have used an alternative method. Research groups have incorporated a double-stranded DNA binding dye, SYBR Green I, to successfully detect *C. jejuni* (Oliveira *et al.* 2005; Rantsiou *et al.* 2010) and *A. butzleri* (González *et al.* 2010) in naturally contaminated poultry products after enrichment. Using this dye classic PCR protocols can be used with slight modifications (Egygor *et al.* 2002), while specificity is determined by the amplicons melting temperature, i.e. temperature at which 50% of the amplicon is in the double stranded form (De Medici *et al.* 2003). RNA has also recently been used to detect *Campylobacter* in retail chicken products. Churruca *et al.* (2007) used a nucleic acid sequence based amplification (NASBA) assay based on molecular beacons. Molecular beacons are small, single stranded nucleic acid, hairpin probes which fluoresce upon binding to the target sites (Tyagi and Kramer 1996). NASBA is a single step PCR assay in which single stranded RNA sequences are targeted by the simultaneous use of three enzymes (avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase) generating a single stranded antisense RNA amplicon at 41^{o}C (Kievits *et al.* 1991 reviewed in Churruca *et al.* 2007). Each NASBA primer set was designed to target a region within the 16S rRNA of either *C. jejuni* or *C. coli* and each probe, in turn, targeting the central region of the *C. jejuni* or *C. coli* NASBA amplicon (Churruca *et al.* 2007). Samples were spiked using three types of cell

suspensions: culturable cells from a 24 h culture; viable but non culturable cells from suspensions stored at 4^{o}C; and autoclaved cells. Five gram chicken breast samples, previously determined to be *Campylobacter* free, were submerged in 20 ml of *C. jejuni* suspension for 10 min and drained on sterile filter pads. RNA was extracted with a kit and used for analysis. Using the realtime NASBA assay, they noted that the presence of rRNA correlated with cell viability even after heat inactivation but not in RNase free cell suspensions. This suggests the efficiency of this realtime NASBA assay as opposed to conventional culturing methods to detect cell viability of *C. jejuni* and *C. coli* in chicken meat even if it is in the viable but non culturable state.

Polymerase Chain Reaction Enzyme Linked Immunosorbent Assay (PCR-ELISA)

Campylobacter, *Arcobacter* and *Helicobacter* elicit antibody responses in the host and these proteins are often used to rapidly detect the presence of the pathogen in a sample (Humphrey *et al.* 2007). Several studies have used a PCR assay based on a hybridization solution which allows for the detection of target microorganisms by a colourimetric reaction (Bolton *et al.* 2002). Using the 16S/23S rRNA intergenic spacer region of *Campylobacter* with DNA oligonucleotide probes as the target, O'Sullivan *et al.* (2000) and Grennan *et al.* (2001) successfully identified *C. jejuni* and *C. coli* in poultry samples after culture enrichment by PCR-ELISA. The sensitivity, reproducibility and rapid ability of PCR-ELISA technique was further demonstrated using raw meat, offal, raw shellfish and artificially contaminated milk

samples (Bolton *et al.* 2002). Hong *et al.* (2003) stumbled upon an additional advantage of PCR-ELISA, the ability of determining the amount of *Campylobacter* present in carcass rinse samples. An increasing number of *Campylobacter* cells in culture correlated with increasing ELISA absorbance (Hong *et al.* 2003). However, as this technique is a PCR based assay it was impossible to determine whether *Campylobacter* present were all dead, alive or a combination of both thus limiting its application in industry.

DNA oligonucleotide array and Loop mediated isothermal Amplification (LAMP) assays

With the development of more sophisticated techniques over recent years, efficient detection of target microorganisms can be done within 12-48 h (Paulsen *et al.* 2005; Quiñones *et al.* 2007; Williams *et al.* 2008; Liu *et al.* 2009). Quiñones *et al.* (2007) produced DNA oligonucleotide arrays to simultaneously detect *Arcobacter* and *Campylobacter* in retail chicken samples. They selected probes specifically targeting housekeeping and virulence associated genes in *A. butzleri*, *C. jejuni* and *C. coli* and the lipooligosaccharide (LOS) membrane protein. Using *C. jejuni* specific primers to label genomic DNA, Quiñones *et al.* (2007) also managed to improve the sensitivity of DNA microarray detection for *C. jejuni* from package liquid of whole chicken carcasses or enrichment broths. Yamazaki *et al.* (2009) developed a loop mediated isothermal amplification (LAMP) and evaluated it against conventional culture methods to detect *C. jejuni* and *C. coli* in naturally contaminated chicken meat after enrichment. The LAMP assay requires fewer

preparation steps and is quicker and more cost effective to perform than other PCR assays. The amount of DNA synthesized is correlated with the amount of by product, an insoluble white precipitate of magnesium pyrophosphate, produced making detection by simple turbidity analysis possible (Yamazaki *et al.* 2009). Using one set each of the outer, inner and loop primers the LAMP assay targets two sequences which either includes an oxidoreductase or a *gufA* gene of *C. jejuni* and *C. coli*, respectively (reviewed in Yamazaki *et al.* 2009). Samples were incubated in Preston broth containing blood, mixed, incubated microaerobically at 42^{o}C for 22-24 h and streaked onto selective media. DNA was extracted from enrichment broth using a three step centrifugation protocol to remove: large debris from chicken samples; inhibitors within the Preston broth; and to concentrate the number of *Campylobacter* cells in a sample. The efficiency of the technique was determined by analyzing 144 chicken meat samples. Compared to conventional culturing methods, the LAMP assay displayed sensitivity and specificity rates of 98.5% (67/68 samples) and 97.4% (74/76 samples), respectively. In contrast, conventional culturing displayed sensitivity rates of 94.1% (64/68) and 79.4% (54/68) on Butzler and modified charcoal cefoperazone deoxycholate media, respectively. The assay required a maximum of 25.5 h from enrichment to detection making the LAMP assay particularly lucrative to industry.

Commercial assays

Kawatsu *et al.* (2010) developed a two step enrichment method combined with an immunochromatographic assay and evaluated it against conventional culturing

followed by selective enrichment and plating to detect *Campylobacter* species in naturally contaminated chicken meat samples. Unspiked samples were enriched in Preston broth in two consecutive steps: one with and one without blood supplementation, each incubated at 42^{o}C for 24 h followed by 5 min incubation at 95^{o}C. The contents were then subjected to a NH Immunocromato *Campylobacter* assay and incubated at room temperature for 15 min. The formation of a red purple colour at both the test and control lines indicated the presence of *Campylobacter*. The method developed by Kawatsu *et al.* (2010) was more efficient in detecting *Campylobacter* (61 of the 68 samples tested) as opposed to conventional culturing. The assay was further evaluated with spiked and unspiked *C. jejuni* samples, half of which were frozen at -20^{o}C for 10 days while others were tested immediately. They found that the detection sensitivity of this method was lower in the freeze stressed samples than the unstressed samples. Due to the short incubation period at -20^{o}C for 10 days it is quite possible that some *Campylobacter* cells may still be viable suggesting the inability of this technique to detect viable but nonculturable *Campylobacter* cells. Another new automated antigen detection system (VIDAS *Campylobacter*) was evaluated by two independent research groups. A comparative study was done by Paulsen *et al.* (2005) using culture enrichment followed by plating on selective agar or culture enrichment and VIDAS analysis. They found the VIDAS system to be rapid, detecting antigen in less than 1½ h (without enrichment) and 24 h (with enrichment); and as sensitive as selective plating but found that differentiation between species was lacking. The combined power of PCR and the VIDAS or other automated systems may add greater value to such systems in

industry. Using multiplex PCR and the VIDAS system, Liu *et al.* (2009) were able to successfully determine the presence of *C. jejuni* and *C. coli* in retail broiler meat but noted that the detection of "true-positive" results was time dependant. They concluded that more *Campylobacter* positive samples were obtained after 48 h enrichment in Bolton broth with or without the addition of blood as opposed to incubation for 24 h. Williams *et al.* (2008) recently used the BAX PCR system with good results. The DuPont Qualicon BAX system is a high throughput, rapid molecular assay that allows the detection of several bacterial species in a range of samples. They performed a comparative study using broiler chicken, fresh chicken carcass rinses as well as gauze socks laden with broiler chicken house matter located on the floor. Samples were plated directly onto selective agar; enriched in Bolton broth supplemented with blood for culture; and in Bolton Broth without blood for analysis with the *Campylobacter* BAX system to detect *C. jejnui* and *C. coli*. They noted that greater detection of *Campylobacter* was achieved using the BAX system as opposed to conventional culturing; that enrichment increased the detection levels and that blood was not required for growth of *C. jejnui* and *C. coli* (Williams *et al.* 2008).

PCR inhibitors

Despite the diverse types of PCR assays available, the presence of PCR inhibitors may prevent the amplification of target fragments in blood, faecal matter, tissues and food (Rådström *et al.* 2004). A range of PCR inhibitors have been identified, including haeme (Akane *et al.* 1994) and leukocyte DNA (Morata *et al.* 1998) in

blood; anticoagulants such as EDTA (Wang *et al.* 1992) and heparin (Satsangi *et al.* 1994); immunoglobulin G in human plasma (Abu Al-Soud *et al.* 2000a); fats, polysaccharides, proteins, glycogen and Ca^{2+} in food (Wilson 1997; Rossen *et al.* 1992); humic acid and heavy metals in environmental samples; constituents of bacterial cells; non-target DNA; and laboratory plasticware (Wilson 1997; Abu Al-Soud and Rådström 1998). The organic and phenolic compounds present in the sample may inhibit cell lysis by denaturing lytic enzymes (Jacobsen and Rasmussen 1992) making DNA unavailable for PCR. Inhibition may occur as a result of pollen (<10 grains) and cellulose that may enzymatically digest an essential reaction component (Bej *et al.* 1991) (St. Pierre *et al.* 1994 reviewed in Wilson 1997) or glove powder which may bind nonspecifically to target DNA (De Lomas *et al.* 1992 reviewed in Wilson 1997). PCR inhibitors could also interfere with cell lysis, a vital process in DNA extraction. As an example, a high salt concentration in the *Listeria* selective media was determined to be the inhibitory factor for unlyzed cells and false-negative reactions by the Accuprobe DNA probe test (Partis *et al.* 1994 reviewed in Wilson 1997) indicative that PCR may be inhibited in a similar way (Wilson 1997). Bacterial cells, debris, proteins or polysaccharides present in a sample may react with the target DNA making it unavailable to the thermostable DNA polymerase (Wilson 1997). In 1993, Lindahl illustrated that hydrolysis; the presence of oxygen; nonenzymatic methylation; enzymatic degradation and inappropriate storage conditions could influence DNA stability and ultimately result in the degradation of target DNA and even primers (Lindahl 1993; Wilson *et al.* 1994). The presence of PCR inhibitors as a result of one or a combination of

mechanisms, discussed above, may result in partial or complete inhibition of the PCR assay (Moreira 1998). These false-negative results suggest the absence of target microorganisms limiting the full diagnostic potential of PCR which may have important implications for clinical and public health investigations, particularly in food and environmental screening (Wilson 1997).

Pre-PCR processing strategies

Over the years, various pre-PCR processing strategies have been developed to remove or reduce the negative effects of PCR inhibitors (Rådström *et al.* 2004). These include: optimizing the sample preparation method; optimizing DNA amplification conditions by using alternative DNA polymerases or by using a combination of these methods. Sample dilution has been used to minimize the effect of PCR inhibitors (Chernesky *et al.* 1997). However, this may be problematic if the target microorganism is already low in number prior to dilution. Olcén *et al.* 1995 used the boiling method to release DNA from bacterial cells. In this way the authors could prevent the contamination of DNA with chemicals required for DNA extraction. However, the boiling method may reduce PCR sensitivity as a result of insufficient separation from structural and DNA-binding proteins (Wilson 1997). Using the aqueous two-phase system composed of 8% (w/v) polyethylene glycol 4000 and 11% (w/w) dextran 40, Lantz *et al.* (1997) successfully removed PCR inhibitors from human faecal samples. The authors reported that majority of the PCR inhibitory components, such as bile salts, were distributed in the polyethylene glycol phase (top); while the target microorganism (*H. pylori*) was successfully

detected by PCR by direct application of the dextran phase (bottom). Lindqvist *et al.* (1997) used density gradient centrifugation to rapidly detect pathogens in food. The authors used Percoll density media to concentrate the target microorganism and to remove PCR inhibitory components of varying densities. This method allows extraction of whole cells of target microorganism which may be used as a PCR template (Lindqvist *et al.* 1997). However, if inhibitory components in the sample matrix display similar densities to the target cells it may be coextracted and inhibit PCR (Rådström *et al.* 2004). Immunological methods based on the use of magnetic beads, precoated with antibodies, have been used with great success by some research groups to extract target DNA from complex samples (Fluit *et al.* 1993; Monteiro *et al.* 2001a). The specificity of the technique is increased as the antibodies target cells with the corresponding antigen. After immunocapture, the sample requires a lysis or a washing step to remove excess antibodies which may inhibit PCR. This technique generally results in an increased concentration of the target microorganism but false-positive reactions may occur as a result of cross-reactivity between antibodies (Rådström *et al.* 2004).

However, most of these interventions are costly; labor intensive; and time consuming to perform, especially if large batches of samples are to be tested. As a result various DNA extraction kits were developed over recent years to rapidly extract high quality DNA for amplification. Most PCR inhibitors are removed using a kit as the DNA is purified using a series of buffers containing chemicals such as EDTA and SDS and stored in Tris-EDTA (TE) buffer (Rådström *et al.* 2004). However, DNA extracted with kits may still contain significant amounts of PCR

inhibitors as illustrated by Moreira in 1998. In some cases, enrichment media (Wernars *et al.* 1991) and filtration (DiMichele and Lewis 1993) together with the use of DNA extraction kits (Ceelen *et al.* 2006; Zanoni *et al.* 2007) have significantly minimized the presence of PCR inhibitors in a sample (Rådström *et al.* 2004).

Various research groups have demonstrated the efficacy of different DNA polymerase such as Hot *Tub*, r*Th*, *Tfl*, *Tli* and *Tth* against a range of PCR inhibitory components (Wiedbrauk *et al.* 1995; Abu Al-Soud and Rådström 1998; Abu Al-Soud and Rådström 2001). Furthermore, Abu Al-Soud and Rådström (2000b) demonstrated that the addition of amplification facilitators such as bovine serum albumin or gp32, in the presence of r*Th* or *Taq* DNA polymerase, could relieve the effect of PCR inhibitors in blood, faeces and meat.

Moreira (1998) and Monteiro *et al.* (2001b) have used an alternative method to remove PCR inhibitors from DNA samples, previously extracted with a commercial kit. In 1998, Moreira developed a technique to efficiently remove PCR inhibitors from DNA samples using agarose embedded DNA preparations. Agarose embedded DNA moulds were prepared by mixing low melting point agarose and DNA in a 1:1 ratio and the mixture aliquoted into rectangular moulds prior to solidification. Thereafter, the agarose blocks were washed in two consecutive steps, either using lysis and TE buffers (Moreira 1998) or TE buffer and distilled water (Monteiro *et al.* 2001b). The difference in size between DNA and the PCR inhibitors such as polysaccharides allowed its efficient removal from the agarose DNA embedded blocks through diffusion during the successive washing steps,

while the genomic DNA (gDNA) remain trapped within the agarose slants (Monteiro *et al.* 2001b). Thereafter, the agarose embedded DNA blocks were used as a template for amplification.

Internal Amplification Control

In a PCR without an internal amplification control (IAC), a negative result (illustrated by the absence of bands) could either suggest the absence of target DNA or the presence of PCR inhibitory components within a reaction tube (Hoorfar *et al.* 2004; He and Shi 2010). In contrast, a PCR with IAC should always produce a control signal even in the absence of target DNA. Thus if no control signal is observed, it is an indication that the PCR has failed (Hoorfar *et al.* 2004). As a result the European Standardization Committee in collaboration with the International Standard Organization has proposed a general guideline for the testing of samples by PCR that requires the use of an IAC in a reaction mix (Lund and Madsen 2006). The IAC can either be constructed using a competitive or noncompetitive strategy (Hoorfar *et al.* 2004). In the competitive IAC strategy, the target fragment and IAC are amplified with a common set of primers in the same PCR tube under the same conditions (Siebert and Larrick 1992). In this strategy, the concentration of the IAC is a critical factor as there is always competition for the amplification of target DNA and the IAC. If target DNA is amplified but not the IAC then it is assumed that the target DNA is present in a greater concentration relative to the IAC (Hoorfar *et al.* 2004). In cases where the competitive IAC strategy was used, a mutagenic primer was often included to produce the IAC. A mutagenic primer is a primer which is

designed to create a deletion from the original target DNA (Denis *et al.* 1999). After amplification of a product using the mutagenic primer, it is cloned into a vector. Once the mutagenic fragments' presence is confirmed by PCR and sequencing, it is used as a competitive IAC (Denis *et al.* 1999). Using this method, Denis *et al.* (2001) detected and identified *C. jejuni* and *C. coli* in French poultry produce. Recently, Randall *et al.* (2010) successfully detected single and double infections of *C. jejuni* and *C. coli* in chicken caecal samples using this strategy.

In the noncompetitive IAC strategy, the target DNA and IAC are amplified with two different sets of primers in two different PCR's but optimized to work using the same PCR conditions. However, this may result in subefficient PCR for one or both reactions (Hoorfar *et al.* 2004). Despite this drawback, this strategy has been used successfully by many research groups to detect *Campylobacter* spp. in chicken carcass rinse and chicken fecal samples with or without enrichment (Lübeck *et al.* 2003a; Lübeck *et al.* 2003b; Josefsen *et al.* 2004; Lund and Madsen 2006).

TYPING

After the successful amplification of strains of *Campylobacter*, *Arcobacter* and *Helicobacter*, isolated from various sources, typing is often required to determine genetic relatedness amongst strains of the same species. To date, many scientists are still debating about the best methods to distinguish strain relatedness which is imperative in tracing the source of an outbreak or illness (Humphrey *et al.* 2007).

Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) was first used by 1998 to characterize isolates of *Neisseria meningitides* but can be applied to most bacterial species (Maiden *et al.* 1998). During MLST typing, fragments ranging in size of 400-500 bp from several genes are amplified and sequenced and grouped according to the variation between the strains tested (Wikipedia 2010). This technique has been used to determine transmission of *H. pylori* strains within a family (Raymond *et al.* 2004) and migration pattern between populations (Yamaoka 2009). In 2009, Miller *et al.* were the first to use MLST on *Arcobacter* species. A year later, Merga *et al.* (2011) reported that a high degree of diversity could be determined among 39 *A. butzleri* isolates using MLST. In addition, MLST has also been used to detect genetic variation among *Campylobacter* isolates of diverse origins, including humans; animals; and the environment (Dingle *et al.* 2001; Sopwith *et al.* 2006; Behringer *et al.* 2011). Recently, the MLST technique was further developed. Lévesque *et al.* (2011) evaluated a high-resolution melting system that is cost-effective and less time-consuming to identify known MLST alleles of *Campylobacter*. This technique uses fluorescent DNA binding dyes to allow a more precise assessment for the variation of sequences based on the analysis of DNA melting curves.

Restriction Fragment Length Polymorphism

Using a rapid two step identification scheme based on a 16S rRNA PCR assay and Restriction Fragment Length Polymorphism (RFLP), Marshall *et al.* (1999) successfully managed to genetically identify many species of *Campylobacter*,

Arcobacter and *Helicobacter*. However, the technique failed to differentiate between *H. cinaedi* and *H. canis*; *C. hyointestinalis* and *C. fetus*; as well as *C. jejuni* and *C. coli*.

Enterobacterial Repetetive Intergenic Consensus and Randomly Amplified Polymorphic DNA PCR

Using enterobacterial repetitive intergenic consensus (ERIC) PCR and randomly amplified polymorphic DNA (RAPD) PCR, Houf *et al.* (2002) assessed the genetic diversity of 255 strains of *Arcobacter*. Twenty-four of the strains were previously isolated from humans and animals and were geographically and epidemiologically unrelated; while three were obtained from an outbreak case in Italy. These were used to assess the discriminatory power of both typing methods. In addition, 228 strains of *Arcobacter* (182 of *A. butzleri* and 46 of *A. cryaerophilus*), previously isolated from poultry neck skins obtained from four different slaughterhouses, were also typed. The discriminatory power of both typing methods was successfully demonstrated with the 24 unrelated *Arcobacter* strains which generated unique profile patterns for each strain; while the three strains obtained from an outbreak case displayed identical profile patterns. With respect to the poultry isolates, the authors found that 86% could be characterized by ERIC-PCR and 98% by RAPD profile patterns. The authors also observed the presence of multiple genotypes of *Arcobacter* in a poultry sample which the authors presumed to be indicative of multiple sources of contamination or the occurrence of genetic recombination among genotypes (Houf *et al.* 2002).

Pulsed Field Gel Electrophoresis

Son *et al.* (2006) used Pulsed Field Gel Electrophoresis (PFGE) to determine the genomic fingerprints of *Campylobacter* and *Arcobacter* strains isolated from broiler carcasses. The authors typed 27 strains of *C. coli* and 188 strains of *C. jejuni* using the *Sma*I restriction enzyme; and 138 strains of *A. butzleri*, 4 strains of *A. cryaerophilus* 1A and 31 strains of *A. cryaerophilus* 1B with the *Kpn*I restriction enzyme. They identified unique PFGE profiles in 57 of 174 strains of *Arcobacter* and five of 215 strains of *Campylobacter*. In contrast, 117 strains of *Arcobacter* were distributed among 25 common PFGE profile patterns; while 210 strains of *Campylobacter* were distributed among eight common PFGE profile patterns. However, using cluster analysis the authors could not identify any associations among strains demonstrating common PFGE profile patterns (Son *et al.* 2006). Other techniques such as DNA amplification fingerprinting (DAF) and arbitrarily primed PCR (AP-PCR), based on the amplification of random genomic fragments by arbitrarily PCR primers, have also been used in strain typing (Reviewed in Vos *et al.* 1995). These techniques allow generation of DNA fragment patterns without any knowledge about the genomic sequence but they are very sensitive to the DNA quality and PCR conditions (Vos *et al.* 1995).

Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) fingerprinting is a reliable technique based on the detection of digested genomic fragments by PCR which may be used for DNA of any origin and complexity (Vos *et al.* 1995). It has been found

to have better discriminatory power and is faster than most typing methods such as PFGE and PCR RFLP (Lindstedt *et al.* 2000). Since its development, in 1995, it has been used by many research groups to generate DNA profiling patterns and determine genetic relatedness or diversity among strains. AFLP may be done using a single (Gibson *et al.* 1998; González *et al.* 2007) or double (Duim *et al.* 1999; On *et al.* 2004; Ceelen *et al.* 2006) restriction enzyme(s). The generation of AFLP profile patterns allowed discrimination of *Campylobacter*, *Arcobacter* and *Helicobacter* strains in poultry, sheep, human, water and food samples (Gibson *et al.* 1998; Duim *et al.* 1999; Gibson *et al.* 1999; Lindstedt *et al.* 2000; On *et al.* 2004; Siemer *et al.* 2004; Ceelen *et al.* 2006; González *et al.* 2007).

Multiple typing techniques

Some groups (Gibson *et al.* 1999; De Boer *et al.* 2000) have used more than one method to type strains of different genera or different strains of the same species (Lindstedt *et al.* 2000) with the aim of conclusively differentiating strains appearing to be genetically related (De Boer *et al.* 2000). Using AFLP and PFGE, Gibson *et al.* (1999) observed distinct genotypes between the human and poultry isolates illustrating a different source of contamination. A close relation was observed among three UK isolates, obtained from chicken carcasses from a processing plant, suggesting a common source of contamination. The authors also observed a similar profiling pattern between a type strain and two other strains, obtained from the same flock, representing a "clonal" origin for *H. pullorum*. Their study further illustrates the possible use of these techniques in epidemiological based studies in identifying

the source of an outbreak. Using AFLP with restriction enzymes *Bgl*II and *Mfe*I, together with PFGE using restriction enzyme *Sma*I and PCR RFLP based on the *fla*A and *flab* genes, Lindstedt *et al.* (2000) genotyped 91 strains of *C. jejuni* subsp. *jejuni* from outbreak and sporadic cases. AFLP was found to be more reliable identifying nine of 10 recognized outbreaks as well as similarities between outbreak and sporadic cases. Siemer and coworkers have shown that numerical analysis of AFLP fingerprinting based on *Mfe*I and *Bsp*DI polymorphisms is useful in correctly identifying *Campylobacter* strains and epidemiological relationships between different hosts (Siemer *et al.* 2005).

Debruyne *et al.* (2010) re-assessed the taxonomy of *A. cryaerophilus* based on AFLP profiling patterns of seven strains previously assigned to subgroup I and 33 strains to subgroup II as well as gene sequence analysis of heat shock protein60 (Hsp60). Their AFLP cluster data displayed multiple cores for strains of *A. cryaerophilus* with no clear grouping observed among the strains previously assigned to subtype I and II. Furthermore, their *hsp*60 gene sequence data indicated the presence of a large clade consisting of most of the *A. cryaerophilus* strains; two small clades consisting of the remainder of the tested *A. cryaerophilus* strains; while the type strain of *A. cryaerophilus* was positioned separately. On the basis of their results, the authors recommended that the current subgrouping nomenclature be abolished and recommend that a more genetically similar strain, one that better represented strains belonging to *A. cryaerophilus*, be used as a type strain in further studies.

SURVIVAL CAPACITY OF *CAMPYLOBACTER*, *ARCOBACTER* AND *HELICOBACTER*

Increased infection rates of *Campylobacter*, *Arcobacter* and *Helicobacter* in humans and animals, has prompted further research in determining their capacity to survive in various sources.

Survival in liquids and on surfaces

A previous report illustrated that the lowest temperature for *Arcobacter* growth was 15ºC (Kiehlbauch *et al.* 1991a). However, studies dating back to the turn of the century have illustrated otherwise. *A. butzleri* has been reported to survive at -20 and 4ºC for at least 21 days in BHI broth (Hilton *et al.* 2001); and 7 days or more at 10ºC in BHI broth (Kjeldgaard *et al.* 2009) and EMJH media (D'Sa and Harrison 2005). Furthermore, Kjeldgaard *et al.* (2009) noted that *A. butzleri* grew better, without any lag in CMJ, while growth in BHI was only noted one day after inoculation. Ohkusa *et al.* (2004) reported that all seven of the tested strains of *H. pylori* could not be cultured after three weeks of storage in saline at -20 and 4ºC; while the type strain could only be cultured after freezing at -80ºC. This either suggests that survival at low and ultra low temperatures may be strain or species dependant; or it may simple imply that the cells are in the viable but nonculturable state. Bohmler *et al.* illustrated that artificially inoculated *H. pylori* could survive in sterile chicken drip and physiological saline up to 48 h at room temperature but dropped below the detection limit after 72 h in *H. pylori* broth. In addition, the pathogen could survive at 7ºC for more than 72 h in all three media (drip, saline and

broth); more than one month in chicken drip and up to one week in saline (Bohmler *et al.* 1996). The authors also spread thin layers of inoculated chicken drip onto a wooden board; as well as on plastic and ceramic tiles and determined that *H. pylori* could be recultured from these surfaces provided that it was still moist. Furthermore, the authors determined that *H. pylori* could not be detected on the wooden board after 30 min of exposure and on plastic and ceramic tiles after 90 min of exposure to air at room temperature. They also reported that at refridgeration temperatures, the administered suspensions of *H. pylori* dried slower, allowing longer survival of *H. pylori*, but could not be cultured from the wooden board after 240 min and after 300 min on plastic or ceramic tiles (Bohmler *et al.* 1996). However, the morphological changes of *H. pylori* were not determined in this study. As a result it is important to note that although *H. pylori* could not be detected it may still have been in the viable but nonculturable state and thus should still be regarded as a potential mode for infection.

Cell viability has previously been shown to decrease at a faster rate at subzero than refrigeration temperatures, presumably due to the formation of ice crystals and freeze thaw damage (Hilton *et al.* 2001). Use of a cryopreservative could minimize cell damage and thus increase survival. Murinda *et al.* (2004) reported that *C. jejuni* could survive for 251 days at -20ºC and up to 70 days at 5ºC in faecal samples using glycerol. Data from the Portner *et al.* (2007) study bears testimony that the viability of freeze dried cell preparations of *C. jejuni* can significantly be increased by using younger cultures, using an adequate lyoprotectant, and optimizing storage and rehydration conditions. The authors

managed to retain longer viability of the *C. jejuni* strain using a 24 h culture, inositol broth with 10% (w/v) trehalose as a lypoprotectant, by storing under vacuum at 4ºC and rehydrating in inositol broth for 30 min at 37ºC.

Other studies have suggested that the survival of a microorganism could be medium dependant. *C. jejuni* strains survived longer in 4% albumin solution as opposed to milk, 4% peptone water, peptone-saline and faeces stored at -60, -20 and 4ºC (Valdés-Dapena *et al.* 1983). Cools *et al.* (2003) reported that *C. jejuni* could survive up to 33 days at 4ºC in pure drinking water. Doyle and Roman noted that the eight tested strains of *C. jejuni* was most stable in brucella broth, inactivated at an intermediate rate in sterile milk and died quicker upon storage at 4ºC in unpasteurized milk (Doyle and Roman 1982). Hilton and coworkers noted that heat stressed *A. butzleri* cells could only be isolated on nonselective media (Hilton *et al.* 2001); while Kjeldgaard *et al.* (2009) reported that chicken meat juice (CMJ) drastically prolonged the survival of *A. butzleri*. Broiler caecal contents with 10^4 cfu g^{-1} of *C. jejuni* were still detectable on mCCDA 6 days after inoculation and storage at 4ºC (Rodgers *et al.* 2010). Van Driessche and coworker reported prolonged survival of *Arcobacter* strains in water containing 1% organic material than in water without organic material. They noted that *A. butzeri* survived significantly longer (up to 250 days at 4 and 7ºC; and 168 days at 20ºC) and were more heat resistant (45, 25 and 15 min at 52, 56 and 60ºC, respectively) than *A. cryaerophilus* and *A. skirrowii* (Van Driessche and Houf 2008). Survival may also be dependant on the microorganism's ability to form biofilms allowing it to persist in a particular environment. Biofilms were formed by *A. butzleri* on the surfaces of microtiter

plates when grown in BHI or MH broth at temperatures ranging between 10 and 37ºC; and on stainless steel at 5; 10; and 21ºC, with the amount of biofilm increasing as the incubation time and temperature increases (Assanta *et al.* 2002; Kjeldgaard *et al.* 2009).

Survival in poultry produce, diary and water

Foods handled or prepared in unhygienic conditions could serve as vehicles for *Campylobacter*, *Arcobacter* and *Helicobacter* transmission in developing countries (Gomes and Martinis 2004). On this basis, various survival studies have been conducted using food products and drinking water. However, the research is mostly based on specific species, namely, *C. jejuni* and *C. coli* (Oyarzabal *et al.* 2010); *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* (Van Driessche and Houf 2008) and *H. pylori* (Zhao *et al.* 2000). Lee *et al.* (1998) found that a virulent strain of *C. jejuni*, inoculated onto 1 cm² chicken pieces, and stored at -20 and -70ºC could withstand repeated freeze thawing cycles; maintain a high degree of viability; and could rapidly replicate to levels exceeding that permitted by Australian food authorities after thawing. Strain variability was further demonstrated among two of the strains of *C. jejuni* in the Oyarzabal *et al.* (2010) study. Oyarzabal and coworkers inoculated retail broiler meat with *C. jejuni* or *C. coli* at 6.4-6.8 log cfu g^{-1} and stored at -20ºC for 84 days, tested on day 0 and every 14 days thereafter; stored for 14 days at 4ºC or 14 days at 12ºC and tested day 0 and every two days thereafter (Oyarzabal *et al.* 2010). The authors observed that *C. jejuni* and *C. coli* displayed similar survival patterns at -20ºC, however fewer viable cells of *C. jejuni* than *C.*

coli were present at 4 and 12ºC. Bhaduri and Cottrell (2004) inoculated ground chicken and chicken skin using 10^9 cfu ml^{-1} of *C. jejuni* which they stored at -20 and 4ºC; and also used combined storage at refrigeration and freezing. The authors noted that three to seven days storage at 4ºC resulted in a 0.34-0.81 log_{10} and 0.31-0.63 log_{10} reduction of *C. jejuni* in ground chicken and chicken skin, respectively; while frozen storage, with and without pre-refridgeration resulted in a 0.56-1.57 log_{10} and 1.38-3.39 log_{10} reduction in ground chicken and chicken skin, respectively, over the two week incubation period. Furthermore, the authors also noted that longer pre-refrigeration periods resulted in lower viable cell counts of *C. jejuni* on chicken skin, however the same phenomenon was not observed in ground chicken which the authors attributed to the microenvironments and composition of the sample (Bhaduri and Cottrell 2004). Speck and Ray illustrated that simple and complex carbohydrates, triglycerides and proteins as well as food viscosity may increase the microorganisms' tolerance to freezing; while acids, ions, inorganic salts, surface active components as well as enzymes may decrease their tolerance to freezing (Speck and Ray 1977). Poms and Tatini (2001) analyzed various samples spiked with 6 x10^4 cfu ml^{-1} *H. pylori*, stored at 4ºC, and determined that viable *H. pylori* could still be recovered from: pasteurized skim milk and tofu up to 5 days; leaf lettuce and raw chicken up to 2 days; however, could not be recovered from yoghurt, perhaps as a result of organic acids produced by lactic acid bacteria (Midolo *et al.* 1995) or competitive exclusion by live cultures present in the yoghurt. Wundt and co-workers noted that *C. jejuni* could survive in milk and drinking water for: 15 and 6 days, respectively at 5ºC; 7 and 4 days, respectively at

22ºC; several weeks in frozen water stored at -20ºC; while survival in sour meat salad (pH 4.2) was limited to a few hours only (Wundt *et al.* 1985). Doyle and Roman (1982) observed strain variability among the eight tested strains after inoculation with 10^7 cfu ml^{-1} *C. jejuni* in unpasteurized milk and storage at 4ºC, of which the most tolerant strain displayed a <2-log_{10} reduction in cell viability after 14 days; a >6-log_{10} reduction in cell viability after 7 days for the most sensitive strain; while one strain was still viable three weeks after inoculation. In contrast, the survival of inoculated *H. pylori* (10^5-10^6 cfu ml^{-1}) was detected for 9-10 days in pasteurized milk (Fan *et al.* 1998; Quaglia *et al.* 2007); 12 days in ultrahigh temperature milk (Quaglia *et al.* 2007); and 4 days in tap water stored at 4ºC with the nonculturable form dominating in the tap water sample (Fan *et al.* 1998). Bohmler and coworkers artificially inoculated high concentrations of *H. pylori* in milk; yoghurt; kefir; and curd cheese with neutral pH and found that the pathogen could only be detected up to three or four days in milk stored at room temperature and 37ºC; three hours in yoghurt; up to 24 h in kefir; and 10 h in neutral pH curd cheese (Bohmler *et al.* 1996). Van Driessche and Houf (2008) analyzed potable water, in compliance with drinking water standards, artificially inoculated with ≥10^5 cfu ml^{-1} of *A. butzleri*, *A. cryaerophilus* or *A. skirrowwi* and stored at 4, 7, 20, 52, 56 and 60ºC. The authors noted that *A. butzleri* survived the longest compared to *A. cryaerophilus* and *A. skirrowwi* at all tested temperatures. As an example, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* survived up to 98, 56 and 42 days in pure drinking water stored at 4ºC, respectively; while at other temperatures *A. butzleri* survived up to 98 days, 91 days, 45 min, 25 min and 15 min at 7, 20, 52, 56 and

60ºC, respectively. Zhao *et al.* (2000) demonstrated that 10^4-10^6 CFU g^{-1} of *C. jejuni* could survive for 13 days in butter without garlic and for 3-24 h in garlic butter at 5ºC. This data clearly demonstrates the survival capacity of these microorganisms in water and a range of food products. Thus the use of effective preservatives such as citric acid, lactic acid, sodium citrate and sodium lactate used alone or in combination with nisin to prevent the proliferation of microorganisms such as *Arcobacter* have been studied (Phillips 1999).

TREATMENT STRATEGIES

The association of *Campylobacter*, *Arcobacter* and *Helicobacter* with human illness has sparked interest in antimicrobial research with the aim of minimizing these microorganisms in foods of animal origin (Pilotto *et al.* 2000; Loc Carrillo *et al.* 2005). Poultry is considered to be a major source of campylobacteriosis in humans (Lin 2009). The introduction of intervention strategies such as antibiotics, bacteriophage and bacteriocin therapies as well as competitive exclusion at farm level, are presumed to significantly reduce *Campylobacteraceae* infections in poultry (Loc Carrillo *et al.* 2005).

Antibiotics

Studies have reported on the emergence of *Campylobacter* isolates resistant to quinolones, commonly used for the treatment of this infection (Piddock 1995; De Wet *et al.* 1999) and species of *Campylobacteraceae* susceptible to aminoglycosides such as kanamycin and streptomycin (Kabeya *et al.* 2004) (Thwaites and Frost 1999

reviewed in Snelling *et al.* 2006). Strains of *A. cryaerophilus* and *A. skirrowii* have been shown to be more susceptible to cephalothin than *A. butzleri*, suggesting the possibility of its use in enrichment or selective media (Kabeya *et al.* 2004). Over the last decade, antibiotic resistant microorganisms including *Campylobacter* and related microorganisms have increased at an alarming rate (Piddock 1995; De Wet *et al.* 1999; Vandeplas *et al.* 2008). It is presumed that antibiotic residues consumed with eggs, milk, meat, honey and other food products could result in resistant bacterial populations in consumers as the same type of antibiotics are often used in animals and humans (Centre for Science and Environment 2010). Evidence for this phenomenon has been provided by the World Health Organization (WHO), in collaboration with the Food and Agriculture Organization (FAO), and other groups identifying links between antibiotic usage in animals; the emergence of antibiotic resistant foodborne pathogens; and antibiotics used in the treatment of human infections (Pezotti *et al.* 2003; Desmonts *et al.* 2004; Luangtongkum *et al.* 2006 reviewed in Vandeplas *et al.* 2008; Centre for Science and Environment 2010). The acquired resistance noted for erythromycin, enrofloxacin and ciprofloxacin among poultry isolates poses a concern as erythromycin and ciprofloxacin are usually used as first line antibiotics for the treatment of *Campylobacteraceae* infections in humans (Houf *et al.* 2004 reviewed in Snelling *et al.* 2006; Centre for Science and Environment 2010). This emphasizes the need for stricter control measures for administering antibiotics, including aminoglycosides; fluoroquinolones; and tetracycline to animals and humans (Kabeya *et al.* 2004; Centre for Science and Environment 2010). In the late 1980s, patients were required to take up to 16 tablets

a day consisting of combinations of bismuth, metronidazole and tetracycline to eradicate *H. pylori* infections (Borody *et al.* 1989 reviewed in Marshall 2002). This made treatment more difficult as many patients did not finish the full course of treatment. During a pilot study, Unge and colleagues noted that the action of amoxicillin was significantly enhanced with the use of omeprazole, a proton pump inhibitor that suppresses gastric acid (Unge *et al.* 1989). Since then, the treatment of *H. pylori* infections has become easier with a one week course of omeprazole and two antibiotics, clarithromycin and amoxicillin or metronidazole. In cases where triple therapy eradication fails, the risk of developing resistance to antibiotics is increased, with up to 50% resistance reported for clarithromycin (Pilotto *et al.* 2000). More severe infections are treated with a quadruple drug regimen consisting of bismuth, tetracycline, metronidazole and omeprazole for 10 days (Bazzoli 1999 and Kung *et al.* 1997 reviewed in Marshall 2002). Although these treatments generally result in a cure rate of between 80-90%, its use has recently become limited as a result of antibiotic resistance with 15% resistance reported for clarithromycin and 50% for metronidazole in some cases (Mégraud and Marshall 2000). Multiple antibiotic resistant strains have been detected in *Campylobacter* and related microorganisms (Sharma *et al.* 2003; Kabeya *et al.* 2004) further emphasizing the need to develop alternate strategies to treat infections. Some strategies have been reviewed, including the use of preservatives; bacteriophage therapy; bacteriocins; competitive exclusion; and culinary products.

Preservatives

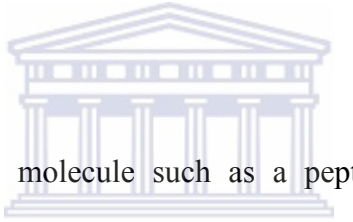
Studies have reported that organic acid concentrations of >0.2% acetic and citric acid as well as short term treatment with trisodium phosphate and EDTA, with or without nisin, significantly reduced *A. butzleri* in culture (Phillips and Duggan 2001; Cervenka *et al.* 2004 reviewed in Snelling *et al.* 2006); while reports on the effect nisin in chicken is contradictory (Phillips and Long 2001; Long and Phillips 2003). At high (0.993) and reduced water activity (a_w), acetic acid and citric acid (>2%) inhibited the growth of *A. butzleri* after 4-5 h incubation in culture (Cervenka *et al.* 2004 reviewed in Snelling *et al.* 2006).

Bacteriophage therapy

Two independent research groups have demonstrated that bacteriophage therapy reduces *C. jejuni* infections in broiler chickens. Loc Carrillo *et al.* (2005) orally administered two phages, CP8 and CP34 shown to have broad host lysis, at different dosages in antacid suspensions to 25-day old broiler chickens. A 0.5-5 $\log_{10}$ cfu g^{-1} reduction in the ceacal contents was noted for *C. jejuni* infected chickens treated with the phage over a five day period. However, these reductions appeared to be dependant on the phage used; dosage applied; and on the time lapsed after administration (Loc Carrillo *et al.* 2005). Wagenaar and coworkers took a preventative and therapeutic approach on phage therapy in treating *C. jejuni* infections in broiler chickens. The preventative group was established by infecting broilers with *C. jejuni* on the fourth day of a 10-day phage treatment; while the therapeutic group was established by treating broilers with the phage for six days, five days after *C. jejuni* colonization had been established; while the control group

received no phage therapy. Treatment was monitored by counting the number of *Campylobacter* colony forming units (CFU) and phage plaque forming units (PFU) from the ceacal contents and compared to the control group. A 3 log decline in CFU was observed in the therapeutic group directly after administering the phage but stabilized five days later at 1 log lower than the control group which the authors presumed to be due to the presence of a resistant subpopulation of *C. jejuni*. The initial decline suggested that phage therapy would be more effective at the early stages of *C. jejuni* colonization (Wagenaar *et al.* 2005). By administering two different phages during therapeutic treatment of broiler chickens approaching the slaughtering age, the authors observed a significant decrease in *C. jejuni* colonization demonstrating that multiple phages could successfully be administered to a host without producing any antagonistic effects. As expected, preventative treatment delayed *C. jejuni* colonization; however after colonization, *C. jejuni* was reduced at a similar rate to that observed in the therapeutic group. Importantly, Wagenaar and colleagues detected no adverse effects in the chickens during phage treatment while viable phages translocated to the gastrointestinal tract suggesting a safer and effective option in treating *C. jejuni* infections in chicken. Undetected phages in a control group receiving phage only for seven days as treatment indicated that phages target specific bacterial hosts without harming the chicken (Wagenaar *et al.* 2005). Using genetic engineering, Cao *et al.* (2000) prevented growth of *H. pylori* in culture and mice. The authors achieved this by developing monoclonal antibodies against the surface antigens of *H. pylori* and displaying the single chain variable fragments (ScFv), derived from murine hybridomas producing monoclonal

antibodies, and expressed as a g3p-fusion protein on a filamentous M13 phage. Cao and colleagues specifically designed the recombinant ScFv-phage to react with a 30 kDa monomeric protein of a *H. pylori* surface antigen. Immunofluorescence microscopy confirmed binding of the phage to both the spiral and coccoid forms of *H. pylori*; while *in vitro* testing illustrated bactericidal and inhibitory activities of the recombinant phage against the growth of all six strains of *H. pylori* (Cao *et al.* 2000). The authors further reported that by pretreating *H. pylori* with the phage for 10 min, prior to oral inoculation, *H. pylori* colonization was significantly reduced in the stomachs of mice (Cao *et al.* 2000).



Bacteriocin treatment

A bacteriocin is a small molecule such as a peptide or protein, produced by microorganisms, that has an inhibitory effect on closely related strains. Stern *et al.* (2005) evaluated a bacteriocin-based treatment to reduce *C. jejuni* in poultry. They used 0.25 g of a previously described purified bacteriocin (secreted by *Paenibacillus polymyxa* NRRL-B-30509) microencapsulated in polyvinylpyrrolidone and mixed it into 1 kg of chicken feed. The authors fed the experimental one-day old chickens with bacteriocin enriched chicken feed and control one-day old chickens with standard chicken feed; thereafter chickens were infected with one of four strains of *C. jejuni*; and further reared in isolation facilities. The chickens were fed with standard chicken feed and water for one week, testing feacal content and the intestines of slaughtered chicken three days later. Stern *et al.* (2005) observed significantly lower colonization of *C. jejuni* in the intestines of experimental

chicken; a mean log 7.2 + 0.3 cfu g^{-1} of *C. jejuni* in faeces of untreated chickens; while *C. jejuni* colonization could not be detected in any of the bacteriocin treated chickens. Similar results were observed in subsequent studies using bacteriocins, purified from *Bacillus*, *Paenibacillus* and *Lactobacillus*, against the colonization of *C. jejuni* and *C. coli* in chickens and turkeys (Cole *et al.* 2006; Stern *et al.* 2006; Stern *et al.* 2010). The effective bacteriocin activity of *Lactobacillus* against *H. pylori*, in culture and in patients; and *H. felis* in mice has also been reported (reviewed in Gotteland *et al.* 2006). These studies suggest an effective measure preventing the transfer of *Campylobacter* and *Helicobacter* from poultry to humans (Stern *et al.* 2005).

Competitive exclusion

Reduction of *Campylobacter* in chicken caeca has been reported using commercial competitive exclusion (CE) Broilact (Aho *et al.* 1992; Hakkinen and Schneitz 1999). Hakkinen and colleague reported a significant reduction of *C. jejuni* positive chicken and colonization in the caeca by orally administering commercial CE Broilact to newly hatched chickens and challenging 23 days later with $5.7x10^4$, 5.4 $x10^4$ and 7.3 $x10^3$ cfu *C. jejuni*. Chaveerach *et al.* (2004) demonstrated the inhibitory effect of a strain of *Lactobacillus fermentum*, previously isolated from a chicken gut, on a total of 10 strains consisting of *C. jejuni* and *C. coli* by the agar diffusion and time killing assays. Their data demonstrated an inhibitory effect against all 10 strains tested with an average reduction of 4.10 ± 2.15 log cfu ml^{-1} recorded during the 24 h of coculture. Chaveerach *et al.* (2004) speculated that this

inhibitory effect was a result of a reduction in pH caused by the production of organic acids. This phenomenon was confirmed by Ryan *et al.* (2008). Two *in vitro* studies have demonstrated that *H. pylori* strains can be inhibited by competitive exclusion (López-Brea *et al.* 2008; Ryan *et al.* 2008). López-Brea *et al.* (2008) used 32 microorganisms, of clinical and commercial origin, to test the effect it would have on *H. pylori* isolates obtained from 35 human gastric biopsy specimens. The diffusion and time killing assays were used to test the *in vitro* activity against the *H. pylori* isolates. In the diffusion method, the authors inoculated a fresh culture of *H. pylori* onto antibiotic free blood agar, followed by the addition of two drops of tested microorganism and microaerobic incubation for two to five days at 37ºC. The authors observed strain dependant inhibition with species of *Lactobacillus*, *Bacillus*, *Enterococcus*, *Saccharomyces*, *Staphylococcus* and some species of Gram-negative microorganisms such as *E. coli*, *Klebsiella*, *Enterobacter*, *Salmonella*, *Acinetobacter* and *Stenotrophomonas*; while all 35 isolates of *H. pylori* were resistant to *Lactococcus*, *Streptococcus* and *P. aeruginosa*. Thereafter, López-Brea *et al.* (2008) used the *Staphylococcus* species with inhibitory activity against all of the tested *H. pylori* isolates in a time killing assay. They used BHI, supplemented with 10% bovine fetal serum and inoculated the experimental tube with viable cells or concentrated freeze dried product of *H. pylori* and *Staphylococcus*; while the killing control was inoculated with viable cells or concentrated freeze dried product of *H. pylori* only and amoxicillin, followed by testing at 0, 1, 2, 6 and 24 h after microaerobic incubation at 37ºC. López-Brea *et al.* (2008) also reported that *Staphylococcus* species could still exhibit bactericidal activity against *H. pylori* after

24 h. Similar results were reported for the 40 *Lactobacillus* strains tested against *H. pylori*, with a strain of *L. salivarius* exhibiting antibacterial activity against antibiotic resistant strains of *H. pylori* (Ryan *et al.* 2008). Furthermore, using pronase or *N*-acetyl cysteine spotted onto the agar plate adjacent to the inoculated paper disc Ryan and coworkers were able to demonstrate that *L. salivarius* inhibition was a result of acid production and thus required live cells (Ryan *et al.* 2008).

In vitro and *in vivo* inhibitory effects of various culinary products

A range of plant extracts have been studied for their antimicrobial abilities. Most of the research in this area has been performed *in vitro* but some *in vivo* studies have been done in poultry and mice.

Garlic and onions belong to the *Allium* family (Benkeblia 2004) are common, accessible, safe, inexpensive spices with antibacterial, antifungal, antiprotozoal and antiviral properties due to the presence of allicin; powerful phenolic; and sulphur compounds (Griffiths *et al.* 2002; Bakri and Douglas 2005). Allicin, the primary biologically active antimicrobial compound (Saeed and Tariq 2006), has been shown *in vitro* to have a broad spectrum of inhibitory activity against Gram-negative and Gram-positive pathogenic microorganisms, yeasts, *Candida species*, *Cryptococcus neoformans*, *H. pylori* and even microorganisms which have gained resistance to antibiotics (Jezowa *et al.* 1966; Rode *et al.* 1989; Cellini *et al.* 1996; Whitemore and Naidu 2000), while supporting the development of natural flora in the host (reviewed by Irfan 2010). An *in vitro* study demonstrated that allicin mainly

exhibits its antimicrobial activity through partial or total inhibition of RNA synthesis suggesting that it is a primary target for allicin (Feldberg *et al.* 1988). Cellini *et al.* (1996) illustrated 90% inhibition against the 16 clinical strains of *H. pylori* with an aqueous extract of garlic at a concentration of 5 mg ml^{-1}. Using a standardized aqueous garlic extract (40 mg of thiosulfinate per litre of water) Sivam *et al.* (1997) were able to demonstrate inhibition among all strains of *H. pylori*. De Wet *et al.* (1999) used a slightly different approach but achieved similar results. They peeled, crushed and homogenized the garlic cloves; mixed a 2:1 ratio of distilled water and garlic pulp for an hour; and incubated it for two hours at 4ºC. The aqueous supernatant was decanted into a sterile centrifuge tube; centrifuged to remove tissue particles; filtered through a 0.45 µm acetate membrane filter; and stored at 4ºC for seven days prior to *in vitro* testing. Using this technique De Wet *et al.* (1999) reported that all 38 clinical isolates of *Campylobacter* and all 32 of *Helicobacter* were susceptible to the aqueous garlic solution. Aqueous extracts of Chinese leek (soft leek) have also been shown to produce strong inhibitory activities against *C. jejuni* subsp. *jejuni* and *C. coli* strains (Lee *et al.* 2004). The inhibitory effect of garlic in perishable food has also been documented. Zhao *et al.* (2000) revealed that *C. jejuni* subsp. *jejuni* could survive in refrigerated butter for up to 13 days at 5ºC and 3 days at 21ºC but populations of 10^3-10^5 CFU g^{-1} in garlic butter were killed within a few hours. Using the rodent model, numerous experimental studies demonstrated that garlic and its components could suppress the development of chemically induced tumours in various organs such as the oesophagus (Wargovich *et al.* 1988), colon (Sumiyoshi *et al.* 1990), mammary gland (Amagase

et al. 1993) and stomach (Wattenberg *et al.* 1989). Epidemiological studies have revealed that the incidence of stomach cancer may be reduced upon daily consumption of *Allium* vegetables such as garlic and onions (You *et al.* 1989; Steinmetz and Potter 1991a ; 1991b; Dorant *et al.* 1996). However, contradictory data was obtained in the *in vivo* study by Graham *et al.* (1999). In their study seven males and five women received test meals in the morning, afternoon and evening consisting of beef, tortillas and a salad to which either 10 sliced cloves of garlic, six freshly sliced jalapeños, two bismuth subsalicylate tablets (positive control) or nothing was added (negative control). Using the urea breath test to detect urease activity of *H. pylori*, they found that neither garlic nor jalapeños were effective against the colonization of *H. pylori* (average urease activity pre and post garlic recorded at 28.5 versus 39.8; and 43.7 versus 46.6 for jalapenos; $P>0.8$) but marked reduction was observed after bismuth ingestion (55.8 versus 14.3; $P<0.001$). Furthermore, two patients reportedly experienced severe diarrhoea and vomiting after eating the jalapenos; while 70% of the patients complained of taste disturbance and body odour after eating garlic.

Ginger contains several bioactive compounds such as borneal, camphene, citral, eucalyptol, linalool, phenylandrene, zingiberine and zingiberol phenols which have been shown to have great therapeutic potential (Hirasa and Takemasa 1998; Ahmad *et al.* 2008 both reviewed in Sunilson *et al.* 2009). Sunilson *et al.* (2009) used petroleum ether, methanol, chloroform and water extracts of ginger which was prepared by vacuum drying fresh ginger rhizome pieces; milling it to a coarse powder; extracting by Soxhlet extraction; and concentrating through vacuum

evaporation. The antimicrobial activity of the extracts were tested, in triplicate, by the disc diffusion method and recorded as an average. Their water extract produced one of the higher inhibitory effects against *C. jejuni* (11.4±0.6 mm) which was only second to their methanol extract (12.3±0.8 mm); while their petroleum ether and chloroform extracts exhibited the lowest inhibition against *C. jejuni*, 9.3±1.7 mm and 8.9±2.2 mm, respectively. Weerasekera *et al.* (2008) dissolved 10 g of ginger powder in 100 ml of distilled water; boiled the suspension for 20 min; filtered through sterile gauze; neutralized the pH; autoclaved and stored the extracts in the dark at -20ºC until required. They reported that all six strains of *H. pylori*, of which five were clinical, were killed within 15 min using the aqueous ginger extract. Mahady *et al.* (2003) conducted an *in vitro* study on 19 strains of *H. pylroi*, of which five were Cag A+, using a methanol extract of the dried powdered ginger rhizome; fractions of the extract; and isolated constituents: 6-, 8- and 10-gingerol; and 6-shogoal. They noted that all 19 pathogenic and Cag A+ strains of *H. pylori* were killed by the direct effects of gingerols and root ginger. The minimum inhibitory concentration (MIC) for the methanol extract and crude extract containing gingerols ranged from 6.25-50 μg ml^{-1} and 0.78-12.5 μg ml^{-1}, respectively. In 2009 Gaus *et al.* administered a methanol extract, based on the technique by Mahady *et al.* (2003), to Mongolian gerbils. They reported that it reduced the bacterial load of *H. pylori* and significantly reduced epithelial cell degeneration and erosion induced by *H. pylori*; cryptitis; as well as acute and chronic inflammation in the mucosa and submucosa but did not increase morbidity or mortality in the Mongolian gerbils. *In vitro* and animal model studies have demonstrated that ginger extracts could play a

direct role in the suppression of key cancer and anti-inflammatory pathways (Mahady *et al.* 2003; Shukla *et al.* 2007) suggesting its use as anti-inflammatory and chemoprotective agents (Gaus *et al.* 2009).

Research on the use of parsley as an inhibitory agent is limited. O'Mahoney *et al.* (2005) and Weerasekera *et al.* (2008) demonstrated that all six clinical strains of *H. pylori* were inhibited by boiled aqueous extracts of fresh parsley.

Thyme has potent antibacterial properties with a wide spectrum of activity against antibiotic resistant strains (Hersch-Martinez *et al.* 2005). An aqueous extract of thyme was found to reduce the growth and potent urease activity of *H. pylori* strains (Tabak *et al.* 1996a). Cervenka *et al.* (2006) used methanol and chloroform extracts to evaluate the inhibitory effect against one strain each of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. They found that the chloroform extracts exhibited greater inhibition against *A. butzleri* and *A. cryaerophilus* (26.0 and 15.8 mm, respectively) than *A. skirrowii* (14.3 mm); while the methanol extract worked best for *A. butleri* and *A. skirrowii* (18.3 and 17.3 mm, respectively).

Preliminary evidence suggests that *Eletteria cardamomum* Maton (Green cardamom) may exhibit greater antibacterial properties than *Amomum subulatum* Roxb (Black cardamom) (Mahady *et al.* 2005; Zaidi *et al.* 2009). The 70% aqueous/ethanol extracts of green cardamom was shown to be ineffective against seven clinical and one reference strain of *H. pylori* even at 500 µg ml^{-1}; whereas the black form displayed complete inhibition at a concentration of 125 µg ml^{-1} (Zaidi *et al.* 2009).

The active chemical components of lemon are limonene, linalool, citral, citronellal, α-terpineol, linalyl, geranylacetate, α-terpinene, β-bisabolene, trans-α-bergamotene, nerol and neral (Saeed and Tariq 2006). In 2006, Fisher and Phillips used the disc diffusion method to evaluate the effect lemon oil and vapour, among others, has on *L. monocytogenes*, *S. aureus*, *B. cereus*, *E. coli* O157 and *C. jejuni*. Their results demonstrated that lemon oil was most effective against *L. monocytogenes* (41 ± 2 mm), followed by *B. cereus* (29 ± 1 mm), *S. aureus* (23 ± 0.6 mm), *E. coli* O157 (21 ± 0.3 mm) and least effective against *C. jejuni* (18 ± 3 mm); while the vapour was ineffective against all species tested. In addition, they also tested the survival of each microorganism, demonstrated to be susceptible in the *in vitro* study, by direct contact on cabbage leaf for 60 s; and on chicken skin for 10 min by direct contact and 24 h by vapour. Fischer and coworker found that the use of citral and linalool vapours resulted in 6 log reductions in *L. monocytogenes*, *S. aureus* and *B. cereus* populations on cabbage leaves after 8-10 h exposure (Fisher and Phillips 2006). A year later, Fisher and colleagues used the protocol to evaluate three strains of *A. butzleri*: a type strain; chicken isolate; and a water isolate (Fisher *et al.* 2007). Of all the oils tested, they noted that lemon oil was the least effective; while citral and linalool oils were highly effective against all three strains of *A. butzleri*. Lemon, citral and linalool vapours were effective over 24 h against the type strain and/or water isolates reducing populations by 8 logs in some cases; however, the chicken isolate was not susceptible to any vapours. Fisher *et al.* (2007) also noted that limonene, as an oil or vapour, exhibited no inhibitory effect on any of the tested strains.

Honey mainly consists of glucose and fructose but may also contain additional medicinal compounds such as essential oils, flavanoids, terpenes and polyphenols depending on the plant from which pollen was obtained (Molan 1992). The antimicrobial mode of action of honey involves several pathways with hydrogen peroxide content; non-peroxide and osmotic effects postulated to aid in the antimicrobial killing of honey (Allen *et al.* 1991; Al Somal *et al.* 1994). Lin *et al.* (2009) demonstrated that 20 strains of *C. jejuni* subsp. *jejuni* and 7 of *C. coli* were sensitive to 1% (v/v) manuka honey. Using the well diffusion assay, Al Somal *et al.* (1994) demonstrated that all five clinical strains of *H. pylori* (from biopsies of gastric ulcers) were inhibited by a 20% (w/v) solution of the dark aromatic Manuka honey from New Zealand. A study performed by Osato *et al.* (1999) demonstrated that the antimicrobial effect against strains of *H. pylori* was not attributed to the presence of hydrogen peroxide but due to the presence nonperoxide and osmotic mechanisms in honey. The saccharolytic effect of honey has also been demonstrated against strains of *H. pylori*. Carbohydrate solutions (≥15%) consisting of glucose; fructose; and glucose-fructose, in a 1:1:23 ratio, were found to display inhibition among all 18 *H. pylori* strains (Osato *et al.* 1999). According to Al Somal *et al.* (1994), neither hydrogen peroxide nor the osmolarity of honey had any inhibitory effect against any of the five clinical strains tested. In their study, *H. pylori* strains were inhibited by a 20% solution but not by a 40% solution of manuka honey which contains a higher content of hydrogen peroxide. Resistance of a *C. jejuni* strain against diluted preparations of natural honey has also been reported (Adebolu 2005). Ali *et al.* (1991) illustrated the antimicrobial activity of 20% honey against

antibiotic resistant isolates of *H. pylori*. However, its antimicrobial effect against infections associated with *Campylobacter* and related microorganisms have been explored in very few *in vivo* cases. In 1985, Haffejee *et al.* observed reduced symptoms in patients suffering from bacterial diarrhoea using honey.

Black cumin seeds, its oil and extracts act as an immune stimulant (Salem 2005) and have antimicrobial activity against several Gram-negative and Gram-positive bacteria, including multidrug resistant microorganisms (Topozada *et al.* 1965; El-Fatatry 1975; Morsi 2000; Aljabre *et al.* 2005). Furthermore, the diethyl extract of black cumin seeds have been shown to have a synergistic or an additive effect with several antibiotics (Hanafi *et al.* 1991). Zuridah *et al.* (2008) have shown that black cumin seeds were ineffective against extended spectrum β-lactamase (ESBL) producers most of which belong to the *Enterobacteriaceae* family such as *K. pneumonia* and some strains of *E. coli*. Mashhadian and Rakhshandeh (2005) have shown that methanol and chloroform extracts of black cumin seeds exhibited high inhibitory effects against standard and hospital strains of *C. albicans*, coagulase positive *S. aureus* and *P. aeroginosa*, while their aqueous extract was ineffective against all tested microorganisms.

Dorantes *et al.* (2000) indicated that the potent antimicromial activity of chillies are due to the presence of cinnamic and *m*-coumaric acids. Recently, investigations have shown that whole chilli or its active compound, capsaicin, can prevent ulcer formation (reviewed in Satyarayana 2006). O'Mahony *et al.* (2005) demonstrated the bactericidal activity of chillies against strains of *H. pylori*; however bacterial resistance to some chillies has previously been reported (Dorantes

et al. 2000). The *in vitro* study of Weerasekera *et al.* (2008) illustrated that an aqueous extract of chillies could kill all six *H. pylori* strains within 60 min. Furthermore, epidemiologic surveys in Singapore have demonstrated that gastric ulcers are three times more common in Chinese than Malaysians or Indians who habitually consume more chillies in their daily diet (reviewed in Satyarayana 2006).

Black pepper contains potent antimicrobials such as piperine. Ground pepper in combination with other spices have been shown to have highly potent inhibitory effects on various microorganisms as ground pepper is known to boost the activity of biochemical active compounds present in a variety of spices by up to several hundred percent (Lambert *et al.* 2004). Lambert and colleagues observed enhanced antimicrobial activity of piperine upon testing of herbal tea in mice (Lambert *et al.* 2004).

The bactericidal activities of cinnamic aldehyde and eugenol, primary antimicrobials of cinnamon, have been proven against various foodborne pathogens (Valero and Frances 2006) including *Campylobacter*, *Arcobacter* and *Helicobacter* (Tabak *et al.* 1996b; Smith-Palmer *et al.* 1998; Cervenka *et al.* 2006). Cervenka *et al.* (2006) tested the antimicrobial activity of chloroform and methanol extracts of cinnamon against one strain each of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. They noted that *A. butzleri* (30.9 mm) and *A. cryaerophilus* (41.2 mm) were greatly inhibited by the chloroform extract as opposed to the methanol extracts (19.4 mm for *A. butzleri* and 21.0 mm for *A. cryaerophilus*, respectively); while a marginal increase in inhibition was noted for *A. skirrowii* using the methanol (23.8 mm) extract as opposed to the chloroform (22.8 mm) one. Tabak *et al.* (1999) used

ethanol and methylene chloride extracts of cinnamon to evaluate the effect of cinnamon against seven strains of *H. pylori* and found that both extracts inhibited all seven strains of *H. pylori*. The methylene chloride extract inhibited growth at a concentration range of common antibiotics; while the ethanol extract was able to counteract the urease activity of *H. pylori in vitro* (Tabak *et al.* 1999). The effect of cinnamon yoghurt was tested *in vitro* on the growth of two clinical strains of *H. pylori* (Behrad *et al.* 2009). They ground barks of cinnamon to a fine powder; prepared a 1:10 ratio of fine cinnamon and distilled water prepared and incubated overnight at 70ºC; centrifuged; and the supernatant filtered through a 0.22 µm membrane filter. Thereafter, they mixed 5 g of starter culture; skim milk; milk powder; and 6% (w/v) cinnamon extract in a litre of milk. Yoghurt mixtures were dispensed into plastic cups and incubated at 41ºC to allow fermentation until pH 4.5 was reached. The *in vitro* inhibition against the growth of *H. pylori* was determined by the agar diffusion and minimum inhibitory concentration methods. A 1 ml aliquot of cinnamon yoghurt was mixed with 9 ml buffered peptone water of which 25 µl was dispensed onto standard 6 mm filter paper. They also monitored changes in pH and total titratable acids and evaluated the viability of probiotic bacteria prior and post refrigeration to determine if the addition of cinnamon could have an adverse effect on yoghurt. The water extract of cinnamon yoghurt was shown to have a strong inhibitory effect (13.5 mm) against both clinical strains of *H. pylori* without altering yoghurt fermentation (Behrad *et al.* 2009). Nir *et al.* (2000) conducted a controlled pilot study testing 15 patients (11 women and four men). Four women and three men were given 40 mg of an ethanol extract of cinnamon (75

mg ml^{-1}); while seven women and one man received placebo. The *H. pylori* colonization was measured by the ^{13}C urea breath test prior and post therapy. They concluded that the ethanol extract of cinnamon was ineffective at a concentration of 80mg day^{-1} in eradicating *H. pylori*.

Research has shown that allspice contain high concentrations of important antioxidants such as proanthocyanidins ensuring protection against a range of diseases (Young and Woodside 2001). Clove essential oil has been shown to exhibit antibacterial activity against a range of methicillin resistant microorganisms (Enzo and Susan 2002). Cervenka *et al.* (2006) tested methanol and chloroform extracts of allspice and cloves against the growth of *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. They reported that the methanol extract of allspice was more effective among the three species tested; while the methanol extract of cloves was more effective against *A. butzleri* and *A. skirrowii*.

Curcumin, a primary component of turmeric, has antibacterial and antifungal properties (Lutomski *et al.* 1974). Goel demonstrated resistance of the tested strain of *C. jejuni* against a hexane extract of turmeric (Goel 2007); while O'Mahony *et al.* (2005) and Weerasekera *et al.* (2008) demonstrated that all strains of *H. pylori* were killed within 15 min of exposure to the boiled aqueous turmeric extracts. Turmeric has also been shown to prevent gastric and colon cancers in rodents (Mahady *et al.* 2002). De *et al.* (2009) demonstrated that curcumin concentrations ranging from 5-50 μg ml^{-1} were able to inhibit the growth of all 65 Indian clinical strains of *H. pylori*. In addition, curcumin was also found to be effective in reducing gastric damage as a result of *H. pylori* infection in C57BL/6 mice (De *et al.* 2009).

Furthermore, Mitsch *et al.* (2004) demonstrated that the proliferation of *C. perfringens*, present in the intestines of live broiler chickens, could be controlled by using two blends of primary components of essential oils. The first blend of their study consisted of thymol; eugenol; curcumin; and piperine which are primary components of thyme; clove; turmeric; and black pepper, respectively. In the second blend, half the thymol was substituted with carvacrol; while all other components remained unchanged. However, some of the primary components tested in the Mitsch *et al.* (2004) study have been investigated against a range of other microorganisms with different success rates (reviewed by Griggs and Jacob 2005).

CONCLUSION

With the help of faster; reliable; and more sophisticated techniques many more species of *Campylobacter*; *Arcobacter*; and *Helicobacter*, some of which are well known food pathogens, have been identified resulting in the rapid expansion of these genera. The techniques would help in accumulating accurate information on these microorganisms' epidemiology and prevalence in various sources. Recent studies demonstrated that *C. jejuni*, *A. butzleri* and *H. pylori* could survive over longer than expected periods in food and water suggesting it as a potential route for infection. The rapid emergence of strains resistant to key antibiotics, used in the treatment of infections, has resulted in alternative strategies being sought for treating infections. Some strategies have shown promising results *in vitro* and should be earmarked for further testing in an *in vivo* setting.

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Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples

ABSTRACT

Aim: To evaluate a new method to remove PCR inhibitors from poultry DNA samples to detect *Campylobacter* by multiplex PCR in the presence of a competitive Internal Amplification Control. **Methods and Results:** Agarose-genomic DNA slants were prepared in a 1:1 ratio; mixed and set at a slant; and washed in three consecutive steps at 50^{o}C with lysis buffer, TE buffer and distilled water, respectively, to remove PCR inhibitors from poultry DNA samples (spiked versus unspiked poultry tissues and blood). The efficacy of this method was tested against two published methods. The target DNA and internal amplification control were successfully amplified by multiplex PCR with the new method but not with the two published methods. **Conclusion:** The new method significantly reduced PCR inhibitors allowing for the successful amplification of target DNA and internal amplification control. **Significance and Impact of Study:** The new method could be used to successfully detect pathogens in poultry.

Keywords: *Campylobacter*, Internal Amplification Control, PCR, poultry.

INTRODUCTION

The consumption of contaminated food is globally recognized as the main cause of human gastroenteritis of which *Campylobacter* species, *C. jejuni* and *C. coli*, are

commonly associated (Friedman *et al.* 2000; Frost 2001). It has been reported that the handling and consumption of undercooked poultry meat are two of the primary sources of human *Campylobacter* infections in industrialized countries such as the U.S.A (Friedman *et al.* 2000). Over the years, microbiological quality controls have been enforced throughout the food supply chain in an effort to minimize the risk of infection to consumers (Mateo *et al.* 2005). However, conventional standardized methods for the detection of food pathogens are primarily based on enrichment procedures; isolation of presumptive colonies on solid media; and biochemical identification of these colonies to the species level, all of which may take weeks (Corry *et al.* 1995; Mateo *et al.* 2005). In some cases the faster growing microorganisms may shield or outgrow the pathogen of interest rendering these isolation procedures ineffective. The food industry requires rapid results to cut the costs of testing for the presence of food pathogens. Since the introduction of the polymerase chain reaction (PCR) this technique has globally been recognized as a rapid, sensitive and specific diagnostic tool for the analysis of microorganisms in clinical, environmental and food samples (Lantz *et al.* 2000). However, PCR sensitivity may drastically be reduced with biological samples such as blood, faeces, tissues and food due to the presence of various PCR inhibitors (Rådström *et al.* 2004). A range of PCR inhibitors have been identified from various sources, including natural components in raw samples such as haeme (Akane *et al.* 1994) and leukocyte DNA (Morata *et al.* 1998) in blood; added anticoagulants such as EDTA (Wang *et al.* 1992) and heparin (Satsangi *et al.* 1994); immunoglobulin G in human plasma (Abu Al-Soud *et al.* 2000); organic and phenolic compounds, fats, polysaccharides, proteins, glycogen and Ca^{2+} in food (Wilson 1997; Rossen *et al.* 1992);

humic acid and heavy metals in environmental samples; constituents of bacterial cells, non-target DNA; glove powder; laboratory plasticware; cellulose and pollen (Wilson 1997). PCR inhibitors can interfere with cell lysis, a vital process of DNA extraction; nucleic acid degradation or capture; block or inactivate the thermostable DNA polymerase (Wilson 1997; Abu Al-Soud and Rådström 1998) resulting in partial or complete inhibition of the PCR assay (Moreira 1998). These false-negative results suggest the absence of target microorganisms limiting the full diagnostic potential of PCR and may have important implications for clinical and public health investigations, particularly in food and environmental screening (Wilson 1997). Various pre-PCR processing strategies have been developed to remove or reduce the negative effects of PCR inhibitors (Rådström *et al.* 2004). These include: aqueous two phase-system (Lantz *et al.* 1994); boiling (Olcén *et al.* 1995); density gradient centrifugation (Lindqvist *et al.* 1997); dilution (Chernesky *et al.* 1997); DNA extraction methods (Klein *et al.* 1997); enrichment media (Wernars *et al.* 1991); filtration (DiMichele and Lewis 1993); immunological methods (Fluit *et al.* 1993; Monteiro *et al.* 2001a); and using various DNA polymerases resistant to PCR inhibitors such as *Tfl* and *Tth* (Wiedbrauk *et al.* 1995; Abu Al-Soud and Rådström 1998). However, most of these interventions are costly to perform. Although the agarose-DNA embedded method, based on Moreira (1998), may be a bit time-consuming it could be more practical for research purposes in developing countries instead of using other expensive immunological methods or costly DNA polymerases such as *Tth*. The difference in size between DNA and PCR inhibitors such as polysaccharides allows their efficient removal from the agarose-DNA embedded slants through diffusion during successive

washing steps, while the genomic DNA (gDNA) remain trapped within the agarose slants (Monteiro *et al.* 2001b). The agarose-embedded DNA slants are then used as a template for amplification. An internal amplification control (IAC) is encouraged as a standard for PCR detection to illustrate that optimal PCR conditions are present in every tube (Lübeck *et al.* 2003; Josefsen *et al.* 2004), thus eliminating the speculation of false-negative results. The IAC may be constructed using a competitive or non-competitive strategy (Hoorfar *et al.* 2004). In the competitive IAC strategy, the target fragment and IAC are amplified with a common set of primers in the same PCR tube under the same conditions. (Siebert and Larrick 1992). Here, the IAC concentration is critical as there is always competition for the amplification of target DNA and the IAC. If target DNA is amplified but not the IAC then it is assumed that the target DNA is present in a greater concentration relative to the IAC (Hoorfar *et al.* 2004). In the non-competitive IAC strategy, the target DNA and IAC are amplified with two different sets of primers in two different PCR's but optimized to work in the same PCR conditions. This may result in sub-efficient PCR for one or both reactions (Hoorfar *et al.* 2004). The aim of this study was to evaluate a new method to remove PCR inhibitors from poultry DNA samples to detect *Arcobacter*, *Campylobacter* and *Helicobacter* by multiplex PCR in the presence of a competitive IAC.

MATERIALS AND METHODS

Preparation of gDNA

DNA from pure cultures of *Campylobacter* were extracted using a modified hexadecyltrimethyl ammonium bromide (CTAB) method (Wilson 1994) and used as a

positive control, while DNA from various tissue and blood samples were extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.) or a DNA extraction technique (Moreira 1998).

Hexadecyltrimethyl ammonium bromide (CTAB) method

The original CTAB method was modified. One to two loopfuls of 2-3 day old pure bacterial culture was resuspended in 567 µl of Tris-EDTA (TE) buffer (Appendix A) in a sterile 2 ml eppendorf tube (Eppendorf, Germany). Thereafter, 30 µl of 10% SDS (Appendix B) and 3 µl of 20 mg ml^{-1} of proteinase K (Appendix B) were added to give a final concentration of 100 µg ml^{-1} of proteinase K in 0.5% SDS. Samples were incubated at 37°C for 1 h using a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) to lyze the bacterial cell walls. A volume of 100 µl of 5 mol l^{-1} NaCl was added to each sample and mixed to prevent a CTAB-nucleic acid precipitate from forming. Thereafter, 80 µl of CTAB/NaCl solution (Appendix B) was added, mixed and incubated for 10 min at 65°C. An equal volume of 24:1 chloroform/isoamyl alcohol (Appendix B) was added, mixed and centrifuged at 10 000 g for 7 min at ambient room temperature (±22°C) (RT) in an Eppendorf centrifuge 5417C to remove CTAB-protein/polysaccharide complexes. Supernatants were transferred to sterile 2 ml eppendorf tubes. Equal volumes of 25:24:1 phenol/chloroform/isoamyl alcohol were added and centrifuged at 10 000 g for 7 min at RT to remove the remaining CTAB precipitate. Supernatants were transferred to sterile 2 ml eppendorf tubes, 0.6 volumes of isopropanol added to precipitate the DNA and centrifuged at 10 000 g for 7 min at RT. The DNA was washed with 200 µl of 70%

EtOH (Appendix B) to remove residual CTAB solution followed by centrifugation at 10 000 g for 10 min at RT. Pellets were resuspended in 100–500 µl of TE buffer, depending on the size of the pellet, for 2 days at 4°C. In rare cases where the pellets were not totally resuspended after the 2 day incubation period, 100 µl of TE buffer was added and incubated at 37°C for 30 min to allow the compact mass of DNA to loosen and resuspend in TE buffer. Tubes were gently pulsed to increase the rate of resuspension and if necessary returned to 37°C for another 30 min. For short-term storage, DNA was stored at 4°C to prevent shearing or degradation of DNA due to consecutive freeze-thaw processes. For long-term storage, the DNA was stored at -20°C.

Sample DNA extractions

DNA from each sample was extracted in duplicate. DNA from chicken liver and "mala" (intestinal part of the chicken) tissue and blood samples were extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.) and a DNA extraction technique (Moreira 1998). In the DNA extraction technique, a 2 ml aliquot of spiked sample or a 2 ml aliquot of unspiked sample, previously homogenized in a Laboratory Blender Stomacher 400 (Seward Medical London SE1 1PP U.K., England), was dispensed into sterile 15 ml tubes (Greiner, Germany) to which 5 volumes of 0.125 mol l^{-1} EDTA (pH 8.0) (Appendix B) were added and allowed to shake overnight at 37°C in a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) to detach the bacterial cells from tissues, blood and organic debris. Thereafter, a low-speed centrifugation step was carried out at 1500 g for 10 min at 37°C to remove remaining

debris. Supernatants were transferred to sterile 15 ml tubes and centrifuged at 10 000 g for 10 min at 37°C to collect all bacterial cells. Supernatants were discarded and pellets resuspended in 100 µl aliquots of 10 mmol l^{-1} Tris-HCl (pH 8.0) (Appendix B) and 1 mol l^{-1} NaCl (Appendix B).

Construction of a competitive Internal Amplification Control (IAC)

The IAC, designed to amplify under multiplex PCR conditions, was constructed by deleting a homologous fragment in the 16S rRNA gene of the *Arcobacter* genus by the strategy of Denis *et al.* (1999). This was achieved by amplifying the 16S rRNA gene with mutagenic primer, primer1, (5'-GTG GAG TAC AAG ACC CGG GAA TTG CGC TCG TTG CGG GAC TTA AC-3') where the underlined sequence corresponds to the Reverse primer of Neubauer and Hess (2006). Multiplex PCR with primers ARCOB1 and primer 1 produced a 580 bp product instead of a 822 bp product. This 580 bp mutagenic product was cloned into the pGEM-T Easy vector (Promega, USA) (Appendix C) and transformed into *Escherichia coli* DH5-α cells. A Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Inc., U.S.A) was used to measure DNA concentration.

Screening of competitive IAC clones

Clones were screened by colony PCR. Positive clones were prepared for midi scale plasmid preparations, reamplified and sequenced at the Core Sequencing Unit at the University of Stellenbosch. Clones displaying the successful insertion of the mutagenic product into the pGEM-T Easy vector were used as a competitive IAC. A 10-fold

dilution series of the competitive IAC was prepared and amplified under multiplex PCR conditions to determine the minimum inhibitory concentration.

Removal of PCR inhibitors

Various techniques are currently used to remove PCR inhibitors from samples but a combination of techniques may prove to be more effective as found in this study.

The adapted Moreira (1998) method entailed using a 50 µl aliquot of each DNA sample dispensed into sterile 2 ml eppendorf tubes to which 50 µl of melted 1.6% Low Melting Point (LMP) (Promega, U.S.A.) agarose (Appendix A) was added, mixed and set in a mould for a few minutes. Aliquots of 1 ml lysis buffer (0.01 mol l^{-1} Tris, 0.5 mol l^{-1} EDTA (pH 9.2), 1% Lauroyl sarcosine and 2 mg ml^{-1} proteinase K solution) (Appendix A) were added and incubated overnight at 50°C with gentle shaking in a G24 Environmental Incubator shaker. Lysis buffer was discarded and followed by two washing steps, each with 2 ml TE buffer gently shaking for 5 h. Fragments of 5 µl were cut with a sterile surgical blade and used as templates for multiplex PCR.

In the Monteiro *et al.* (2001b) method, the agarose-embedded DNA moulds were transferred to sterile 15 ml Greiner tubes and washed in 10 ml TE buffer overnight at 50°C with gentle shaking. TE buffer was discarded and followed with a second wash consisting of 5 ml distilled water for 2 h at 50°C with gentle shaking. Thereafter, 5 µl fragments were used as templates for multiplex PCR.

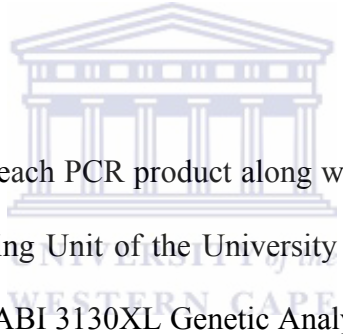
In the new method a 1:1 ratio of LMP agarose and gDNA was prepared, mixed and allowed to set at a slant in 2 ml round-bottomed eppendorf tubes. The agarose-embedded DNA slants were transferred to sterile 15 ml Greiner tubes and washed in

three consecutive steps. A volume of 1 ml of lysis buffer was added and incubated overnight at 50°C with gentle shaking in a G24 Environmental Incubator shaker. Lysis buffer was discarded and replaced with 10 ml TE buffer per slant and incubated overnight at 50°C with gentle shaking. Lastly, TE buffer was replaced with 5 ml distilled water and incubated for 2 ½ h at 50°C with gentle shaking. Thereafter, 5 µl fragments were each cut with a sterile surgical blade and used as template in multiplex PCR.

Multiplex PCR

Multiplex 16S rRNA gene primers were designed by Neubauer and Hess (2006) for the simultaneous detection of *Arcobacter*, *Campylobacter*, and *Helicobacter* in a reaction tube. The forward and reverse primers, listed below, were tested to simultaneously detect *Arcobacter*, *Campylobacter*, and *Helicobacter* by multiplex PCR (figure 1). This was done to detect possible multiple infections of the target microorganisms in poultry. Reactions were performed in a 50 µl mixture of 0.8 mmol l^{-1} dNTP (Promega, U.S.A.), 1x Flexi buffer (Promega, U.S.A.), 1.5 mmol l^{-1} $MgCl_2$ (Promega, U.S.A.), 0.2 µmol l^{-1} of each forward (ARCOB1, CAMPCJL1 and HELIP2) and reverse (Reverse and primer 1) primer and 0.025 U of GoTaq Flexi DNA polymerase (Promega, U.S.A.). A positive control (*C. jejuni*) or sample DNA (spiked or unspiked) and 3.4 ng $µl^{-1}$ of competitive IAC DNA were added to the designated reaction tubes and mixed. Multiplex PCR was performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Singapore) with the following programme: one cycle of 5 min at 94°C; 30 cycles, each, consisting of 2 min at 94°C, 1 min at 65°C, 1 min at 72°C; and a final extension step of

10 min at 72°C (Neubauer and Hess 2006). Aliquots of 10 µl of each PCR product were mixed with 0.5 µl of 6x loading dye; loaded into wells of a 2% EtBr-agarose gel (12.5 ng ml^{-1}) and electrophoresed at 85 V for 1 h alongside a molecular weight marker. A final concentration of 2 ng ml^{-1} of Ethidium bromide (EtBr) was mixed with 1x TBE buffer, at the bottom-end of the submerged gel, to ensure its upward transfer and efficient intercalation within DNA. DNA was visualized under an ultra violet light source of 302 nm in a MultiImage Lightcabinet and photographed using an AlphaImager HP camera (AlphaInnotech, U.S.A.) and the AlphaEase FC AlphaImager 3400, Ink for Windows (AlphaInnotech, U.S.A.) programme.



Sequence analysis

A volume of 25–35 µl of each PCR product along with 1.1 µmol l^{-1} of each primer was sent to the Core Sequencing Unit of the University of Stellenbosch where all samples were sequenced using an ABI 3130XL Genetic Analyzer (Applied Biosystems, U.S.A.). A post-PCR cleanup was requested and performed by the sequencing unit on each PCR sample prior to sequencing. DNA sequences were manually corrected using the Bio Edit sequence alignment editor programme (version 5.0.2., 1999). The BLAST tool function for nucleotide sequences (blastn) in the National Centre for Biotechnology Information (NCBI) database was used to determine whether the DNA of *Campylobacter* or the mutagenic fragment of *Arcobacter* (IAC) was amplified. Positive products displayed the following sizes: 946 bp and 580 bp fragments for the *Campylobacter*-spiked samples and IAC, respectively.

RESULTS

Ability to detect possible multiple infections of *Arcobacter, Campylobacter* and *Helicobacter* in poultry DNA samples

Figure 1 illustrates the ability of the primers, designed by Neubauer and Hess (2006), to detect multiple infections of *Arcobacter*, *Campylobacter* and *Helicobacter* in poultry samples.

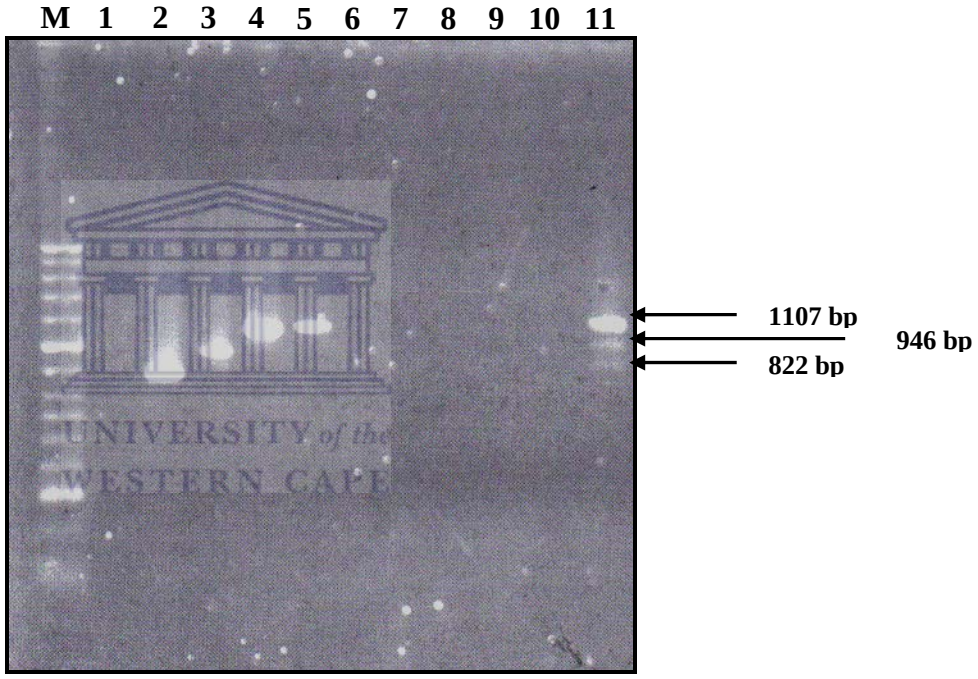


Fig. 1: A 2% agarose gel depicting the amplification of the reference fragments of *Arcobacter* (822 bp), *Campylobacter* (946 bp) and *Helicobacter* (1107 bp). Lane M: Hyperladder II marker; lane 1: negative control; lane 2: *A. butzleri*; lane 3: *C. jejuni*; lane 4: *H. fennelliae*; lane 5: spiked poultry sample with *H. fennelliae*; lanes 6-10: unspiked samples; lane 11: spiked poultry sample with *A. butzleri*, *C. jejuni* and *H. fennelliae*. The gel was electrophoresed at 85 V for 1 h.

Minimum Inhibitory Concentration (MIC) for the competitive IAC

A 10-fold serial dilution was performed to determine the MIC for the competitive IAC when coamplified with a target fragment. Figure 2 illustrates the amplification of the

580 bp fragment of IAC in lanes 2-7, while lanes 2 and 3 display coamplification of the target fragment of *C. jejuni* (946 bp) and IAC (580 bp). These lanes correspond to the undiluted and 10^{-1} dilution of the IAC corresponding to 34.0 ng μl^{-1} and 3.4 ng μl^{-1} of the IAC, respectively. Figure 2 suggests that 3.4 ng μl^{-1} of the IAC would be the best concentration to use to allow coamplification of the target fragment and IAC. The ± 700 bp fragments, coamplified in lanes 4-7, appear to be a result of non-specific amplification.

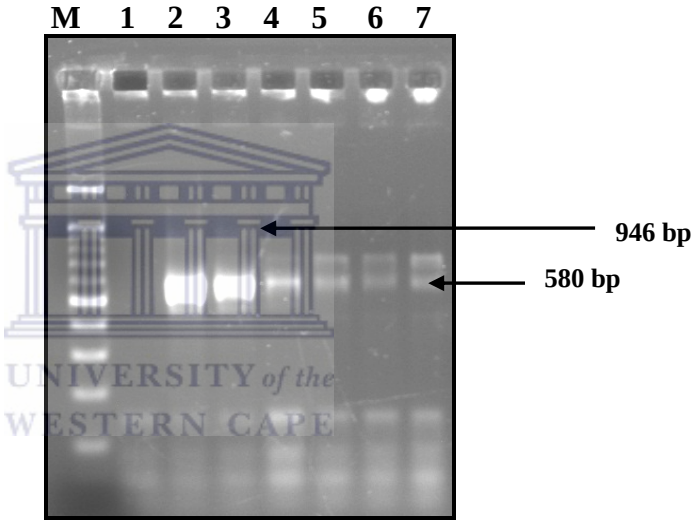


Fig. 2: A 2% agarose gel depicting the amplification of the 580 bp fragment of the competitive IAC in lanes 2-7, while the target fragment (946 bp of *Campylobacter*) was only amplified in lanes 2 and 3. Lane M: 100 bp marker; lane 1: negative control; lane 2: undiluted IAC; lane 3: 10^{-1} IAC; lane 4: 10^{-2} IAC; lane 5: 10^{-3} IAC; lane 6: 10^{-4} IAC and lane 7: 10^{-5} IAC. The gel was electrophoresed at 85 V for 1 h.

Amplification of gDNA samples

A concentration of 3.4 ng μl^{-1} of IAC was added to each raw sample (spiked and unspiked), multiplex PCR performed and electrophoresed on a 2% agarose gel. Figure 3 illustrates the successful amplification of the IAC in the raw gDNA samples, extracted

with a Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.); however, no target DNA was amplified due to the presence of PCR inhibitors.

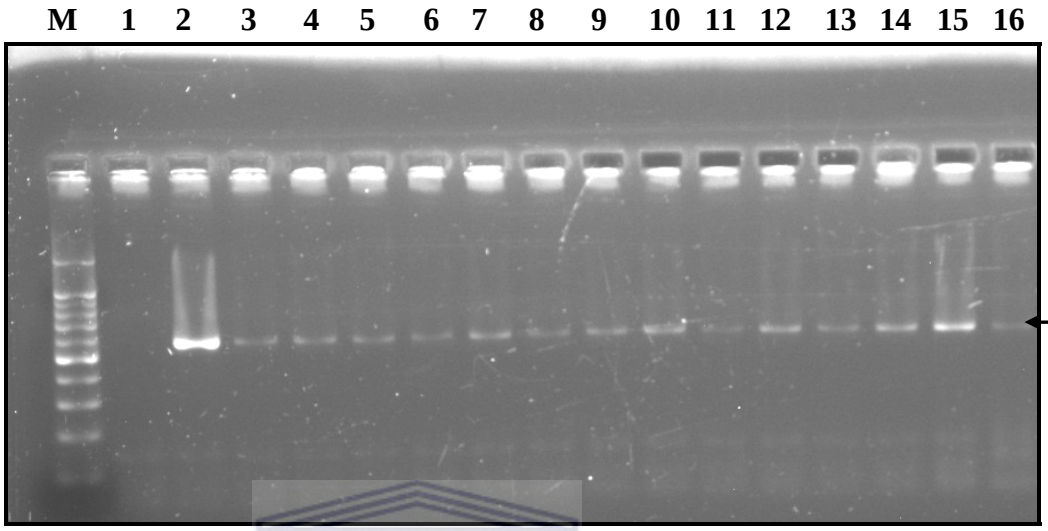


Fig. 3: Representative 2% agarose gel illustrating the effect of PCR inhibitors on the amplification of DNA extracted with a Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.). Only the 580 bp product of the IAC has been amplified. Lane M: 100 bp marker; lane 1: negative control; lane 2: 3.4 ng μl^{-1} of IAC. The following lanes 3-16 illustrate the amplification of the IAC only in the IAC/sample tubes. Lane 3: spiked "mala" sample; lane 4: spiked "mala" contents sample; lane 5: spiked chicken liver sample; lane 6: spiked chicken blood sample: lanes 7-10: unspiked "mala" samples; lanes11-12: unspiked "mala" contents sample; lanes 13- 14: unspiked chicken liver samples; and lanes 15-16: unspiked chicken blood samples. The gel was electrophoresed at 85 V for 1 h.

Removal of PCR inhibitors

The same samples were tested for each of the methods. Using the Moreira (1998) method it can be seen, in figure 4, that although the IAC was successfully amplified, no target DNA was detected in any of the spiked samples suggesting the presence of PCR inhibitors.

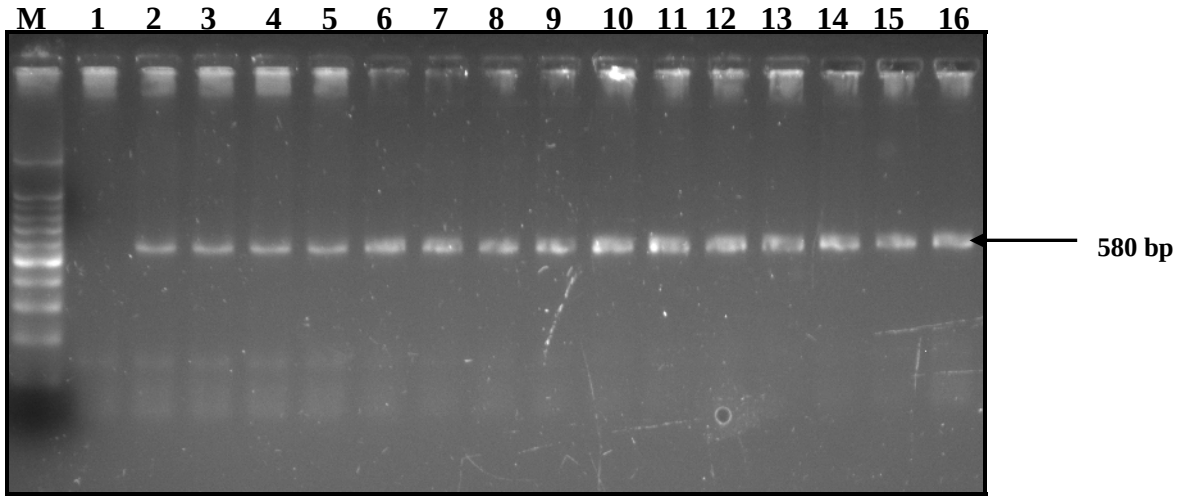


Fig. 4: Representative 2% agarose gel illustrating the effect of PCR inhibitors on the amplification of DNA extracted with the Moreira method. Only the 580 bp fragment of the IAC was successfully amplified. Lane M: 100 bp marker; lane 1: negative control; lane 2: 3.4 ng μl^{-1} of IAC; lane 3: spiked "mala" sample; lane 4: spiked "mala" contents sample; lane 5: spiked chicken liver sample; lane 6: spiked chicken blood sample: lanes 7-10: unspiked "mala" samples; lanes11-12: unspiked "mala" contents sample; lanes 13-14: unspiked chicken liver samples; and lanes 15-16: unspiked chicken blood samples. The gel was electrophoresed at 85 V for 1 h.

Results from the Monteiro method, figure 5, illustrates the successful amplification of the IAC in all samples but again no target DNA was amplified suggesting the presence of PCR inhibitors.

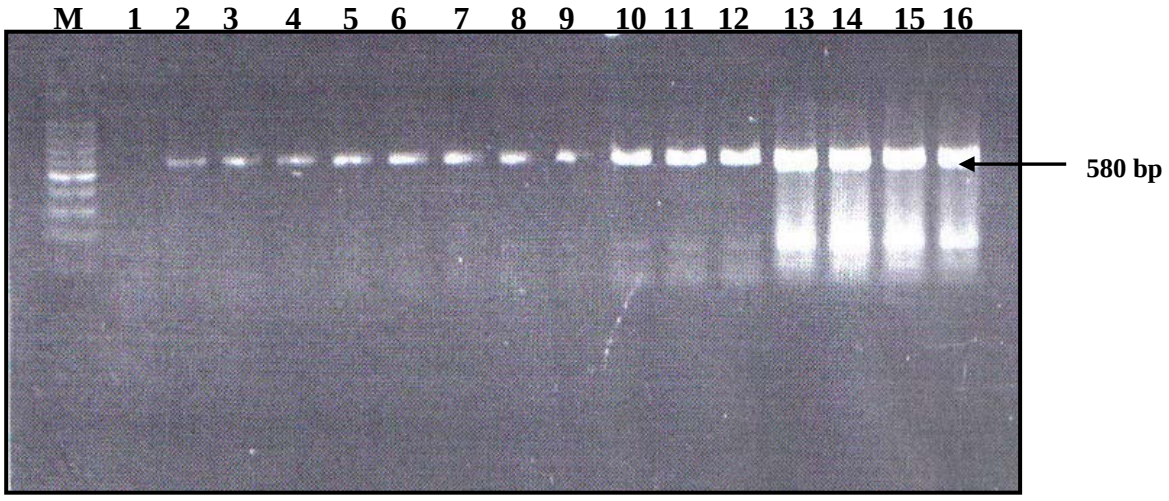


Fig. 5: A 2% agarose gel illustrates the effect of PCR inhibitors on DNA extracted by the Monteiro method; only the 580 bp product of the IAC was amplified. Lane M: 100 bp marker; lane 1: negative control; lane 2: 3.4 ng μl^{-1} of IAC; lane 3: spiked "mala" sample; lane 4: spiked "mala" contents sample; lane 5: spiked chicken liver sample; lane 6: spiked chicken blood sample: lanes 7-16: unspiked samples. The gel was electrophoresed at 85 V for 1 h.

The new method, figure 6, illustrates the removal of PCR inhibitors from the extracted DNA samples by the successful amplification of the IAC (580 bp) in all samples; as well as the target DNA (*C. jejuni*: 946 bp) in the spiked samples.

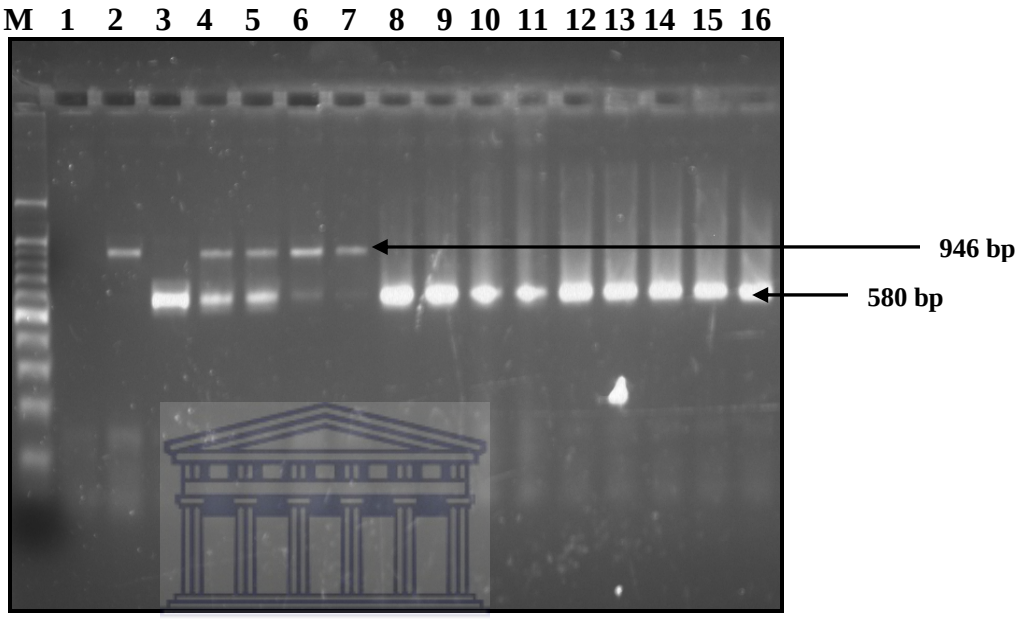


Fig. 6: A 2% agarose gel illustrating the removal of PCR inhibitors using the new method. This method allowed successful amplification of the IAC (580 bp) as well as the target fragment of *C. jejuni* (946 bp) in the spiked samples. Lane M: 100 bp marker; lane 1: negative control; lane 2: (positive control) 1.92 µg μl^{-1} of *C. jejuni* gDNA; lane 3: 3.4 ng μl^{-1} of IAC; lane 4: spiked "mala" sample; lane 5: spiked "mala" contents sample; lane 6: spiked chicken liver sample; lane 7: spiked chicken blood sample: lanes 8-16: unspiked samples. The gel was electrophoresed at 85 V for 1 h.

DISCUSSION

PCR inhibitors may interfere with PCR, at several stages, resulting in partial or complete inhibition of the amplification of samples (Moreira 1998). PCR inhibitors can interfere with cell lysis, a vital process of DNA extraction; nucleic acid degradation or capture; block or inactivate the thermostable DNA polymerase (Wilson 1997; Abu Al-Soud and Rådström 1998) resulting in partial or complete inhibition of the PCR assay

(Moreira 1998). This study suggests that the thermostable DNA polymerase activity could have been blocked by the PCR inhibitors in poultry blood and chicken products as the samples were only amplified using the new method as opposed to the Moreira (1998) and Monteiro *et al.* (2001b) methods.

False-negative results cannot always be linked to the presence of PCR inhibitors as internal amplification controls are seldom incorporated in each reaction tube (Wilson 1997). The competitive IAC strategy, used in this study, may have resulted in a decreased PCR efficiency and lowered detection limit of the target DNA (Hoorfar *et al.* 2004) as illustrated in figure 2. In theory, when two targets of varying sizes are coamplified; the reaction kinetics should be driven towards the amplification of the smaller product (Sachadyn and Kur 1998). This was confirmed in this study. However, studies by Brightwell *et al.* (1998) and Abdulmawjood *et al.* (2002) have provided evidence indicating that the amplification of the target fragment is not hindered when using an IAC of less than 500 bp. It was also possible to use the non-competitive IAC strategy where the target DNA and IAC are coamplified with two different sets of primers in two different PCR's and optimized to work in the same PCR conditions. However, this strategy is more difficult to optimize and often results in sub-efficient PCR for one or both reactions (Hoorfar *et al.* 2004).

The agarose-embedded DNA strategy, based on Moreira (1998), requires few steps and manipulations to remove PCR inhibitors from samples thereby reducing the risk of contamination by foreign DNA (Monteiro *et al.* 2001b) and false-negative results.

The successful amplification of target fragments, in the spiked samples after using the new method to clean up PCR inhibitors in the samples (figure 6) confirm that the large-size gDNA fragments were retained within the agarose matrix, while the smaller fragments and soluble contaminants diffused out of the agarose-DNA slants (Moreira 1998). Furthermore, this study suggests that the new method (figure 6) efficiently removes PCR inhibitors from poultry DNA samples allowing the successful detection of target DNA in comparison to the Moreira (1998) and Monteiro *et al.* (2001b) methods, shown in figures 4 and 5, respectively. However, it is important to note that the samples used in these studies were different to those of this study. In the Moreira (1998) study, soil samples were analyzed; while the Monteiro *et al.* (2001b) study tested human stool samples for the presence of *H. pylori*. The type of PCR inhibitors present is determined by the samples tested. Soil samples are naturally contaminated with polysaccharides and humic acids (Moreira 1998), while faecal samples contain PCR inhibitors such as acid polysaccharides, metabolic products and abundant non-target DNA (Monteiro *et al.* 1997). The poultry samples of this study may have contained PCR inhibitors such as haeme (Akane *et al.* 1994) and leukocyte DNA (Morata *et al.* 1998) in poultry blood; and organic compounds, fats, proteins, polysaccharides, glycogen, non-target DNA and constituents of bacterial cells (Wilson 1997; Rossen *et al.* 1992) in the chicken products such as liver and "mala". Failure of the Monteiro *et al.* (2001b) method could have been contributed by the anticoagulant, EDTA, present in the TE buffer used to wash the agarose-DNA embedded slants from poultry blood and chicken products (Wang *et al.* 1992) together with other PCR

inhibitors. It may also be that more stringent washing agents were required to successfully remove PCR inhibitors from poultry blood and chicken products.

It would be interesting to test if the new technique could successfully be applied to different foods including: poultry, beef, seafood, diary products; blood; as well as to different water and environmental samples.

In conclusion, this new method efficiently removed PCR inhibitors from spiked chicken products and poultry blood thus allowing the detection of target DNA and the IAC in these samples. This technique could be used to ensure the safety of poultry by adequately detecting pathogens which may ultimately result in a reduced risk of contracting various foodborne illnesses and infections.

Acknowledgements

Sincere gratitude is extended to Mr. C. Jacobs for the competent cells. Sincere gratitude is also extended to the Department of Biotechnology of the University of the Western Cape, National Research Foundation (NRF) of South Africa, Muslim Hands of South Africa and the South African National Zakah Fund (SANZAF) for funding.

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Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken

ABSTRACT

Aim: To detect and isolate *Arcobacter* and *Campylobacter* in chicken products and blood. **Methods and Results:** A total of 670 retail chicken products (livers, gizzards, "mala" (intestines), "mala" contents and neck skins); 25 chlorine-washed "free-range"/organic chicken livers; 25 chlorine-free "free-range"/organic chicken livers; 50 "Free-range"/organic chicken blood; 60 "battery" chicken blood; and 30 ostrich blood samples were analyzed by conventional culturing methods and multiplex polymerase chain reaction (PCR) in the presence of an internal amplification control (IAC) after PCR-inhibitor removal. Positive samples and isolates were identified by 16S rRNA gene sequencing and amplified fragment length polymorphism (AFLP) analysis. Three *Arcobacter* species were detected in chicken: *A. cibarius* in liver; *A. cryaerophilus* in caeca; and *A. butzleri* in "mala" contents and "free-range"/organic chicken blood. *A. butzleri* was also isolated from one "free-range" chlorine-free organic chicken liver sample; while two *C. jejuni* subsp. *jejuni* biotype II isolates were isolated from "free-range"/organic chicken blood samples. No *Campylobacteraceae* species were found in any of the ostrich blood samples. **Conclusion:** Although, no *Campylobacter* species were found in any of the retail chicken products tested; three species of *Arcobacter* were. The isolation of *C. jejuni* subsp. *jejuni* from chicken blood is a novel finding, indicating a possible reservoir. **Significance and Impact of Study:** This study suggests that

chicken may be a reservoir to various species of *Arcobacter*. The isolation of two *C. jejuni* subsp. *jejuni* biotype II isolates suggests the potential of "free-range"/organic chicken blood as a reservoir.

Keywords: *Arcobacter*, blood, *Campylobacter*, chicken, isolate, PCR.

INTRODUCTION

Microorganisms belonging to the genera of *Campylobacter* and *Arcobacter* are Gram-negative and display a spiral or rod shape (Prescott *et al.* 1996). *Campylobacter* species grow under microaerophilic conditions, requiring low O_2 levels in the range of 2-10% for growth (Prescott *et al.* 1996). *Arcobacter* species are aerotolearant capable of growing at normal atmospheric conditions and at temperatures ranging between 15-30ºC (Vandamme *et al.* 1991). Due to similarities within the phylogenetic and phenotypic characteristics of *Campylobacter* and *Arcobacter*, they are collectively placed within the *Campylobacteraceae* family (Vandamme and De Ley 1991). Over the years *Campylobacter* species have increasingly become a significant public health threat (Skirrow *et al.* 1993; Mead *et al.* 1999; Ethelberg *et al.* 2005; Sinclair *et al.* 2005; Stewart *et al.* 2005; Valliant *et al.* 2005). *C. jejuni* is noted as the main bacterial pathogen causing diarrhoea in humans (Corry and Atabay 2001). Although the frequency of campylobacteriosis in humans is increasing, infections are seldom fatal. However, some infections may result in severe complications such as Guillain Barré syndrome, neuropathy and reactive arthritis (Park *et al.* 1991; Lastovica 2006). *Arcobacter* species such as *A.*

butzleri and *A. skirrowii* have been associated with bacteraemia, chronic diarrhoea and enteritis (Vandamme *et al.* 1992a; Vandamme *et al.* 1992b; Lastovica and Skirrow 2000; Wybo *et al.* 2004). The handling and consumption of raw or undercooked poultry meat is recognized as the main source of human campylobacteriosis (Deming *et al.* 1987; Potter *et al.* 2003; Luber *et al.* 2005). Some *Arcobacter* infections may be transmitted in a similar manner (Corry and Atabay 2001; Phillips 2001; Ceelen *et al.* 2006) as these microorganisms have mainly been recovered from poultry meat (Atabay and Corry 1997; Atabay *et al.* 1998; Wesley and Baetz 1999; Houf *et al.* 2005). Species of *Campylobacteraceae* have been identified in ostrich: livers, cloacal swabs, carcasses (Stephens *et al.* 1998; Ley *et al.* 2001; Cuomo *et al.* 2007); avian intestines (Oyarzabal *et al.* 1995) in chicken: skins, livers, carcases, caeca and in the colon (Wempe *et al.* 1983; Atabay and Corry 1997; Atabay *et al.* 1998; Houf *et al.* 2005). They have also been found in other sources, including: milk, beef, pork, seafood, sewage/sludge effluent as well as ground and stagnant water (Blaser 1982; Lovett *et al.* 1983; Stampi *et al.* 1993; Collins *et al.* 1996; de Boer *et al.* 1996; Wesley 1996; Endtz *et al.* 1997; Gude *et al.* 2005; Scullion *et al.* 2006). Isolation procedures are often time-consuming with limited results. Routine identification of these microorganisms is difficult as they have fastidious growth requirements (Kiehlbauch *et al.* 1991). Due to several morphological and biochemical similarities, incorrect identification of species of *Campylobacteraceae* may occur when solely relying on conventional plating techniques and phenotypic tests (González *et al.* 2000). Due to the specificity; reliability; and rapid ability of the polymerase chain reaction (PCR), the

technique is extensively applied as an alternative to conventional microbiological culture methods to detect specific microorganisms in water, food and environmental samples (Hill 1996). However, in some cases it is essential to isolate the microorganism to do further analysis particularly when a new species is speculated and antibiotic susceptibility information is required. Thus it is advisable to use PCR concurrently with isolation procedures to correctly determine the species of microorganism present in a sample. The natural presence of inhibitory compounds in a sample may affect the PCR resulting in false-negative results (Cone *et al.* 1992; Dickinson *et al.* 1995; Wilson 1997). Hence, the use of an internal amplification control in PCR is recommended to avoid false-negative results (Ursi *et al.* 1992; Ballagi-Pordany and Belak 1996; Lübeck *et al.* 2003; Josefsen *et al.* 2004). The aim of this study was to detect and isolate *Arcobacter* and *Campylobacter* in chicken produce and blood.

MATERIALS AND METHODS

Revitalization of reference strains

A list of the reference strains used in this thesis is shown in Appendix E. A100 µl aliquot of sterile 2xYeast Tryptone (2xYT) broth (Oxoid, U.K.) (Appendix B) was added to 2 ml sterile Eppendorf tubes (Eppendorf AG, Germany) to which three PL160M Microbank Beads (Microbank™ Pro-Lab Diagnostics, Canada) of clinical strains of *A. butzleri*, *C. jejuni* subsp. *jejuni* biotype I or *C. jejuni* subsp. *jejuni* biotype II were added using a sterile loop. The Eppendorf tubes were allowed to shake at 37ºC in a G24 Environmental Incubator shaker (New Brünswick Scientific

Co., Inc., U.S.A.) for 10-15 min to lift the bacteria from the beads into the 2xYT broth. The suspension was aseptically dispensed; drop wise, onto tryptose blood agar (TBA) (Oxoid, U.K.) (Appendix B) plates, incubated at ambient room temperature (±22ºC) (RT) for 30 min to allow the drops to dry. The TBA plates were inverted and incubated for 2-3 days at 37ºC in a H_2-enriched microaerophilic atmosphere generated by an Oxoid BR 0038B gas generating kit (Oxoid, U.K.).

Samples Tested

A total of 495 "battery" retail chicken samples were tested from one company, in the Western Cape region of South Africa, consisting of: 210 livers, 25 gizzards, 160 "mala" (intestines) and 100 "mala" contents. In contrast, chicken tissue samples from another company, in the Western Cape region of South Africa, accumulated to a total of 175 samples consisting of: 160 livers and 15 neck skins. Twenty-five chlorine-washed "free-range"/organic and 25 chlorine-free "free-range"/organic chicken livers were also tested. In addition, 30 ostrich; 50 "battery" chicken; and 60 "free-range"/organic chicken blood samples were analyzed.

Isolation of *Campylobacteraceae*

Membrane filters and 1x Phosphate Buffer Saline (PBS) (pH 7.2) (Appendix B) were tested for possible sources of contamination prior to all experiments. All experimental and positive control tissue and "mala" contents samples were prepared for testing using a combination of two techniques (Ceelen *et al.* 2006; Lastovica 2006). In the experiment, a 20% (w/v) suspension of sample and sterile 1xPBS (pH

7.2) were prepared; while the positive controls were prepared in a similar manner and spiked with a 10 µl loopful of 2-3 day old *A. butzleri* and *C. jejuni* subsp. *jejuni* culture.

The chicken tissue samples were analyzed in duplicate, one without enrichment and one using an enrichment step. All experiments were based on the techniques of Ceelen *et al.* (2006) and Lastovica (2006), briefly described below. All experimental and positive control samples were homogenized in a Laboratory Blender Stomacher 400 (Seward Medical London SE1 1PP U.K., England) for 2 min on normal blending speed. Thereafter, 500 µl of homogenate was filtered through a sterile 0.6 µm cellulose acetate membrane filter (Schleicher and Schuell MicroScience, Germany), pre-absorbed onto a TBA plate. Without inversion, the TBA plates with filters were incubated for 1 h at 37ºC with 5% CO_2, generated by a CO_2 Gen sachet (Oxoid, U.K.) in a gas jar in a Scientific Series 9000 incubator (LASEC: Laboratory and Scientific Equipment Co., Pty, Ltd., R.S.A.). The filters were removed, filtrates streaked with a sterile loop, plates inverted and incubated at 37^{o}C in the presence of a BR 0038B gas generating kit (Oxoid, U.K) for 2-3 days.

Chicken tissue samples analyzed by an enrichment step were firstly homogenized in a Laboratory Blender Stomacher 400 for 2 min on normal blending speed. This was followed by an enrichment technique whereby a 1/100 dilution of homogenate and *Arcobacter* enrichment broth (AEB) (Oxoid, U.K.) (Appendix B) was prepared for each sample and incubated with overnight shaking at 250 rpm in sterile 200 ml glass bottles in a G24 Environmental Incubator shaker. A volume of 200 µl of AEB was aseptically dispensed onto the surfaces of each semi-dried TBA

plate and spread across the surfaces with a sterile glass spreader. These plates were allowed to air-dry in a laminar flow for 15-20 min. Thereafter, 500 µl volumes of enriched culture were filtered through 0.6 µm cellulose acetate membrane filters, pre-absorbed onto allocated TBA plates. All filters were removed within 20 min, the filtrates allowed to dry on the TBA surfaces, plates inverted and incubated as described above.

In the analysis of chicken "mala" contents, the experimental samples consisted of a volume of 10 ml of "mala" contents squeezed into a sterile 50 ml tube (Greiner, Germany), using two sterile forceps and topped up to 50 ml of 1xPBS (pH 7.2) to make a 20% (v/v) suspension. Samples were mixed thoroughly by gently inverting the tubes by hand, followed by filtration and incubation (described above).

All blood experiments were only analyzed post-treatment of TBA plates with AEB. For the analysis of ostrich and chicken blood, 9 ml blood samples were transported with ice-packs by courier from laboratories in Oudtshoorn (ostrich blood) and Stellenbosch (chicken blood) to Cape Town in LH Lithium Heparin tubes (Greiner bio-one, Germany). A total of 30 ostrich and 110 chicken (50 "battery' and 60 organic) blood samples were analyzed. Four methods were used to analyze the blood samples: spreading; streaking; filtration and squirting to determine the technique that was best suited to test the blood samples. One TBA plate was provided per control and experimental blood sample. A 200 µl aliquot of AEB was added and spread across the surfaces of each TBA plate and allowed to dry for 30 min at RT prior to adding the sample. Two positive controls were used in each technique. For each positive control, a 10 µl sterile loop was used to scrape

together colonies of either 2-3 day old *A. butzleri* or *C. jejuni* subsp. *jejuni* strain grown on TBA plates, and added to 1.5 ml of blood in allocated 2 ml eppendorf tubes. Homogeneous mixtures of each control were created by vortexing for a few seconds.

In the spreading technique a volume of ± 250 µl of positive control (described above) or experimental blood sample was dispensed onto and spread across the surface of a TBA plate using a sterile glass spreader. The plates were allowed to dry before inversion and incubated at 37°C in a H_2-enriched microaerophilic atmosphere generated by a BR 0038B gas generating kit for 2-3 days.

In the streaking technique a 10 µl sterile loop was swirled around in the positive control (described above) or experimental blood samples and streaked onto the allocated TBA plates.

In the squirting technique a volume of 500 µl of experimental or positive control (described above) blood sample was squirted onto the surfaces of the TBA plates, allowed to dry at RT for 30 min and incubated 37°C in a H_2-enriched microaerophilic atmosphere generated by a BR 0038B gas generating kit for 2-3 days.

For the direct filtration technique, each positive control (described above) and experimental blood sample was directly passed through 0.6 µm cellulose acetate membrane filters onto the surfaces of allocated TBA plates.

Phenotypic and Biochemical characterization of isolates

The "Cape Town protocol" (Lastovica 2006) (Appendix D) was used to isolate and identify species of *Campylobacteraceae* from retail chicken produce and "free-range"/organic chicken blood. Based on the 16S rRNA gene sequencing results of the isolates, the expected results of the following species of *Arcobacter* and *Campylobacter* were used as a guide: *A. butzleri* for isolate 10.1; and *C. jejuni* subsp. *jejuni* biotypes I and II for isolates 7.2 and 7.4. During phenotypic characterization, colonies resembling the known morphology of *Campylobacter* and *Arcobacter* were picked and streaked on fresh TBA plates for single colonies. Generally, *Campylobacter* colonies appear beige in colour and grow in H_2-enriched microaerophilic conditions, while *Arcobacter* can grow in aerobic conditions and appear to be lighter in colour than *Campylobacter* (Lastovica 2006). Selected colonies were cultured and subsequently analyzed by Gram staining and biochemical tests to determine if the bacteria belonged to the genera of *Campylobacter* or *Arcobacter*. The latter was determined by biochemical analysis of each cultured isolate.

The following biochemical tests were performed on each cultured isolate to identify them to the species level: indoxyl acetate; nitrate reductase; catalase; pyrazinamide; aryl sulfatase; oxidase; hippurate; rapid H_2S and TSI Agar. In addition, the following antibiotics were also tested against the growth of the cultured isolates: cephalothin (30 μg $disk^{-1}$) and nalidixic acid (30 μg $disk^{-1}$).

The growth of pure cultures were maintained every second or third day and incubated at 37ºC in a H_2-enriched microaerophilic atmosphere generated by using

an Oxoid BR 0038B gas generating kit placed in a clean gas jar. Cultures intended for long-term storage were stored on beads in Microbank tubes as per manufacturer's instructions (Microbank^{TM} Pro-Lab Diagnostics, Canada).

Preparation of genomic DNA (gDNA)

The gDNA from pure cultures of *Campylobacter* and *Arcobacter* were extracted using a modified hexadecyltrimethyl ammonium bromide (CTAB) method (Wilson 1999), described below, and used as a positive control. The gDNA from various tissue and blood samples were extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.) or by a DNA extraction technique based on Moreira (1998).

CTAB method

The original CTAB method was modified. One to two loopfuls of 2-3 day old pure bacterial culture was resuspended in 567 µl of Tris-EDTA (TE) buffer (Appendix A) in a sterile 2 ml eppendorf tube (Eppendorf, Germany). Thereafter, 30 µl of 10% SDS (Appendix B) and 3 µl of 20 mg ml^{-1} of proteinase K (Appendix B) were added to give a final concentration of 100 µg ml^{-1} of proteinase K in 0.5% SDS. Samples were incubated at 37ºC for 1 h using a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) to lyze the bacterial cell walls. A volume of 100 µl of 5 mol l^{-1} NaCl (Appendix B) was added to each sample and mixed to prevent a CTAB-nucleic acid precipitate from forming. Thereafter, 80 µl of CTAB/NaCl solution (Appendix B) was added, mixed and incubated for 10 min at 65ºC. An equal volume of 24:1 chloroform/isoamyl alcohol (Appendix B) was

added, mixed and centrifuged at 10 000 g for 7 min at RT in an Eppendorf centrifuge 5417C to remove CTAB-protein/polysaccharide complexes. Supernatants were transferred to sterile 2 ml eppendorf tubes. Equal volumes of 25:24:1 phenol/chloroform/isoamyl alcohol (Appendix B) were added and centrifuged at 10 000 g for 7 min at RT to remove the remaining CTAB precipitate. Supernatants were transferred to sterile 2 ml eppendorf tubes, 0.6 volumes of isopropanol added to precipitate the gDNA and centrifuged at 10 000 g for 7 min at RT. The gDNA was washed with 200 µl of 70% EtOH (Appendix B) to remove residual CTAB solution followed by centrifugation at 10 000 g for 10 min at RT. Pellets were resuspended in 100-500 µl of TE buffer, depending on the size of the pellet, for 2 days at 4ºC. In rare cases where the pellets were not totally resuspended after the 2 day incubation period, 100 µl of TE buffer was added and incubated at 37ºC for 30 min to allow the compact mass of gDNA to loosen and resuspend in TE buffer. Tubes were gently pulsed to increase the rate of resuspension and if necessary returned to 37ºC for another 30 min. For short-term storage, gDNA was stored at 4ºC to prevent shearing or degradation of gDNA due to constant freeze-thaw processes. For long-term storage, the gDNA was stored at -20ºC.

Sample gDNA extractions

The gDNA from each sample was extracted in duplicate. The gDNA from various tissue and blood samples were extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, U.S.A.) and a DNA extraction technique (Moreira 1998). DNA extracted using the Qiagen DNeasy Blood and Tissue kit were carried out as per

manufacturers manual (Qiagen, U.S.A.). In the Moreira-based (1998) DNA extraction technique, 20% (w/v) suspensions of sample and 1x PBS (pH 7.2) (Appendix B) were prepared and homogenized in a Laboratory Blender Stomacher 400 (Seward Medical London SE1 1PP U.K., England). Thereafter, 2 ml aliquots of spiked and unspiked samples were dispensed into allocated sterile 15 ml tubes (Greiner, Germany) to which 5 volumes of 0.125 mol l^{-1} EDTA (pH 8.0) (Appendix B) were added. These tubes were incubated, shaking overnight, at 37ºC in a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) to detach the bacterial cells from tissues, blood and organic debris. A low-speed centrifugation step was carried out at 1500 g for 10 min at 37ºC to remove remaining debris. Supernatants were transferred to sterile 15 ml tubes and centrifuged at 10 000 g for 10 min at 37ºC to collect all bacterial cells. Supernatants were discarded and pellets resuspended in 100 µl aliquots of 10 mmol l^{-1} Tris-HCl (pH 8.0) (Appendix B) and 1 mol l^{-1} NaCl (Appendix B).

Construction of a competitive Internal Amplification Control (IAC)

The IAC, designed to amplify under multiplex PCR conditions, was constructed by deleting a homologous fragment in the 16S rRNA gene of the *Arcobacter* genus by the strategy of Denis *et al.* (1999). This was achieved by amplifying the 16S rRNA gene with mutagenic primer, primer1: 5'-GTG GAG TAC AAG ACC CGG GAA TTG CGC TCG TTG CGG GAC TTA AC-3', where the underlined sequence corresponds to the Reverse primer (Neubauer and Hess 2006). Multiplex PCR with primers ARCOB1 and primer 1 produced a 580 bp product instead of a 822 bp

product. This 580 bp mutagenic product was cloned into the pGEM-T Easy vector (Promega, USA) (Appendix C) and transformed into competent *Escherichia coli* DH5-α cells (supplied by Mr. C. Jacobs). A Nanodrop^{TM} 1000 spectrophotometer (Thermo Fisher Scientific, Inc., U.S.A) was used to measure DNA concentration.

Screening of competitive Internal Amplification Control (IAC) clones

Clones were screened by colony PCR. Positive clones were prepared for midi scale plasmid preparations, reamplified and sequenced at the Core Sequencing Unit at the University of Stellenbosch. Clones displaying the successful insertion of the mutagenic product into the pGEM-T Easy vector were used as a competitive IAC. A 10-fold dilution series of the competitive IAC was carried out and amplified together with positive samples, under multiplex PCR conditions, to determine the minimum inhibitory concentration of the IAC.

Removal of PCR inhibitors

This method was based on the Moreira (1998) and Monteiro *et al.* (2001) techniques. It entailed using a 50 µl aliquot of each gDNA sample dispensed into sterile 2 ml eppendorf tubes to which 50 µl of melted 1.6% Low Melting Point (LMP) agarose (Promega, U.S.A.) (Appendix A) was added. These were mixed thoroughly and allowed to solidify at a slant for a few minutes. The slants were transferred to sterile 15 ml Greiner tubes and washed in 1 ml lysis buffer (0.01 mol l^{-1} Tris, 0.5 mol l^{-1} EDTA (pH 9.2), 1% Lauroyl sarcosine and 2 mg ml^{-1} proteinase K solution) (Appendix A) overnight at 50ºC with gentle shaking in a G24

Environmental Incubator shaker. Lysis buffer was discarded and replaced with 10 ml TE buffer per slant and incubated overnight at 50ºC with gentle shaking. TE buffer was replaced with 5 ml distilled water and incubated for 2 ½ h at 50ºC with gentle shaking. Thereafter, 5 µl fragments were cut with sterile surgical blades and used as gDNA templates in multiplex PCR.

Multiplex PCR

Multiplex 16S rRNA gene primers were designed by Neubauer and Hess (2006) for the simultaneous detection of *Campylobacter*, *Arcobacter* and *Helicobacter* in a reaction tube. However, the concentrations of the PCR reagents used in this study differed to those used in the Neubauer and Hess (2006) study. Reactions were performed in a 50 µl mixture of 0.8 mmol l^{-1} dNTP (Promega, U.S.A.), 1x Flexi buffer (Promega, U.S.A.), 1.5 mmol l^{-1} $MgCl_2$ (Promega, U.S.A.), 0.2 µmol l^{-1} of each forward (HELIP2, CAMPCJL1 and ARCOB1) and reverse (Reverse and primer 1) primer and 0.025 U of GoTaq Flexi DNA polymerase (Promega, U.S.A.). Positive control (*C. jejuni* subsp. *jejuni*) or sample gDNA (chicken tissue or poultry blood) and 3.4 ng μl^{-1} competitive IAC DNA was added to designated reaction tubes and mixed. Multiplex PCR was performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Singapore) with the following programme: one cycle of 5 min at 94ºC; 30 cycles, each, consisting of 2 min at 94ºC, 1 min at 65ºC, 1 min at 72ºC; and a final extension step of 10 min at 72ºC (Neubauer and Hess 2006). Aliquots of 10 µl of each PCR product were mixed with 0.5 µl of 6x loading dye; loaded into wells of a 2% Ethidium Bromide (EtBr)-agarose gel (12.5 ng ml^{-1})

and electrophoresed at 85 V for 1 h alongside a molecular weight marker. A final concentration of 2 ng ml^{-1} of EtBr was mixed with 1x Tris-Borate EDTA (TBE) buffer, at the bottom-end of the submerged gel to ensure its upward transfer and efficient intercalation within DNA. DNA was visualized under an ultra violet light source of 302 nm in a MultiImage Lightcabinet and photographed using an AlphaImager HP camera (AlphaInnotech, U.S.A.) and the AlphaEase FC AlphaImager 3400, Ink for Windows (AlphaInnotech, U.S.A.) programme. Positive products displayed the following sizes: 580 bp; 946 bp; and 822 bp fragments for the IAC, *Campylobacter* and *Arcobacter*-spiked samples, respectively.

Sequence analysis

A volume of 25-35 µl of each PCR product along with 1.1 µmol l^{-1} of each primer was sent to the Core Sequencing Unit of the University of Stellenbosch where all samples were sequenced using an ABI 3130XL Genetic Analyzer (Applied Biosystems, U.S.A.). A post-PCR cleanup was requested and performed by the sequencing unit on each PCR sample prior to sequencing. DNA sequences were manually corrected using the Bio Edit sequence alignment editor programme software version 5.0.2. (1999). The nucleotide BLAST tool function for nucleotide sequences (blastn) in the National Centre for Biotechnology Information (NCBI) database was used to determine whether the DNA of *Campylobacter* or *Arcobacter* genera was amplified. Thereafter, the 16S rRNA gene sequences of the reference strains and positive DNA samples were aligned and Bootstrap N-J phylogenetic trees generated using the ClustalX software version 2.0.11 (Larkin *et al.* 2007) and

Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1 (Tamura *et al.* 2007) programmes.

Amplified Fragment Length Polymorphism analysis of samples

The Amplified Fragment Length Polymorphism (AFLP) technique is based on the selective amplification of genomic DNA fragments that have been digested with restriction enzyme(s) (Vos *et al.* 1995). This technique incorporates three steps: digestion of DNA with restriction enzyme(s) and the ligation thereof with oligonucleotide adapters; selective amplification of restriction digested fragments; and gel analysis of the amplified fragments (Vos *et al.* 1995). In essence, the reliability of the Restriction Fragment Length Polymorphism (RFLP) technique (Botstein *et al.* 1980; Tanksley *et al.* 1989) is combined with the rapid, sensitive PCR technique (Mullis and Faloona 1987; Saiki *et al.* 1988; Ehrlich *et al.* 1991; Vos *et al.* 1995) The AFLP fingerprinting protocol of this study was adapted from Gibson *et al.* (1998) and González *et al.* (2007b). Briefly, 2 µg of gDNA was digested with 5 U of *Hind*III (Roche Diagnostics GmbH, USA) in SuRE/Cut buffer B (10 mmol l^{-1} Tris-HCl, 5 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} 2-Mercaptoethanol) (pH 8.0) provided with the enzyme; 5 mmol l^{-1} spermidine trihydrochloride (Sigma, Switzerland); and sterile distilled water, all of which were added in a final volume of 20 µl and incubated at 37ºC for 2 h in a Scientific Series 9000 incubator. A 5 µl aliquot of digested gDNA was incubated with 10 µmol l^{-1} of each adapter: ADH1 (5' ACGGTATGCGACAG 3') and ADH2 (5' AGCTCTGTCGCATACCGTGAG 3') (Gibson *et al.* 1998); 3 U of *T4*-DNA ligase

(10 mmol l^{-1} Tris-HCl (pH 7.4), 50 mmol l^{-1} KCl, 1 mmol l^{-1} DTT, 0.1 mmol l^{-1} EDTA, 50% glycerol) (Promega, USA) in the ligation buffer (300 mmol l^{-1} Tris-HCl (pH 7.8), 100 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} DTT, 10 mmol l^{-1} ATP) provided; and sterile distilled water, all of which were added in a final volume of 20 µl and incubated at 37ºC for 3 h in a Scientific Series 9000 incubator. Thereafter, the ligation mix was incubated in a water bath at 80ºC for 10 min to inactivate the *T4*-DNA ligase enzyme. A volume of 5µl of ligated gDNA sample was used, as template, in a PCR mix containing: 10x PCR buffer; 2.5 mmol l^{-1} $MgCl_2$, dNTPs solution (10 mmol l^{-1} each), 10 µmol l^{-1} of HIG primer (5' GGTATGCGACAGAGCTTG 3') (Gibson *et al.* 1998) and 1.25 U of GoTaq DNA Polymerase (Promega, U.S.A.) made up to a final volume of 50µl with autoclaved sterile distilled water. The PCR parameters were as follows: an initial denaturation step at 94ºC for 4 min; followed by 33 cycles, each consisting of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2.5 min; a final primer extension step at 72ºC for 2 min and a 4ºC storage step to avoid DNA degradation. Aliquots of 10 µl of each PCR product were mixed with 0.5 µl of 6x loading dye; loaded into wells of a 2% EtBr-agarose gel (12.5ng μl^{-1}) and electrophoresed at 85 V for 1 h alongside a molecular weight marker. A final concentration of 2 ng ml^{-1} of EtBr was mixed with 1x TBE buffer, at the bottom-end of the submerged gel to ensure its upward transfer and efficient intercalation within DNA. DNA was visualized under an ultra violet light source of 302 nm in a MultiImage Lightcabinet and photographed using an AlphaImager HP camera (AlphaInnotech, U.S.A.) and the AlphaEase FC AlphaImager 3400, Ink for Windows (AlphaInnotech, U.S.A.) programme.

RESULTS

One *A. butzleri* isolate from a chlorine-free "free-range"/organic chicken liver and two *Campylobacter jejuni* subsp. *jejuni* biotype II isolates from four "free-range"/organic chicken blood samples were obtained. All were isolated on TBA plates pre-treated with AEB.

Phenotypic and Biochemical characterization of isolates

Gram staining; indoxyl acetate; nitrate reductase; catalase; pyrazinamide; aryl sulfatase; oxidase; hippurate; rapid H_2S and TSI agar tests were done. Motility of the strains was not tested in this study. Based on sequencing of the 16S rRNA gene, results were compared to those expected for *A. butzleri*, *C. jejuni* subsp. *jejuni* biotypes I and II (Lastovica 2006) to identify the species of each isolate. Single antibiotic disks were used to assess the effects against the growth of all isolates. All isolates were Gram negative.

Table 1: Identification of isolates according to the "Cape Town" Protocol (Lastovica 2006)

Isolate/ Reference Strain	Ind. Ac.	N.R.	Cat.	Pyraz.	Aryl Sulf.	Oxid.	Hipp.	Rapid H_2S	TSI Agar	Antibiotic Testing
C. jejuni jejuni biotype I	+	+	+	+	-	+	+	-	-	KF^R; Nal^S
C. jejuni jejuni biotype II	+	+	+	+	-	+	+	+	+	KF^R; Nal^S
Isolate 7.2	+	+	+	+	-	+	+	+	+	KF^S; Nal^S
Isolate 7.4	+	+	+	+	-	+	+	+	+	KF^R; Nal^S
A. butzleri	+	+	+	-	-	+	-	-	+	KF^R; Nal^R
Isolate 10.1	+	+	+	-	-	+	-	-	+	KF^R; Nal^S

Key:

Ind. Ac: Indoxyl acetate	Oxid.: Oxidase	^{R}: Resistant
N.R.: Nitrate Reductase	Hipp.: Hippurate	^{S}: Sensitive
Cat.: Catalase	TSI: Triple Sugar Iron	
Pyraz.: Pyrazinamide	KF: Cephalothin	
Aryl Sulf.: Aryl Sulfatase	NA: Nalidixic Acid	

Multiplex PCR of samples with competitive IAC

The IAC was co-amplified with naturally contaminated *Arcobacter* or *Campylobacter* samples by multiplex PCR in retail chicken produce (tissue) and blood samples. The minimal detection limit for the IAC was determined to be 3.4 ng μl^{-1} (data not shown). Figure 1 depicts the *Arcobacter*-positive amplified products in the tissues and blood; as well as the *Arcobacter butzleri* isolate (10.1) isolated from a chlorine-free "free-range"/organic chicken liver sample. Although some non-specific bands were present in lanes 4-6 of figure 1, these did not interfere with the sequencing reaction. Figure 2 illustrates the positive amplified products of *Campylobacter* isolates (7.2 and 7.4) in chicken blood.

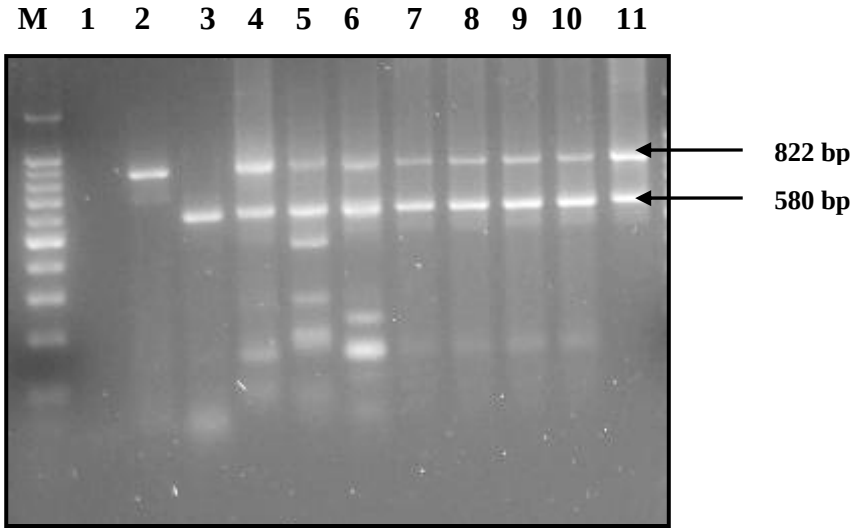

Fig. 1: A 2% agarose gel depicting the amplification of competitive IAC and target fragment of *Arcobacter*. The 580 bp fragment of the IAC was amplified in lanes 3-11, while the 822 bp target fragment of *Arcobacter* was amplified in lanes 2 and 4-11. Lane M: 100 bp marker; lane 1: negative control; lane 2: positive control (*A. butzleri*); lane 3: IAC; lane 4: MC8+IAC; lane 5: L2+IAC; lane 6: cc1+IAC; lane 7: R5+IAC; lane 8: W3+IAC; lane 9: W4+IAC; lane 10: W5+IAC and lane 11: Isolate 10.1+IAC. The gel was electrophoresed at 85 V for 1 h.

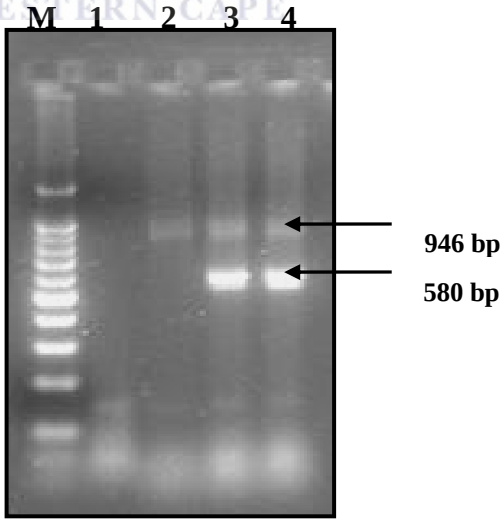

Fig. 2: A 2% agarose gel depicting the amplification of the competitive IAC and target fragment of *Campylobacter*. The 580 bp fragment of the competitive IAC was detected in lanes 3 and 4, while the 946 bp target fragment of *Campylobacter* was detected in lanes 2-4. Lane M: 100 bp marker; lane 1: negative control; lane 2: positive control (*C. jejuni* subsp. *jejuni*); lane 3: isolate 7.2+IAC and lane 4: isolate 7.4+IAC. The gel was electrophoresed at 85 V for 1 h.

Phylogenetic tree based on the 16S rRNA gene of *Arcobacter*

Figure 3, illustrates the phylogenetic tree based on the 16S rRNA gene sequences of *Arcobacter*. PCR-positive samples from retail chicken produce; a chlorine-free "free-range"/organic chicken liver isolate and some *Arcobacter* reference strains were grouped with *W. succinogenes* ATCC 29543 set as the root.

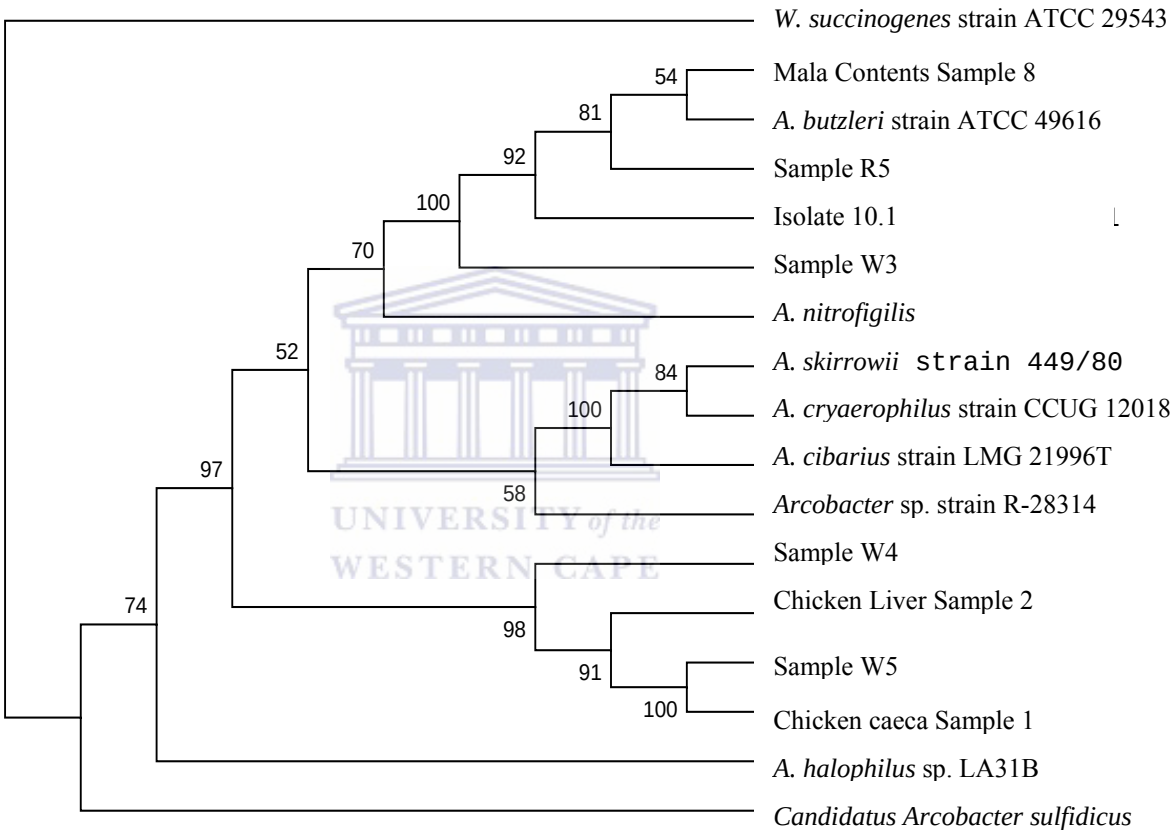

Fig. 3: A 16S rRNA phylogenetic tree of *Arcobacter*. The phylogenetic tree includes multiplex PCR-positive *Arcobacter* tissue (mala contents sample 8; chicken liver 2; chicken caeca 1) and blood samples (R5; W3 and W4); isolate 10.1 and some *Arcobacter* reference strains obtained from the NCBI database. *W. succinogenes* ATCC 29543 was set as the root.

Phylogenetic tree based on the 16S rRNA gene of *Campylobacter*

Figure 4, illustrates the 16S rRNA gene phylogenetic tree of *Campylobacter*. The *C. jejuni* subsp. *jejuni* biotype II isolates, 7.2 and 7.4, were grouped separately from the NCBI *Campylobacter* reference strains.

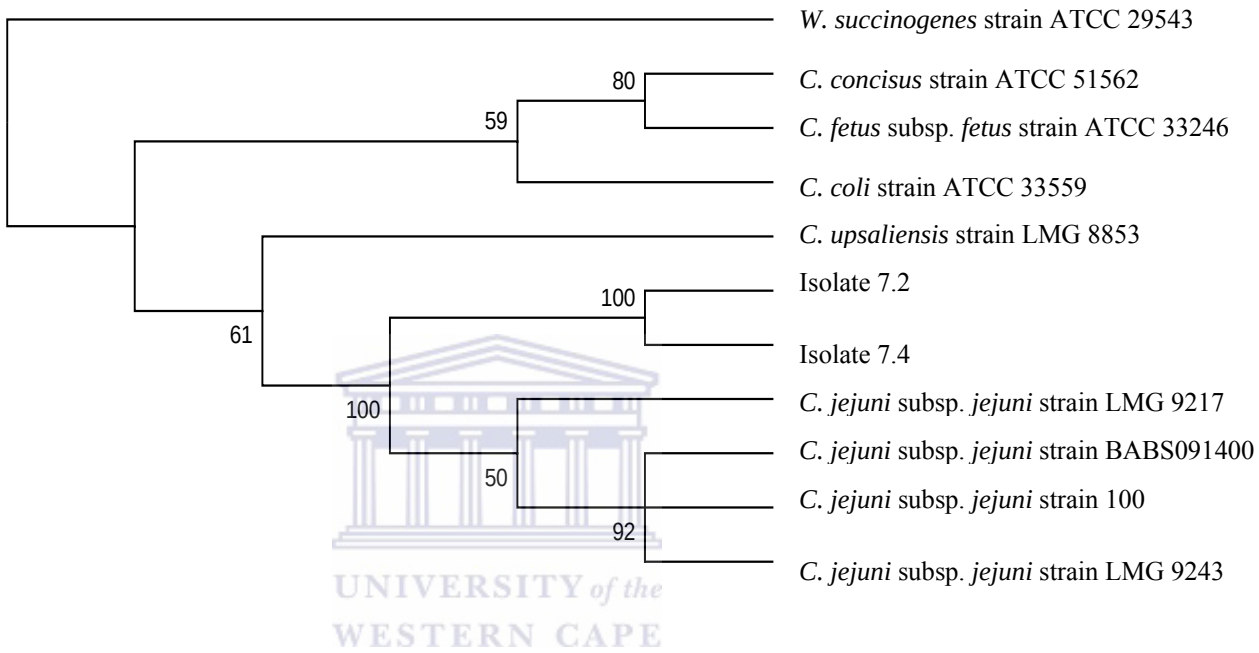

Fig. 4: A 16S rRNA phylogenetic tree of *Campylobacter*. The phylogenetic tree includes the two *Campylobacter jejuni* subsp. *jejuni* isolates (7.2 and 7.4) and some *Campylobacter* reference strains obtained from the NCBI database. *W. succinogenes* ATCC 29543 was set as the root.

AFLP analysis of positive samples and isolates from chicken products and blood

The AFLP profile patterns in figures 5A and 5B were generated using a single restriction enzyme, *Hind*III. The AFLP profile patterns of *Arcobacter* identified in chicken tissues; free-range/organic chicken blood samples; and reference strains of *A. butzleri*, *A. buzleri*-like and *A. cryaerophilus* are illustrated in figure 5A. These patterns consist of 4-11 fragments, ranging in size from 400 to ±1520 bp. Figure 5B

depicts the AFPL profile patterns of two *Campylobacter jejuni* subsp. *jejuni* isolates from free-range/organic chicken blood and reference strains of *C. jejuni* subsp. *jejuni* biotype I and *C. jeuni* subsp. *jejuni* biotype II. These patterns consisted of 4-8 fragments, ranging from ±400-1200 bp.

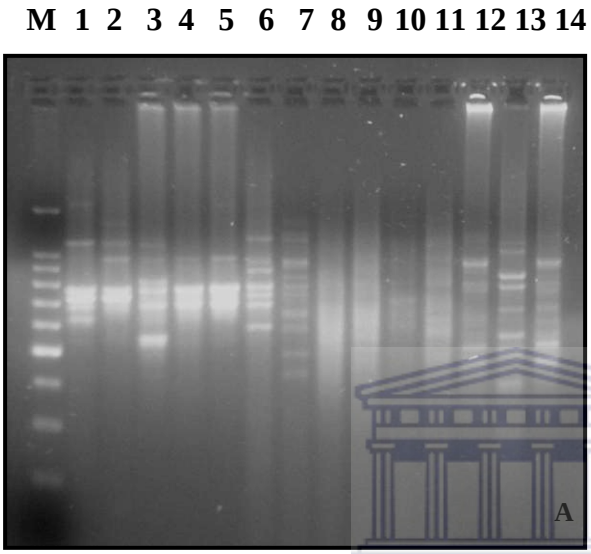

Fig. 5A: A 2% agarose gel illustrating the AFLP profile patterns of *Arcobacter* reference strains and multiplex PCR-positive samples. Lane M, 100 bp marker; lane 1, *A. butzleri* reference strain; lane 2, *A. butzleri* isolate 10.1; lanes 3-6, *A. butzleri*-like reference strains (7, 8, 10 and 12); lane 7, sample MC8; lane 8, sample R5; lane 9, sample W3; lane 10, sample W4; lane 11, sample W5; lane 12, sample cc1; lane 13, *A. cryaerophilus* reference strain; and lane 14, sample L2. AFLP products were loaded in wells of a 2.5% agarose gel and electrophoresed at 80 V for 1½ h.

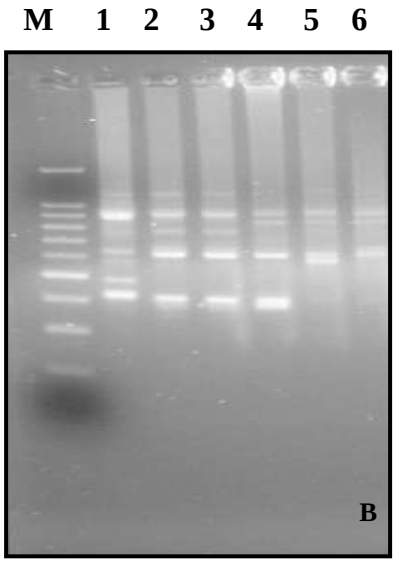

Fig. 5B: A 2% agarose gel illustrating the AFLP profile patterns of *Campylobacter* reference strains and *Campylobacter* isolates (7.2 and 7.4). Lane M, 100 bp marker; lane 1, *C. jejuni* subsp. *jejuni* biotype I reference strain; lanes 2-3, *C. jejuni* subsp. *jejuni* chicken blood isolates (7.2 and 7.4); lane 4, *C. jejuni* subsp. *jejuni* biotype II reference strain; and lanes 5-6, *C. jejuni* subsp. *jejuni* biotype I reference strains. AFLP products were loaded in wells of a 2.5% agarose gel and electrophoresed at 80 V for 1½ h.

DISCUSSION

The term "free-range" organic chicken refers to chicken reared/fed outdoors, free to move anywhere in an allocated area (en.wikipedia.org/wiki/Free_range) and are not treated with antibiotics, hormones or artificial ingredients (Wallace 2008).

In total, three isolates were isolated from chicken. Biochemical results, based on that of the "Cape Town protocol" (Lastovica 2006), indicated that the chlorine-free "free-range"/organic chicken liver isolate was an *Arcobacter butzleri*. It also indicated that the two isolates from the "free-range"/organic chicken blood samples belonged to the *Campylobacter jejuni* subsp. *jejuni* biotype II group (Lastovica 2006).

It was possible to obtain the isolates from these samples as no inhibitory agents were present, allowing *Arcobacter* and *Campylobacter* to proliferate and survive in chicken. It is generally difficult to isolate viable *Arcobacter* and other related microorganisms from retail chicken produce due to the use of chlorine washes (Hinton *et al.* 2007). Studies have shown that the culturability of *A. butzleri* and *C. jejuni* cells is lost in as little as 5 min in chlorinated water due to cell membrane damage (Blaser *et al.* 1986; Rice *et al.* 1999; Moreno *et al.* 2004). Li *et al.* (2002) has shown that high-temperature chlorine sprays (at 55ºC and 60ºC) were much more effective than similar treatments at lower temperatures (20ºC) to significantly reduce *C. jejuni* in chicken.

In some retail chicken produce ("mala" contents sample 8; caeca sample 1; liver sample 2) and "free-range"/organic chicken blood samples (R5; W3; W4 and W5) of this study, species of *Arcobacter* (represented by a 822 bp fragment in figure

1) could only be detected but not isolated. This either suggests that the viability of the *Arcobacter* cells had been lost or that the microorganism had been killed. Although *Arcobacter* was detected in one chicken caeca sample (cc1) as well as in a "mala" (intestinal) contents sample (MC8), the species identified by 16S rRNA sequencing in each sample was different. This may either suggest co-infection with two different species of *Arcobacter* in different sites of the same bird or it may be a result of two species of *Arcobacter* each infecting two different birds. The latter is more likely as only one species of *Arcobacter* was detected in each sample.

A comparison of the "mala" contents (MC8) and chicken caeca (cc1) 16S rRNA gene sequences to known sequences in the NCBI database indicated that each sample was homologous to a different species of *Arcobacter*. Sequencing results from the "mala' contents sample (MC8) illustrated a 98% homology to *A. butzleri*. The phylogenetic tree in figure 3, illustrated the grouping of MC8 with a NCBI reference strain of *A. butzleri* (ATCC 49616) suggesting that sample MC8 contained *A. butzleri* DNA. To our knowledge, this is the first time that *A. butzleri* had been detected in "mala" (intestinal) contents and in chicken blood.

The 16S rRNA gene sequencing data for the positive caeca sample displayed a greater homology to *A. cryaerophilus* than to other species of *Arcobacter*. A Pubmed search suggests that the detection of *A. cryaerophilus* in a chicken caeca sample may be a novel finding as it has only been identified in wastewater (González *et al.* 2007a; González *et al.* 2010), chicken carcasses (Atabay *et al.* 2006) and from chicken cloacal swabs (Atabay *et al.* 2006). Although the entire 16S rRNA gene sequence of a chicken caeca sample (cc1) was not homologous to *A.*

cryaerophilus, 70% of the sequence was highly homologous to it. However, species identification cannot solely be determined based on the 16S rRNA gene sequence as indicated by the contradictory results of the NCBI blastn analysis and the phylogenetic tree in figure 3.

Furthermore, 16S rRNA gene sequencing analysis suggested that the amplified product of the chicken liver sample (L2) was highly homologous (±90%) to that of *Arcobacter* sp. R-2834 strain R-2834 (NCBI database) but the phylogenetic tree (fig. 3) contradicts this. A blastn search on the NCBI database of the 16S rRNA gene of isolate 10.1 illustrated a 99% homology to *A. butzleri*. This match was confirmed by group clustering of 10.1 and *A. butzleri* reference strain in figure 3. The scattering effect of various positive tissue (chicken liver sample: L2; chicken caeca: cc1) and blood (W4 and W5) samples suggests that the 16S rRNA gene sequences may be too short for analysis resulting in the improper grouping of these samples within the phylogenetic tree (fig. 3). This is in agreement with a study where aligned sequences consisting of ±960 nucleotides were used to generate a phylogenetic tree (Gueneau and Loiseaux-De Goër 2002). This study fell short of generating bigger fragments for *Arcobacter* as the primers of Neubauer and Hess (2006) could only amplify a maximum of 822 bp.

The two *C. jejuni* subsp. *jejuni* biotype II isolates were obtained using two different techniques: one through filtration and the other by streaking onto TBA plates pre-treated with AEB. As all of the isolates (one of *A. butzleri* and two of *C. jejuni* subsp. *jejuni* biotype II) were isolated using these plates, it suggests that AEB

media may be used to enhance the chances of recovery of both *Arcobacter* and *Campylobacter* species. However, further work is required to confirm this.

The presence of *C. jejuni* subsp. *jejuni* in chicken blood is a novel finding. Both *Campylobacter* isolates were isolated from "free-range"/organic chicken blood but none were isolated from any of the "battery" chicken or ostrich blood samples. This may be a direct effect of antibiotic treatment of "battery" chickens and ostriches in an effort to prevent disease (Wallace 2008). Preliminary *Campylobacter* isolates were confirmed by multiplex PCR (fig. 2). Although the sequencing data, based on the 16S rRNA gene, could clearly identify the isolates as *C. jejuni* subsp. *jejuni* using the NCBI blastn search function, it could not identify the biotype class of the isolates. Both of the *C. jejuni* subsp. *jejuni* isolates, identified by biochemical testing, are grouped together but separately from the *C. jejuni* subsp. *jejuni* reference strains obtained from the NCBI database (fig. 4). This may suggest that all of *C. jejuni* subsp. *jejuni* reference strains belong to the biotype I class. However, Vandamme *et al.* (2000) have stressed that caution should be taken when identifying an isolate based on its 16S rRNA gene sequencing analysis. Previous isolates have been misidentified in this manner, thus as a precautionary measure the sequencing data should be used in conjunction with other molecular techniques such as pulsed-field gel electrophoresis (PFGE), polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) or amplified fragment length polymorphism (AFLP) fingerprinting. Although, PFGE has been proven to be an effective tool in epidemiological studies of many bacterial strains, it may take several days to obtain a conclusive result (On *et al.* 2004). PFGE profiles of *Campylobacter jejuni*, for

example, are susceptible to genetic events such as recombination and genomic rearrangement that may complicate the interpretation of the results (Wassenaar *et al.* 2000). AFLP has been used to ascertain genetic relatedness among bacterial strains of the same species (Duim *et al.* 1999; reviewed by Savelkoul *et al.* 1999; On and Harrington 2000; Siemer *et al.* 2004); is less expensive; quicker to perform; better suited to type many isolates; and is also much easier to perform than other whole-genome molecular techniques such as multi-locus sequence typing (MLST) and PFGE (De Boer *et al.* 2000; Schouls *et al.* 2003; On *et al.* 2004). AFLP allows the analysis of DNA fragments, distributed over the entire genome of a microorganism; while other molecular techniques such as PCR-RFLP are restricted to the analysis of restriction site changes in single genes (Gibson *et al.* 1998). For these reasons it was decided to use AFLP instead of PFGE or other typing methods to analyze positive samples and isolates of this study.

Although some genetic relatedness was observed between the reference strain of *A. butzleri* and isolate 10.1 and sample MC8, differences were also observed between them (fig. 5A). Isolate 10.1 appeared to be the most identical of the two to the reference strain of *A. butzleri*, while sample MC8 consisted of up to seven bands more. This suggests genetic diversity among *A. butzleri*. This is in agreement with other studies where multiple genotypes of *A. butzleri* have been found to coexist in a single location over several months (Amisu *et al.* 2003), in a single sample and in a single animal (Hume *et al.* 2001; Atabay *et al.* 2002; Houf *et al.* 2002; On *et al.* 2004). The AFLP profile patterns of samples MC8, a "mala" contents sample, and W5, "free-range"/organic chicken blood sample, appeared to

be similar suggesting close genetic relatedness between the two strains. Despite the many attempts to analyze the "free-range"/organic chicken blood samples (R5; W3; W4: and W5) by AFLP, it was not as successful as the analysis of other samples.

Various molecular techniques, including AFLP fingerprinting are known to have some drawbacks. The major disadvantages are that the technique is very sensitive to PCR conditions, quantity and quality of the DNA as well as to PCR temperature profiles (Riedy *et al.* 1992; Ellsworth *et al.* 1993; Muralidharan *et al.* 1993; Micheli *et al.* 1994; Caetano-Anolles *et al.* 1992). The feint, smeared AFLP profile patterns in lanes 8-10 of figure 5A, suggest that gDNA may have degraded due to consecutive freeze-thawing of the samples. Furthermore, gDNA of these samples were embedded in agarose-moulds (mixed in a 1:1 ratio); cleaned to remove PCR inhibitors; cut in 5µl fragments and digested with the *Hind*III restriction enzyme. Successful AFLP fingerprinting thus also depends on the amount of DNA present in the excised portion of the mould.

In this study, some diversity was also noted among *A. butzleri*-like reference strains (fig. 5A) but to a far lesser degree than for *A. butzleri*. The number and distribution of the AFLP fragments of the reference strain of *A. cryaerophilus* and that of sample cc1 varied suggesting high diversity among *A. cryaerophilus* strains. Conclusions cannot be drawn solely based on this study as only two strains of *A. cryaerophilus* were tested. Nevertheless, our data is in agreement with a recent study (Debruyne *et al.* 2010) indicating that *A. cryaerophilus* is a heterogeneous species. In contrast, our data also indicates genetic relatedness between samples cc1 and L2 (fig. 5A) suggesting that the DNA in both samples might have belonged to

the same species of *Arcobacter* (*Arcobacter* sp. R-2834 strain R-2834). However, this could not be confirmed with AFLP profiling as DNA from this specific reference strain (*Arcobacter* sp. R-2834 strain R-2834) was not available at any culture bank.

The two strains of *C. jejuni* subsp. *jejuni* (7.2 and 7.4), in figure 5B, isolated from the same "free-range"/organic chicken blood sample shared the same AFLP profile. This confirms the suitability of AFLP fingerprinting in assessing epidemiological relatedness among strains (González *et al.* 2007b). These isolates and a *C. jejuni* subsp. *jejuni* biotype II reference strain also displayed similar AFLP profile patterns, with the exception of two bands, illustrating genetic relatedness among them. This suggests that both isolates belong to the biotype II class and is in agreement with classification according to the "Cape Town" protocol in table 1. Classification solely based on the AFLP profiles of strains of *C. jejuni* should be avoided as genetic diversity, due to genomic rearrangements and horizontal gene transfer, is well recognized (Wassenaar *et al.* 2000).

In conclusion, our study has demonstrated that chicken may be a reservoir to various species of *Arcobacter*. Furthermore, the novel finding of two *C. jejuni* subsp. *jejuni* biotype II strains from "free-range"/organic chicken blood suggest that chicken blood may be a potential reservoir and thus play a role in transmission. The AFLP fingerprinting technique was highly informative and discriminatory in identifying related strains of *Arcobacter* and *Campylobacter*, in comparison to the 16S rRNA phylogenetic trees. This study confirms the suitability of AFLP profiles in epidemiological studies and may be used to identify virulent strains.

The presence of *C. jejuni* subsp. *jejuni* in "free-range"/organic chicken blood samples has sparked interest in further work. An *in vitro* study could be conducted to determine how long strains of different species of *Campylobacteraceae* and *Helicobacter* can survive at varying temperatures in "free-range"/organic chicken blood.

ACKNOWLEDGEMENTS

A special thank you is extended to Mr. C. Jacobs for the competent cells. Sincere gratitude is extended to the Department of Biotechnology of the University of the Western Cape, National Research Foundation (NRF) of South Africa, Muslim Hands of South Africa and the South African National Zakah Fund (SANZAF) for financial support.

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In vitro Survival test of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures

ABSTRACT

Aim: To determine how long strains of *Campylobacter*, *Arcobacter* and *Helicobacter* can survive in "free-range"/organic chicken blood at varying temperatures. **Methods and Results:** Fifty strains: two of *A. butzleri*; four *A. butzleri*-like; 10 of *C. jejuni* subsp. *jejuni* biotype I; five of *C. jejuni* subsp. *jejuni* biotype II; nine of *C. coli*; one each of *C. doylei*, *C. lari*, *C. concisus*, *C. fetus* subsp. *fetus* and *C. curvus*; two of *C. upsaliensis*; three each of *H. pylori* and *H. cinaedi*; and seven of *H. fennelliae* were tested in duplicate. "Free-range"/organic chicken blood (2 ml) was dispensed into sterile 2 ml eppendorf tubes and each tested strain generously inoculated (a 10 µl loopful) into five allocated tubes using a sterile loop. Tubes were gently mixed by vortexing a few seconds at a time to avoid damage to the cells. Prior to incubation and thrice weekly thereafter, blood inoculums were tested to confirm strain viability by filtration and differential streaking. Tubes were incubated either at -20ºC, 4ºC, ambient room temperature (±22ºC) (RT), 37ºC or 42ºC. *Campylobacter* and *Arcobacter* strains survived much longer than those of *Helicobacter*. Most notably, *A. butzleri* was able to survive up to 297 days in blood stored at 4ºC. Strain viability could be detected for a longer time through differential streaking than filtration. **Conclusion:** This novel *in vitro* study indicated that *Campylobacter*, *Arcobacter* and *Helicobacter* strains were able to survive at -20, 4, 37 and 42ºC as well as at ambient room temperature in chicken blood.

Significance and Impact of Study: The prolonged survival of each strain was unexpected and demonstrated the possibility of chicken blood as a reservoir but an *in vivo* study is required to confirm this. These results would be relevant in chicken slaughtering and processing plants as well as in supermarkets where the prevention of infections caused by foodborne pathogens is of importance.

Keywords: *Campylobacter*, *Arcobacter*, *Helicobacter*, blood, chicken

INTRODUCTION

The genera of *Campylobacter*, *Arcobacter* and *Helicobacter* form a distinct group within the epsilon sub-division of *Proteobacteria* (Vandamme *et al.* 1991). The taxonomic diversity of the group is matched by the broad spectrum of disease it is associated with, as well as the diverse ecological niches in which the microorganisms survive (On 2001). These microorganisms are Gram-negative and spiral-shaped (Wesley 1997). *Campylobacter* and *Helicobacter* are microaerophilic (Prescott *et al.* 1996), while *Arcobacter* are aerotolerant microorganisms (reviewed by Lastovica 2006). *Campylobacteraceae* (*Campylobacter* and *Arcobacter*) and *Helicobacter* species are associated with various diseases in humans and animals and may be potential foodborne and waterborne pathogens as well as possible zoonotic agents (Newell and Fearnley 2003; Fox 2002; Solnick 2003; Diergaardt *et al.* 2004; Ho *et al.* 2006).

Campylobacter form part of the normal enteric flora in animals such as poultry, pigs and cattle and may be transmitted to humans through the consumption or handling of contaminated foods (Stern *et al.* 2003; Gallay *et al.* 2007). The handling and consumption of raw or undercooked poultry meat is

identified as the major source of human campylobacteriosis (Deming *et al.* 1987; Neimann *et al.* 2003; Potter *et al.* 2003; Friedman *et al.* 2004; Luber and Bartelt 2007). *Campylobacter jejuni* has also been associated with septicaemia, meningitis, the Guillain-Barré syndrome, neuropathy and reactive arthritis (reviewed by Lastovica 2006).

A. butzleri, *A. cryaerophilus* and *A. skirrowii* have been associated with gastric ulcers (Suarez *et al.* 1997), mastitis (Logan *et al.* 1982) and reproductive disorders (On *et al.* 2002) in livestock; while *A. cibarius* was isolated from the skins of broiler carcasses (Houf *et al.* 2005). *A. cryaerophilus* have also been associated with bacteraemia and diarrhoea in humans which occasionally resulted in chronic illness and death in the elderly and children (Hsueh *et al.* 1997; Mansfield and Forsythe 2000; Yan *et al.* 2000; Woo *et al.* 2001; Lau *et al.* 2002; Vandenberg *et al.* 2004).

Various *Helicobacter* species have been associated with bacteraemia in immune-competent adults and children (Vandamme *et al.* 1990; Orlicek *et al.* 1994; Trivett-Moore *et al.* 1997; Tee *et al.* 1998; Uckay *et al.* 2006). These cases emphasize the ability of these microorganisms to cause disease in blood and using blood as a possible mode of transmission to other hosts. To our knowledge, there is no data indicating the survival of these microorganisms in chicken blood. The aim of this novel *in vitro* study was to determine how long strains of *Campylobacter*, *Arcobacter* and *Helicobacter* could survive in "free-range"/organic chicken blood at varying temperatures. This study would be relevant for the implementation of good storage practices at home and at supermarkets; as well as chicken slaughtering and processing plants where the prevention of infections caused by foodborne pathogens is of importance.

MATERIALS AND METHODS

Revitalization of strains

A list of reference strains used in this thesis is shown in Appendix E. Pure cultures of strains of various species of *Campylobacter*, *Arcobacter* and *Helicobacter*, stored at -80ºC on PL160M Microbank Beads (Microbank™ Pro-Lab Diagnostics, Canada), were revitalized as follows. A 100 µl aliquot of sterile 2x Yeast Tryptone (YT) broth (Oxoid, U.K.) (Appendix B) was added to 2 ml sterile eppendorf tubes (Eppendorf AG, Germany). Thereafter, three PL160M Microbank Beads of a specific strain were added to allocated eppendorf tubes using a sterile loop. The eppendorf tubes were allowed to shake at 37ºC in a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) for 10-15 min to lift the bacteria from the beads into the 2xYT broth. Thereafter, the entire volumes of broth were aseptically dispensed; drop wise, onto allocated tryptose blood agar (TBA) (Oxoid, U.K.) (Appendix B) plates. The TBA plates remained at ambient room temperature (RT) for approximately 30 min to allow the drops to dry. The TBA plates were then inverted and incubated for 2-3 days at 37ºC in a H_2-enriched microaerophilic atmosphere generated by an Oxoid BR 0038B gas generating kit (Oxoid, U.K.).

Maintenance of pure cultures

Revitalized cultures were grown on 10% TBA plates for 5-7 days and checked every 2-3 days. Once revitalized, Gram staining and biochemical tests were performed to confirm purity. The cultures were maintained through streaking every second or third day on fresh TBA plates and re-incubated at 37ºC under a H_2-enriched microaerophilic atmosphere, generated by an Oxoid BR 0038B gas

generating kit. Pure cultures intended for long-term storage involved the "sweeping up" of healthy cells of 2-3 day old cultures, using a sterile loop, from TBA plates. Bacteria were sterilely deposited into a Microbank tube containing broth and beads. The Microbank tube was shaken gently but sufficiently to ensure that the bacterial mass was broken down and evenly displaced throughout the broth to allow the bacteria to enter the hole in the centre of the bead and adhere to the rest of the bead. Thereafter, the broth was swiftly removed using a sterile Pasteur pipette after flaming the mouth of the Microbank tube. Tubes were immediately snap-frozen and stored at -80ºC.

Inoculation and incubation of strains in chicken blood

Fresh chicken blood samples were obtained from a "free-range"/organic farm in the Western Cape region of South Africa; transported on ice in 9 ml LH Lithium Heparin tubes (Greiner bio-one, Germany); stored at 4ºC and used within 24 h. Experiments were performed in duplicate for each strain tested at -20ºC, 4ºC, RT, 37ºC and 42ºC. A total of 50 strains of two species of *Arcobacter* (two *A. butzleri* and four *A. butzleri*-like strains); eight species of *Campylobacter* (10 of *C. jejuni* subsp. *jejuni* biotype I; five of *C. jejuni* subsp. *jejuni* biotype II; nine of *C. coli*; one each of *C. doylei*, *C. lari*, *C. concisus*, *C. fetus* subsp. *fetus* and *C. curvus*; and two of *C. upsaliensis*); and three species of *Helicobacter* (three strains each of *H. pylori* and *H. cinaedi*; and seven of *H. fennelliae*) were tested. Aliquots of 2 ml of blood were dispensed into sterile 2 ml eppendorf tubes. The inoculation process entailed the gentle sweeping up (compact loopful) of 2-3 day old healthy-growing pure cultures of species of *Campylobacteraceae* and *Helicobacter* with a sterile 10 µl loop. Pure cultures were gently deposited,

through swirling, into allocated eppendorf tubes containing 2 ml of "free-range"/organic chicken blood. Five tubes were prepared, in duplicate, for each strain of each species. The blood inoculates were vortexed for a few seconds, at a time, to prevent cell damage but ensure thorough mixing of each sample. The tubes were placed in five tube-racks, in duplicate, for incubation at -20ºC (freezer), 4ºC (fridge), RT (on laboratory work-bench), as well as at 37ºC and 42ºC in a Scientific Series 9000 incubator (LASEC: Laboratory and Scientific Equipment Co., Pty, Ltd., R.S.A.).

Determining the survival of strains at tested temperatures

The first reading was taken immediately after inoculation and prior to incubation to confirm the presence of the microorganism. Thereafter, blood inoculums were tested thrice a week. Frozen samples, stored at -20ºC, were thawed, tested at RT and refrozen at -20ºC until the next test. Similarly, blood inoculums at RT; 4; 37; and 42ºC were tested at RT and returned to the respective temperatures for incubation until the next test. Blood inoculums were tested in two ways: one using a cellulose acetate membrane filter and the other by differential streaking.

Using a sterile blade, sterile 0.6 µm cellulose acetate membrane filters (Schleicher and Schuell MicroScience, Germany) were cut in quarters in a sterile petri dish. The sterile quartered 0.6 µm cellulose acetate membrane filters were strategically placed onto pre-dried quartered 10% TBA plates with sterile tweezers and allowed to absorb to the surfaces. Volumes of 100 µl of inoculated blood were gently dispensed in the centres of the quartered filters to avoid overflow. The blood inoculates were allowed to filter through onto the surfaces of the TBA plates, removing the filters within 20 min. A quartered 0.6 µm

cellulose acetate membrane filter, without blood inoculate, was placed onto the TBA surface as a negative control to confirm that the filters were not contaminated. All quartered filters were gently removed with sterile tweezers and discarded.

Using differential streaking, a sterile 10 µl loop was gently swirled in the blood inoculated sample and streaked for single colonies onto allocated pre-dried TBA plates.

All TBA plates were inverted and incubated overnight at 37ºC with BR 0038B gas generating kits. Presumptive *Campylobacter*, *Arcobacter* or *Helicobacter* colonies were confirmed by Gram-staining and biochemical testing. These included the following tests: indoxyl acetate; catalase; oxidase; nitrate reductase; arylsulfatase; pyrazinamide and hippurate.

RESULTS

Survival experiments were each performed in duplicate. The average survival time, shown in table 1, of every species tested was determined at -20ºC; 4ºC; RT; 37ºC; and 42ºC. The survival of all strains was observed for a longer period through streaking than filtration. *Campylobacter*, *Arcobacter* and *Helicobacter* species displayed specific survival patterns. On average, *C. jejuni* subsp. *jejuni* biotype II strains survived longer, at every temperature, than other *Campylobacter* strains, including those of *C. jejuni* subsp. *jejuni* biotype I. *C. doylei* survived similar periods at most temperatures but survived the longest at 4ºC. Similar patterns were observed for *C. lari* and *C. concisus* at most temperatures, except that they survived the shortest time at 42ºC. *C. coli* displayed a uniform survival pattern at all temperatures. An interesting

observation was made for one of the *A. butzleri* isolates. Growth was detected by filtration and streaking on day 0 (day of inoculation); no growth from days two to eight; followed by growth from day nine to day 297. This may indicate a possible viable but nonculturable state of the *A. butzleri* strain as a means of adapting to its external environment. On average, *A. butzleri* and *A. butzleri*-like strains displayed similar survival patterns. Similar survival patterns were noted at RT and 37ºC and 42ºC for *A. butzleri* and *A. butzleri*-like strains. However, the latter species survived better at -20ºC; while the former species survived longest at 4ºC. All strains of *Helicobacter* survived for much shorter periods than every other *Campylobacter* or *Arcobacter* strain tested at various temperatures.

Table 1: Total days *Campylobacter, Arcobacter* and *Helicobacter* strains survived in "free-range"/organic chicken blood incubated at various temperatures

Microorganism/ Isolate	No. of strains	Source	-20°C (days)		4°C (days)		RT (days)		37°C (days)		42°C (days)	
			Streaking	Filtration	Streaking	Filtration	Streaking	Filtration	Streaking	Filtration	Streaking	Filtration
C. jejuni subsp. *jejuni* biotype I	10	Clinical, Ostrich	48-62	30-43	28-62	18-44	35-62	34-51	28-62	34-53	28-60	34-56
C. jejuni subsp. *jejuni* biotype II	5	Clinical, Chicken	56-67	35	62	45	62	35	62	35	49-62	35
C. doylei	1	Clinical	41	30	53	44	41	34	41	34	41	34
C. lari	1	Clinical	40	33	35	28	42	35	49	35	28	21
C. coli	9	Clinical	35-49	21-33	35-49	33-44	37-43	34-40	35-43	34-45	28-42	21-34
C. concisus	1	Clinical	40	35	35	28	42	35	47	33	28	21
C. fetus subsp. *fetus*	1	Clinical	45	35	42	35	35	28	42	33	35	25
C. curvus	1	Clinical	49	31	42	35	35	28	42	33	35	25

Table 1 (continued): Total days *Campylobacter, Arcobacter* and *Helicobacter* strains survived in "free-range"/organic chicken blood incubated at various temperatures

Microorganism/ Isolate	No. of strains	Source	-20°C (days)		4°C (days)		RT (days)		37°C (days)		42°C (days)	
			Streaking	Filtration	Streaking	Filtration	Streaking	Filtration	Streaking	Filtration	Streaking	Filtration
C. upsaliensis	2	Clinical	34-49	N/T	28-33	N/T	25-31	N/T	33-41	N/T	23-27	N/T
A. butzleri	2	Clinical	33-49	30-41	66-*297	>60	62	44	62	54	42	54
A. butzleri- like	4	Sewage/ sludge	48-62	30-50	65	48	62	42	62	49	56-62	48-52
H. pylori	3	Clinical	20-35	N/T	12-14	N/T	12-14	N/T	14	N/T	12-14	N/T
H. cinaedi	3	Clinical	11-39	N/T	14	N/T	9-11	N/T	13-14	N/T	13-14	N/T
H. fennelliae	7	Clinical	11-35	N/T	14	N/T	11	N/T	13	N/T	13	N/T

Key:

- N/T indicates that the strains could not be tested by filtration
- The symbol * indicates the total number of days an *A. butzleri* strain was able to survive at 4°C.

DISCUSSION

This is a novel study in evaluating the survival of *Campylobacteraceae* and *Helicobacter* strains in "free-range"/organic chicken blood at different temperatures. However, the survival of well known species such as *C. jejuni*, *A. butzleri* and *H. pylori* has been evaluated in some media and various food products (Valdés-Dapena and Adám 1983; Alvin *et al.* 1998; Fan *et al.* 1998; Zhao *et al.* 2000; Saumya and Cottrell 2004; Quaglia *et al.* 2007; Driessche and Houf 2008).

The presence of protective chemical components such as proteins in blood may have contributed to the survival of strains of *Campylobacter*, *Arcobacter* and *Helicobacter* at -20ºC by inhibiting the formation of compact ice crystals (Lee *et al.* 1998; Hilton *et al.* 2001; Palmfeldt *et al.* 2003; Driessche and Houf 2008).

At -20ºC, the survival rates of *C. jejuni* subsp. *jejuni* strains were only second to *Arcobacter* but displayed the longest survival rate among all *Campylobacter* strains illustrating its ability to withstand repeated freeze thawing cycles (Alvin *et al.* 1998). This is in agreement with a recent study (Kjeldgaard *et al.* 2009). In addition, the *C. jejuni* subsp. *jejuni* biotype II strains were able to survive longer at -20ºC than the *C. jejuni* subsp. *jejuni* biotype I strains. This implies that the biotype II strains are good survivors in chicken blood which could contribute to its pathogenicity, as observed in humans suffering from the Guillian-Barré syndrome. *C. jejuni* subsp. *jejuni* biotype II strains have previously been implicated in the Guillian-Barré syndrome in South Africa (Lastovica *et al.* 1997). Humphrey and Cruickshank have shown that sub-lethal injury of *C. jejuni* may occur during freezing or heating as a result of

damages caused to the outer membrane components. This may explain the lower survival rate of *C. jejuni* subsp. *jejuni* biotype I strains at -20ºC and further explains the lower survival rate of some *Campylobacter* strains as temperature increases.

Campylobacter species grow optimally at 37-42ºC and previously thought not to grow below 30ºC (Lee *et al.* 1998). However, data from this (table 1) and previous studies (Skirrow and Blaser 1995; Hazeleger *et al.* 1998) demonstrated the ability of *C. jejuni* to survive at 4ºC. Except for *C. doylei* and *C. coli*, the survival rate of *Campylobacter* species in this study were generally in agreement with previous studies indicating a lower survival rate at 4ºC than at -20ºC (Oyarzabal *et al.* 2010), albeit to a lesser degree.

Most *Arcobacter* strains in this study survived better at 4ºC than at -20ºC. This suggests the susceptibility of most *Arcobacter* strains to intracellular ice crystal formation followed by additional cell membrane damage during thawing (Hilton *et al.* 2001).

Strain variability among *Campylobacter*, *Arcobacter* and *Helicobacter* was noted in this study as some strains were better able to survive at certain temperatures than others (table 1). However, this phenomenon of intraspecies strain variability was not observed for *Arcobacter* (Driessche and Houf 2008) but was observed for *Campylobacter* (Oyarzabal *et al.* 2010) and *Helicobacter* (Ohkusa *et al.* 2004). One *A. butzleri* strain was able to survive for up to 297 days at 4ºC (table 1), in agreement with a previous study recording survival by enrichment up to 250 days in water containing organic material (Driessche and Houf 2008).

In the current study the prolonged survival rate of *Campylobacter*, *Arcobacter* and *Helicobacter* strains may have been a result of the combined protective roles of blood and an anaerobic environment created by the sealed eppendorf tubes. The presence of blood has been shown to allow optimal growth and rehydration conditions for repair of *Campylobacter* and related microorgainsms (Humphrey 1986). Portner *et al.* (2007) determined this optimum temperature for rehydration of *Campylobacter* to be 37ºC; while Kjeldgaard *et al.* (2009) has shown that *A. butzeri* forms biofilms, as a protective mechanism, at temperatures ranging between 10 and 37ºC. This may have further influenced the survival of strains of *Campylobacter* and related microorganisms of this study. *Campylobacter* is microaerophilic and the surrounding environment, i.e. blood, in the sealed eppendorf tubes could have prolonged survival of these microorganisms by ensuring protection against oxidative stress (Rogers 1914; Stead and Park 2000).

The pH of the chicken blood could have increased the survival of the strains of *Campylobacter* and related microorganisms (Palumbo and Williams 1991). Pubmed literature searches were done to attempt to identify the pH of normal chicken blood but it was unsuccessful. The pH of the chicken blood was not determined in this study. In 1995, Skirrow and Blaser illustrated that pH values below 5.0 and above 9.0 hindered *Campylobacter* growth.

In this study, *Helicobacter* strains could not survive for long periods at various temperatures. The data of this study is in agreement with previous studies illustrating the short survival periods of *H. pylori* at various temperatures (Fan *et al.* 1998; Poms and Tatini 2001; Ohkusa *et al.* 2004; Quaglia *et al.* 2007). *Campylobacter* and *Helicobacter* are also known to form viable but non-

culturable cells when exposed to environmental stresses such as high temperatures, low nutrient availability, desiccation, aeration of media, exposure to antimicrobial agents, long ineffective incubation times or the presence of free-radical scavengers (Rollins and Colwell 1986; Gomes and Martinis 2004). In this study (data not shown) and other studies (Fan *et al.* 1998; Ohkusa *et al.* 2004) *Helicobacter* cells were converted from viable to non-culturable form within days. This suggests that *Helicobacter* strains cannot cope with the different temperatures and changing environment, during incubation, as effectively as *Campylobacter* and *Arcobacter* strains.

The survival of *Campylobacter* and related microorganisms also seem to be dependent on the availability of good nutrients, suitability of the environment and medium in which it thrives (Doyle and Roman 1982; Valdés-Dapena and Adám 1983; Wundt *et al.* 1985; Fan *et al.* 1998; Poms and Tatini 2001; Quaglia *et al.* 2007; Driessche and Houf 2008).

In conclusion, most of the strains tested were able to survive at various temperatures for many days. The presence of adequate amounts of these microorganisms in blood could thus serve as a route for transmission, particularly from bird to bird or bird to human as a result of an unattended wound or laceration; act as a reservoir and a source of continual re-infection as *Campylobacter* and related microorganisms have been shown to survive in blood for long periods (table 1). Black *et al.* (1988) demonstrated that a minimum of 800 *Campylobacter* cells are required to cause diarrhoeal illnesses in humans. This data further demonstrates the need for the implementation of good sanitary practices at home; supermarkets; flock-houses; as well as at poultry slaughter and processing plants. Many butchers and supermarkets have recently started

selling marinated poultry products and herb-and-spice-flavoured foods. The spices and herbs have a twofold purpose, adding flavour to foods and thought to limit the proliferation of viable infectious microorganisms such as *Campylobacter*, *Arcobacter* and *Helicobacter*.

ACKNOWLEDGEMENTS

Sincere gratitude is extended to the Department of Biotechnology at the University of the Western Cape, National Research Foundation (NRF), Muslim Hands (South Africa) and the South African National Zakah Fund (SANZAF) for financial support.

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Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

ABSTRACT

Aim: The effect of some culinary products (non-irradiated spices; herbs; fresh lemon juice; fynbos honey; garlic, ginger and onion) was tested on the growth of *Campylobacter*, *Arcobacter* and *Helicobacter* species in comparison to ciprofloxacin and other antibiotics. **Methods and Results:** A total of forty-five strains: 29 strains of six species of *Campylobacter*; two strains of *Arcobacter butzleri*; four *Arcobacter butzleri*-like strains; and 10 strains of four species of *Helicobacter*, from various sources, were tested. Test ingredients were added to sterile distilled water, incubated at 85ºC for 35 min and dispensed, in 100 µl aliquots, into the designated wells of TBA plates. Antibiotic discs were strategically placed on streaked TBA plates. The growth of all *Campylobacteraceae* and *Helicobacter* strains tested were potently inhibited by garlic; ginger; fynbos honey; cinnamon; allspice; and cloves, while the inhibitory effects of other culinary products and antibiotics appeared to be species or strain dependant. **Conclusions:** Garlic; ginger; fynbos honey; cinnamon; allspice; and cloves proved to be potent growth inhibitors against all of the *Campylobacteraceae* and *Helicobacter* strains tested. No antibiotic tested could display similar inhibition. **Significance and Impact of Study:** This study suggests the use of garlic; ginger; fynbos honey; cinnamon; allspice; and cloves as possible food additives for the prevention against infections caused by *Campylobacteracaeae* and *Helicobacter* species.

Keywords: Antibiotics, *Campylobacteraceae*, *Helicobacter*, culinary products, inhibitory effect.

INTRODUCTION

Campylobacter, *Arcobacter* (*Campylobacteraceae*) and *Helicobacter* species are spiral-shaped, Gram-negative microorganisms. *Campylobacter* and *Helicobacter* are microaerophilic microorganisms, requiring low O_2 levels in the range of 2-10% for growth as their cells are damaged by the normal atmosphere level of 20% O_2 (Prescott *et al.* 1996). *Arcobacter* are capable of growing at normal atmospheric conditions. *Campylobacteraceae* is the family name proposed to include both genera, *Arcobacter* and *Campylobacter*, based on the similarities within their phylogenetic and phenotypic characteristics (Vandamme and De Ley 1991). Species of *Campylobacteraceae* and *Helicobacter*, which are foodborne pathogens, are known to be the causative agent of a range of diseases affecting both humans and animals. Some *Arcobacter* species have been associated with enteritis, bacteraemia and chronic diarrhoea (Vandamme *et al.* 1992a; Vandamme *et al.* 1992b; Lastovica and Skirrow 2000; Wybo *et al.* 2004). The most widely studied *Campylobacter* species, *C. jejuni*, is recognized as one of the major causes of gastroenteritis and diarrhoea in humans and animals (Griffiths and Park 1990; Allos 2001; Lastovica 2006). It has also been associated with septicaemia, meningitis, the Guillain-Barré syndrome and reactive arthritis (reviewed by Lastovica 2006). There are at least 35 *Helicobacter* species, most of which have been identified in human and animal digestive tracts and thought to be associated with human and animal disease of gastric, enteric or systemic origin (Totten *et al.* 1985; Parsonnet *et al.* 1994; Fox

et al. 1998; De Groote *et al.* 1999; Tee *et al.* 2001; Gueneau and Loiseaux-De Goër 2002). Species of *Campylobacteraceae* and *Helicobacter* have also been detected in drinking water (Diergaardt *et al.* 2004); retail meats including; pork, beef and poultry (Stanley *et al.* 1994; Kabeya *et al.* 2004); poultry processing plants (Son *et al.* 2006); raw milk (Lovett *et al.* 1983; Dore *et al.* 2001; Scullion *et al.* 2006), etc. Some strains of some species of *Campylobacteraceae* and *Helicobacter* have acquired resistance to a range of antimicrobial agents including those used in the treatment of diarrheal illnesses (Mansfield and Forsythe 2000; Ceelen *et al.* 2005; Gallay *et al.* 2007). Generally, microorganisms may either be eradicated or reduced using a combination of chemical preservatives (Phyllips and Duggan 2001) in food or by the physical treatment of food such as temperature change or irradiation (Collins *et al.* 1996; Hilton *et al.* 2001). However these may either be too costly; ineffective; the microorganism may gradually gain resistance to the treatment; or the treatments may, over the long term, have adverse effects on human health. Various spices and herbs have been used for centuries as preservatives, medicine and in cooking to add flavour and enhance the palatability of food (Dog 2006). Although, some research is available determining the effect of spices and herbs on *Campylobacteraceae* (Diker *et al.* 1991; Smith-Palmer *et al.* 1998; Cervenka *et al.* 2006) and *Helicobacter* (Annuk *et al.* 1999; Stamatis *et al.* 2003; Tabak *et al.* 1996a and 1996b), these studies have focused on herbal medicines; tea; plant essential oils; and on plant extracts many of which are very costly. The aim of this study was to determine the effect of some culinary products (non-irradiated spices; herbs; fresh lemon juice; fynbos honey; and onion), purchased from three

different retail stores, on the growth of *Campylobacteraceae* and *Helicobacter* species versus the effect of selected antibiotics.

MATERIALS AND METHODS

Strains tested

Forty-five strains of seven species of *Campylobacteraceae* and four species of *Helicobacter* comprising of two *A. butzleri*, four *A. butzleri*-like; 10 *C. jejuni* subsp. *jejuni* biotype I, five *C. jejuni* subsp. *jejuni* biotype II, one *C. fetus* subsp. *fetus*, one *C. lari*, nine *C. coli*, one *C. curvus*, two *C. upsaliensis*, two *H. cinaedi*, one *H. pamatensis*, two *H. pylori* and five *H. fennelliae* strains were tested. All strains, of pure culture, previously stored at -80ºC on PL160M Microbank Beads (Microbank™ Pro-Lab Diagnostics, Canada) were revitalized as follows. A volume of 100 µl of sterile 2xYeast Tryptone (YT) broth (Appendix B) was added to each 2 ml sterile Eppendorf tube (Eppendorf AG, Germany). Five to seven PL160M Microbank Beads, of a specific strain, was added to the Eppendorf tubes using a sterile loop. Eppendorf tubes were allowed to shake at 37ºC in a G24 Environmental Incubator shaker (New Brünswick Scientific Co., Inc., U.S.A.) for 10-15 min to lift the bacteria from the beads and into the 2xYT broth. The entire volume of broth was aseptically dispensed; drop wise, onto allocated tryptose blood agar (TBA) (Appendix B) plates. The TBA plates remained at ambient room temperature (±22ºC) (RT) for 30 min to allow the drops to dry. TBA plates were inverted and incubated for three days at 37ºC in a H_2-enriched microaerophilic atmosphere generated by an Oxoid BR 0038B gas generating kit (Oxoid, U.K.). Cultures were maintained by streaking the cultures onto fresh TBA plates and replacing the gas generating kits twice a week.

Optimal method to analyze the effect of a few culinary products on the growth of *Campylobacteraceae* and *Helicobacter* strains

Optimal conditions including: the preparation of culture, the quantity of microorganism and optimal technique had to be determined to correctly analyze the effect of the culinary products (non-irradiated spices; herbs; fresh lemon juice; onion; and fynbos honey) on the growth of the strains.

Three preparation techniques: streaking; enrichment 10^{-1} dilution series; and direct 10^{-1} dilution series were tested with the disc diffusion technique, described below. In the streaking procedure, a sterile 10 µl loop was used to sweep up a single colony of pure culture and streaked across the surface of an allocated TBA plate. In the enrichment 10^{-1} dilution series technique, each strain was streaked for single colonies on TBA plates and incubated overnight at 37ºC. Thereafter, a single colony was mixed into 10 ml of *Arcobacter* enrichment broth (AEB) (Appendix B) and incubated overnight at 37ºC. This was followed by a 10^{-1} dilution series to determine the optimal dilution for analysis. The direct 10^{-1} dilution series technique was the method of choice for this study and consisted of the complete resuspension of each strain in 1x Phosphate Buffer Saline (PBS) (pH 7.2) (Appendix B), vortexing a few seconds at a time to create starting cultures of 10^{7}-10^{8} cfu ml^{-1}. As a confirmatory guide, a McFarland nephelometer tube number 0.5 was prepared and used as a standard to estimate the bacterial density. This was done by mixing 9.95 ml of 1% Sulphuric acid in Mueller-Hinton broth and 0.05 ml of 1% Barium chloride in distilled water (Saeed *et al.* 2005).

Two techniques were used to determine the effect culinary products have on the growth of each strain: the disc diffusion and well-diffusion techniques. In

the disc diffusion technique, circles of approximately 0.5 cm in diameter were punched out of 150 mm Whatman no. 1 filter paper discs, placed into a 50 ml sterile beaker covered with foil; sterilized by autoclaving; and dried overnight at 50ºC. Each starting culture of 10^7-10^8 cfu ml^{-1} was spread onto TBA plates and allowed to dry at RT for 30 min. Thereafter, single; double; and triple discs were randomly placed onto the TBA surfaces to which 10, 20 and 30 µl, respectively, of spice sample (paste, crushed or aqueous) was added making sure that the sample did not overflow onto or directly touch the surfaces of the TBA. In addition, a double disc was completely submerged into the aqueous spice sample and strategically placed onto the TBA after allowing the excess solution to drip off. However, despite these attempts the disc diffusion technique appeared to be ineffective.

In the well-diffusion technique (the technique of choice in this study) starting cultures of 10^7-10^8 cfu ml^{-1}, equivalent to the bacterial density of a McFarland nephelometer tube number 0.5 suspension, were spread onto TBA media with a sterile glass spreader and allowed to dry in a laminar flow at RT for 30 min. A maximum of four wells were created into the solidified TBA using the broader part of a sterile 200 µl pipette tip. Thereafter, 100 µl aliquots of each culinary product, described below, were directly dispensed into designated wells while absolute EtOH was used as a positive control on each TBA plate.

Preparation and testing of various culinary products

All culinary products were purchased from three different retail stores and tested in duplicate. The culinary products were randomly selected based on the fact that many are used as ingredients in various traditional cuisines. These included:

garlic, ginger, dried parsley, dried thyme, cardamom, fresh lemon juice, fynbos honey, crushed onion, crushed chillies, ground pepper, cinnamon, allspice, cloves and turmeric. Black cumin seeds were also tested due to its health benefits. The samples were analyzed in the following forms: paste (ginger and garlic), crushed (ginger, garlic and chillies) and juice (ginger and garlic). The paste refers to the ginger or garlic paste (processed product) which has previously been prepared by the manufacturer and sold in various retail outlets and spice shops. The crushed product refers to the raw garlic, ginger, onion or chillies which have manually been crushed, at room temperature, using a porcelain pestle and mortar to release important antimicrobials protected within the tissues (garlic, ginger and onion) or pits (chillies). While the product is crushed at room temperature, as described above, some liquid is released as a by-product and referred in this chapter as the "juice". Each prepared sample was dispensed in 100 µl aliquots into the designated wells in TBA. Aqueous samples were prepared by incubating a 1:1 ratio of sample [ginger pieces, garlic pieces, dried parsley, dried thyme, ground pepper (whole pepper ground in a liquidizer), ground black cumin seeds (whole black cumin seeds ground in a liquidizer), stick cinnamon, fine cinnamon (purchased product), whole allspice, fine allspice (purchased product), whole cloves, fine cloves (purchased product) or crushed cardamom (crushed with a metal pestle and mortar as finely as possible to release the protected antimicrobials within the seeds and pits)] and sterile distilled water in 50 ml Greiner tubes (Greiner Bio-one, Germany) and incubated at 85ºC for 35 min in an attempt to diffuse some essential elements into the water. The samples were only heated for 35 min as this was the minimal time required for the antimicrobials to diffuse into the water (indicated by a murky

solution). In addition, the aqueous samples were also tested by incubating a 1:1 ratio of the above sample and sterile distilled water in 50 ml Greiner tubes and incubated at room temperature ranging from one h to 24 h but the results were unsatisfactory thus subsequent experiments were only performed at 85˚C for 35 min. All samples were tested within 3 h of preparation. All strains were spread onto duplicate TBA plates as described in the well-diffusion technique, above. A volume of 100 µl of aqueous sample was added per well. A 2:1 ratio of distilled water and turmeric was used to form a homogenous suspension and incubated at 85ºC for 35 min to heat the suspension and allow the diffusion of essential components into the water in a natural manner without the use of toxic solvents such as ethanol or methanol. A volume of 100 µl was immediately dispensed into the wells. The effect of fresh lemon juice; fynbos honey and onion was also tested. Fresh lemon juice was squeezed into a sterile 50 ml Greiner tube and dispensed in 100 µl aliquots into the wells of TBA plates. A volume of 5 ml of fynbos honey (from three different manufacturers) was dispensed into a sterile 50 ml Greiner tube for transportation to the lab and tested within 3 h. Without dilution, aliquots of 100 µl of fynbos honey were dispensed into the designated wells of the TBA plates. Pieces of freshly chopped *Allium cepa* (onion) were dispensed in 100 µl aliquots into wells in TBA plates. After all ingredients were loaded into the wells, the TBA plates were incubated as described below. In addition to testing the inhibitory effects of various culinary products, a range of antibiotics were also tested to determine the effect it had on the growth of the isolates.

Antibiotic Testing

A selection of antibiotic discs was tested against the growth of all strains. Erythromycin is commonly used for treatment of campylobacteriosis in humans (Chan 2007), however, erythromycin-resistant strains of *Campylobacter* (Chan 2007), *Arcobacter* (Houf *et al.* 2004) and *Helicobacter* (Oyedeji *et al.* 2009) are on the increase. As a result, it was decided to test the effect of other antibiotics on the growth of strains of *Campylobacter*, *Arcobacter* and *Helicobacter*. The single antibiotic discs tested included: gentamicin (CN: 10 µg $disc^{-1}$), cephalothin (KF: 30 µg $disc^{-1}$), penicillin G (P: 10 units), nalidixic acid (NA: 30 µg $disc^{-1}$), ciprofloxacin (CIP: 5 µg $disc^{-1}$), teicoplanin (TEC: 30 µg $disc^{-1}$), oxacillin (OX: 1 µg $disc^{-1}$), amikacin (AK: 30 µg $disc^{-1}$) and clindamycin (DA 2 µg $disc^{-1}$). One double-treatment antibiotic disc, namely, sulphamethoxazole/trimethoprim (SXT: 25 µg $disc^{-1}$) was also tested. Antibiotic testing was performed, in duplicate, by streaking strains for heavy and even growth on the surfaces of allocated TBA plates. A total of four antibiotic discs were strategically placed onto the streaked TBA surfaces. All experimental TBA plates were incubated at 37ºC, without inversion, for 24 h. The degree of sensitivity, represented by the inhibition zones, was determined by measuring the radii on either side of the wells or antibiotic disc; the average calculated; and recorded in mm for each strain. All strains displaying inhibition zones of diameters < 5 millimeters (mm) around the wells or antibiotic discs were regarded as resistant; intermediate sensitivity was indicated by a 5-9 mm inhibition zone; and sensitive strains were indicated by inhibition zones ≥10 mm.

RESULTS

Optimal method to analyze the effect of a few culinary products on the growth of *Campylobacteraceae* and *Helicobacter* strains

The streaking and enrichment 10^{-1} dilution series techniques resulted in an overgrowth of the strains and thus the correct effect of the culinary products could not be determined. The direct 10^{-1} dilution series, used to obtain starting cultures of 10^{7}-10^{8} cfu ml^{-1}, and the well-diffusion techniques were determined to be the optimal methods for testing the effect of various culinary products against the growth of the tested strains.

Effects of culinary products versus antibiotics on the growth of *Campylobacteraceae* and *Helicobacter* strains

The effects of garlic; ginger; dried parsley; dried thyme; cardamom; fresh lemon juice; fynbos honey; crushed onion; crushed chillies; ground pepper; cinnamon; allspice; cloves; turmeric; and black cumin seeds were determined on the growth of 44 strains of seven species of *Campylobacteraceae* and four species of *Helicobacter* and recorded in Tables 1-3. The results for the antibiotic testing are shown in table 4. All experiments were done in duplicate. A representative photograph, figure 1, illustrates the inhibitory effect of the different forms of garlic (paste, crushed and juice) on the growth of *C. curvus*.

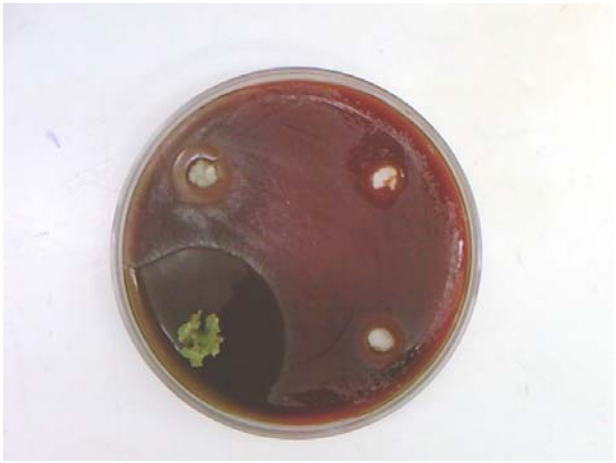

Fig. 1 The effect of garlic on the growth of *Campylobacter curvus* is illustrated. Volumes of 100 µl of Absolute ethanol, a positive control (upper right well); garlic paste (upper left); crushed garlic (bottom left); and garlic juice (bottom right) were loaded into wells of a 10% TBA plate.

Table 1: Inhibition zones, in mm, for species of *Campylobacter*, *Arcobacter* and *Helicobacter* for garlic, ginger and absolute ethanol

Strains (number tested)	Garlic Paste (mm)		Crushed Garlic (mm)		Aqueous Garlic Solution (mm)		Garlic Juice (mm)		Ginger Paste (mm)		Crushed Ginger (mm)		Aqueous Ginger Solution (mm)		Ginger Juice (mm)		Abs. EtOH (mm)
A. butzleri **(2)**	0^R; 2^S	30	0^R; 2^S	50	0^R; 2^{IS}	5	0^R; 2^S	30	0^R; 2^S	18	0^R; 2^{IS}	5	0^R; 2^S	10	1^R; 1^S	10	15
A. butzleri-like **(4)**	0^R; 4^S	23	0^R; 4^S	63	0^R; 4^{IS}	5	0^R; 4^S	43	0^R; 4^S	29	0^R; 4^S	13	0^R; 4^{IS}	8	0^R; 4^{IS}	6	15
C. jejuni subsp. *jejuni* I **(10)**	0^R; 10^S	23	0^R; 10^S	40	0^R; 10^{IS}	5	0^R; 10^S	26	0^R; 10^S	12	0^R; 10^S	22	0^R; 10^{IS}	9	0^R; 10^S	10	15
C. jejuni subsp. *jejuni* II **(5)**	0^R; 5^S	38	0^R; 5^S	56	0^R; 5^{IS}	5	0^R; 5^S	35	0^R; 5^{IS}	8	0^R; 5^S	20	0^R; 5^{IS}	5	0^R; 5^{IS}	7	16
C. coli **(9)**	0^R; 9^S	22	0^R; 9^S	24	0^R; 9^{IS}	5	0^R; 9^S	14	0^R; 9^S	18	0^R; 9^S	26	0^R; 9^{IS}	5	0^R; 9^{IS}	5	15
C. lari **(1)**	0^R; 1^S	15	0^R; 1^S	30	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^{IS}	5	0^R; 1^S	10	0^R; 1^{IS}	5	0^R; 1^{IS}	5	20
C. fetus subsp. *fetus* **(1)**	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^{IS}	5	0^R; 1^{IS}	5	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^{IS}	5	0^R; 1^S	10	15
C. curvus **(1)**	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^{IS}	5	0^R; 1^S	10	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^{IS}	5	0^R; 1^{IS}	5	10
C. upsaliensis **(2)**	0^R; 2^S	30	0^R; 2^S	40	1^R; 1^{IS}	5	0^R; 2^S	25	0^R; 2^S	11	0^R; 2^S	19	0^R; 2^S	10	0^R; 2^{IS}	6	10
H. cinaedi **(2)**	0^R; 2^S	35	0^R; 2^S	45	0^R; 2^{IS}	5	0^R; 2^S	20	0^R; 2^S	18	0^R; 2^S	21	0^R; 2^{IS}	5	0^R; 2^S	13	13
H. fennelliae **(5)**	0^R; 5^S	28	0^R; 5^S	34	0^R; 5^{IS}	8	0^R; 5^S	15	0^R; 5^S	12	0^R; 5^S	26	0^R; 5^{IS}	5	0^R; 5^{IS}	5	10
H. pylori **(2)**	0^R; 2^S	30	0^R; 2^S	50	0^R; 2^{IS}	5	0^R; 2^S	23	0^R; 2^S	10	0^R; 2^S	22	0^R; 2^{IS}	5	0^R; 2^{IS}	5	15
H. pamatensis **(1)**	0^R; 1^S	40	0^R; 1^S	60	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^S	10	0^R; 1^S	25	0^R; 1^{IS}	5	0^R; 1^{IS}	6	10

The bold number, in brackets, denotes the number of isolates tested.

Superscript: R (^{R}) = Resistance: <5 mm inhibition zone Superscript: IS (^{IS}) = Intermediate Sensitivity: 5-9 mm inhibition zone Superscript: S (^{S}) = Susceptible: ≥10 mm inhibition zone

The number preceding the superscript denotes the number of isolates resistant or susceptible to the sample tested

N.B. The diameters of all inhibition zones exclude the well

Table 2: Inhibition zones, in mm, for species of *Campylobacter*, *Arcobacter* and *Helicobacter* for dried parsley, dried thyme, cardamom, fresh lemon juice, fynbos honey, crushed onion, black cumin seeds, crushed chillies and absolute ethanol

Strains (number tested)	Dried Parsley (mm)		Dried Thyme (mm)		Cardamom (mm)		Fresh Lemon Juice (mm)		Fynbos Honey (mm)		Crushed Onion (mm)		Black Cumin Seeds (mm)		Crushed Chillies (mm)		Abs. EtOH (mm)
A. butzleri **(2)**	0^{R}; 2^{S}	10	1^{R}; 1^{IS}	5	1^{R}; 1^{IS}	5	0^{R}; 2^{IS}	5	0^{R}; 2^{S}	10	1^{R}; 1^{IS}	5	0^{R}; 2^{S}	20	2^{R}; 0^{S}	R	15
A. butleri-like **(4)**	0^{R}; 4^{S}	11	0^{R}; 4^{IS}	8	2^{R}; 2^{IS}	5	0^{R}; 4^{S}	45	0^{R}; 4^{IS}	9	0^{R}; 4^{S}	14	4^{R}; 0^{S}	R	3^{R}; 1^{IS}	5	15
C. jejuni subsp *jejuni* I **(10)**	4^{R}; 6^{S}	17	2^{R}; 8^{IS}	8	5^{R}; 5^{IS}	9	5^{R}; 5^{S}	11	0^{R}; 10^{S}	21	4^{R}; 6^{IS}	6	6^{R}; 4^{S}	15	1^{R}; 9^{IS}	9	15
C. jejuni subsp. *jejuni* II **(5)**	1^{R}; 4^{S}	14	0^{R}; 5^{S}	14	0^{R}; 5^{IS}	7	2^{R}; 3^{S}	16	0^{R}; 5^{S}	23	1^{R}; 4^{IS}	8	1^{R}; 4^{S}	13	0^{R}; 5^{IS}	7	15
C. coli **(9)**	2^{R}; 7^{IS}	7	4^{R}; 5^{IS}	5	3^{R}; 6^{IS}	6	1^{R}; 8^{S}	24	0^{R}; 9^{S}	18	1^{R}; 8^{S}	11	3^{R}; 6^{IS}	7	0^{R}; 9^{IS}	7	15
C. lari **(1)**	0^{R}; 1^{S}	30	0^{R}; 1^{S}	40	0^{R}; 1^{S}	20	1^{R}; 0^{S}	R	0^{R}; 1^{S}	20	0^{R}; 1^{S}	10	0^{R}; 1^{S}	30	0^{R}; 1^{S}	20	15
C. fetus subsp. *fetus* **(1)**	0^{R}; 1^{S}	20	0^{R}; 1^{S}	10	0^{R}; 1^{S}	10	0^{R}; 1^{IS}	5	0^{R}; 1^{S}	10	0^{R}; 1^{S}	10	0^{R}; 1^{S}	10	0^{R}; 1^{IS}	5	20
C. curvus **(1)**	1^{R}; 0^{S}	R	1^{R}; 0^{S}	R	1^{R}; 0^{S}	R	0^{R}; 1^{S}	20	0^{R}; 1^{IS}	5	0^{R}; 1^{S}	10	0^{R}; 1^{IS}	5	1^{R}; 0^{S}	R	10
C. upsaliensis **(2)**	0^{R}; 2^{S}	10	1^{R}; 1^{S}	10	0^{R}; 2^{IS}	8	0^{R}; 2^{S}	26	0^{R}; 2^{S}	13	1^{R}; 1^{S}	20	0^{R}; 2^{IS}	8	1^{R}; 1^{S}	10	10
H. cinaedi **(2)**	1^{R}; 1^{S}	10	1^{R}; 1^{IS}	5	0^{R}; 2^{IS}	8	1^{R}; 1^{S}	25	0^{R}; 2^{S}	31	1^{R}; 1^{S}	20	0^{R}; 2^{S}	23	0^{R}; 2^{S}	10	10
H. fennelliae **(5)**	2^{R}; 3^{S}	10	1^{R}; 4^{IS}	5	1^{R}; 4^{IS}	5	0^{R}; 5^{S}	25	0^{R}; 5^{S}	30	0^{R}; 5^{S}	19	0^{R}; 5^{IS}	9	0^{R}; 5^{S}	12	15
H. pylori **(2)**	0^{R}; 2^{IS}	5	0^{R}; 2^{S}	10	0^{R}; 2^{IS}	5	0^{R}; 2^{S}	25	0^{R}; 2^{S}	20	0^{R}; 2^{S}	10	0^{R}; 2^{S}	20	0^{R}; 2^{S}	13	15
H. pamatensis **(1)**	0^{R}; 1^{S}	5	0^{R}; 1^{S}	30	0^{R}; 1^{S}	10	0^{R}; 1^{S}	30	0^{R}; 1^{S}	10	0^{R}; 1^{IS}	5	0^{R}; 1^{S}	60	0^{R}; 1^{S}	40	10

The bold number, in brackets, denotes the number of isolates tested.

Superscript: R (^{R}) = Resistance: <5 mm inhibition zone Superscript: IS (^{IS}) = Intermediate Sensitivity: 5-9 mm inhibition zone Superscript: S (^{S}) = Susceptible: ≥10 mm inhibition zone

The number preceding the superscript denotes the number of isolates resistant or susceptible to the sample tested

N.B. The diameters of all inhibition zones exclude the well

Table 3: Inhibition zones, in mm, for species of *Campylobacter*, *Arcobacter* and *Helicobacter* for ground pepper, stick-cinnamon, fine cinnamon, whole allspice, fine allspice, whole cloves, fine cloves, turmeric powder and absolute ethanol

Strains (number tested)	Ground Black Pepper (mm)		Stick-cinnamon (mm)		Fine Cinnamon (mm)		Whole Allspice (mm)		Fine Allspice (mm)		Whole Cloves (mm)		Fine Cloves (mm)		Aqueous Turmeric (mm)		Abs. EtOH (mm)
A. butzleri **(2)**	0^R; 2^S	10	0^R; 2^S	15	0^R; 2^S	40	0^R; 2^S	10	0^R; 2^S	15	0^R; 2^S	10	0^R; 2^S	20	0^R; 2^S	10	10
A. butzleri-like **(4)**	4^R; 0^S	R	0^R; 4^S	23	0^R; 4^S	45	0^R; 4^S	11	0^R; 4^S	23	0^R; 4^S	10	0^R; 4^S	25	0^R; 4^{IS}	6	15
C. jejuni subsp. *jejuni* I **(10)**	0^R; 10^S	16	0^R; 10^S	13	0^R; 10^S	34	0^R; 10^S	14	0^R; 10^S	32	0^R; 10^S	12	0^R; 10^S	20	0^R; 10^{IS}	7	15
C. jejuni subsp. *jejuni* II **(5)**	0^R; 5^S	18	0^R; 5^S	20	0^R; 5^S	46	0^R; 5^S	10	0^R; 5^S	26	0^R; 5^S	11	0^R; 5^S	22	0^R; 5^{IS}	5	15
C. coli **(9)**	0^R; 9^S	17	0^R; 9^S	25	0^R; 9^S	29	0^R; 9^S	14	0^R; 9^S	24	0^R; 9^S	18	0^R; 9^S	21	1^R; 8^{IS}	7	10
C. lari **(1)**	0^R; 1^S	20	0^R; 1^S	15	0^R; 1^S	30	0^R; 1^S	30	0^R; 1^S	40	0^R; 1^S	20	0^R; 1^S	40	0^R; 1^{IS}	5	15
C. fetus subsp. *fetus* **(1)**	0^R; 1^S	30	0^R; 1^S	10	0^R; 1^S	40	0^R; 1^S	10	0^R; 1^S	30	0^R; 1^S	10	0^R; 1^S	30	0^R; 1^{IS}	5	20
C. curvus **(1)**	0^R; 1^S	10	0^R; 1^{IS}	5	0^R; 1^S	10	0^R; 1^{IS}	5	0^R; 1^S	10	0^R; 1^{IS}	5	0^R; 1^S	20	0^R; 1^{IS}	5	15
C. upsaliensis **(2)**	1^R; 1^S	20	0^R; 2^S	18	0^R; 2^S	35	0^R; 2^{IS}	8	0^R; 2^S	18	0^R; 2^{IS}	8	0^R; 2^S	15	0^R; 2^S	13	10
H. cinaedi **(2)**	0^R; 2^S	10	0^R; 2^{IS}	8	0^R; 2^S	23	0^R; 2^{IS}	8	0^R; 2^S	25	0^R; 2^S	13	0^R; 2^S	30	0^R; 2^{IS}	5	10
H. fennelliae **(5)**	0^R; 5^{IS}	9	0^R; 5^S	16	0^R; 5^S	26	0^R; 5^{IS}	8	0^R; 5^S	16	0^R; 5^{IS}	5	0^R; 5^S	16	0^R; 5^{IS}	5	15
H. pylori **(2)**	0^R; 2^S	15	0^R; 2^S	16	0^R; 2^S	30	0^R; 2^S	10	0^R; 2^S	20	0^R; 2^S	10	0^R; 2^S	20	0^R; 2^S	13	20
H. pamatensis **(1)**	0^R; 1^S	30	0^R; 1^S	40	0^R; 1^S	70	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^S	10	0^R; 1^S	20	0^R; 1^S	10	10

The bold number, in brackets, denotes the number of isolates tested.

Superscript: R (^{R}) = Resistance: <5 mm inhibition zone Superscript: IS (^{IS}) = Intermediate Sensitivity: 5-9 mm inhibition zone Superscript: S (^{S}) = Susceptible: ≥10 mm inhibition zone

The number preceding the superscript denotes the number of isolates resistant or susceptible to the sample tested

N.B. The diameters of all inhibition zones exclude the well

Table 4: Inhibitory effect of some antibiotics against the growth of strains of *Campylobacter, Arcobacter* and *Helicobacter*

Strains (number tested)	CN (10µg disk^{-1}) (mm)		KF (30µg disk^{-1}) (mm)		P (10µg disk^{-1}) (mm)		NA (30µg disk^{-1}) (mm)		CIP (5µg disk^{-1}) (mm)		TEC (30µg disk^{-1}) (mm)		OX (1µg disk^{-1}) (mm)		AK (30µg disk^{-1}) (mm)		DA (2µg disk^{-1}) (mm)		SXT (25µg disk^{-1}) (mm)	
A. butzleri **(2)**	0^R; 2^S	20	2^R; 0^S	R	2^R; 0^S	R	1^R; 1^S	10	0^R; 2^S	35	2^R; 0^S	R	1^R; 1^{IS}	5	0^R; 2^S	25	1^R; 1^{IS}	5	2^R; 0^S	R
A. butzleri-like **(4)**	0^R; 4^S	13	1^R; 3^S	23	2^R; 2^S	10	1^R; 3^S	10	0^R; 4^S	35	4^R; 0^S	R	4^R; 0^S	R	0^R; 4^S	28	4^R; 0^S	R	4^R; 0^S	R
C. jejuni subsp. *jejuni* I **(10)**	1^R; 9^S	19	8^R; 2^S	20	5^R; 5^S	11	5^R; 5^S	26	4^R; 6^S	32	9^R; 1^S	10	9^R; 1^S	5	1^R; 9^S	33	3^R; 7^S	29	8^R; 2^S	18
C. jejuni subsp. *jejuni* II **(5)**	0^R; 5^S	22	2^R; 3^{IS}	8	2^R; 3^S	15	1^R; 4^S	29	1^R; 4^S	36	4^R; 1^S	10	5^R; 0^S	R	0^R; 5^S	38	1^R; 4^S	31	4^R; 1^S	30
C. coli **(9)**	1^R; 8^S	22	9^R; 0^S	R	9^R; 0^S	R	4^R; 5^S	13	5^R; 4^S	24	9^R; 0^S	R	9^R; 0^S	R	1^R; 8^S	20	2^R; 8^S	14	9^R; 0^S	R
C. lari **(1)**	0^R; 1^S	10	0^R; 1^S	30	0^R; 1^S	40	1^R; 0^S	R	0^R; 1^S	40	0^R; 1^S	20	1^R; 0^S	R	0^R; 1^S	10	0^R; 1^S	60	1^R; 0^S	R
C. fetus subsp. *fetus* **(1)**	0^R; 1^S	20	1^R; 0^S	R	0^R; 1^{IS}	5	1^R; 0^S	R	0^R; 1^S	30	1^R; 0^S	R	1^R; 0^S	R	0^R; 1^S	30	1^R; 0^S	R	1^R; 0^S	R
C. curvus **(1)**	0^R; 1^S	15	0^R; 1^S	40	0^R; 1^S	40	0^R; 1^S	7	0^R; 1^S	14	0^R; 1^S	16	0^R; 1^S	31	0^R; 1^{IS}	8	0^R; 1^S	21	1^R; 0^S	R
C. upsaliensis **(2)**	0^R; 2^S	18	0^R; 2^S	32	0^R; 2^S	21	1^R; 1^S	40	0^R; 2^S	32	1^R; 1^S	20	1^R; 1^S	21	1^R; 1^S	40	0^R; 2^S	15	2^R; 0^S	R
H. cinaedi **(2)**	0^R; 2^S	16	1^R; 1^S	21	1^R; 1^S	30	0^R; 2^S	16	0^R; 2^S	37	2^R; 0^S	R	2^R; 0^S	R	0^R; 2^S	20	2^R; 0^S	R	1^R; 1^S	20
H. fennelliae **(5)**	0^R; 5^S	34	2^R; 3^S	27	0^R; 5^S	27	1^R; 4^S	26	2^R; 3^S	38	5^R; 0^S	R	5^R; 0^S	R	1^R; 4^S	50	2^R; 3^S	25	5^R; 0^S	R
H. pylori **(2)**	0^R; 2^S	30	1^R; 1^S	20	0^R; 2^S	37	1^R; 1^S	15	0^R; 2^S	53	2^R; 0^S	R	2^R; 0^S	R	0^R; 2^S	50	0^R; 2^{IS}	5	2^R; 0^S	R
H. pamatensis **(1)**	0^R; 1^S	20	1^R; 0^S	R	1^R; 0^S	R	0^R; 1^S	20	*	*	*	*	*	*	*	*	*	*	*	*

The bold number, in brackets, denotes the number of isolates tested.

Superscript: R (^{R}) = Resistance: <5 mm inhibition zone ^{IS} = Intermediate Sensitivity: 5-9 mm inhibition zone ^{S} = Susceptible: ≥10 mm inhibition zone

The number preceding the superscript denotes the number of isolates resistant or susceptible to the sample tested

CN: Gentamicin KF: Cephalothin P: Penicillin G NA: Nalidixic Acid
CIP: Ciprofloxacin TEC: Teicoplanin Ox: Oxacillin AK: Amikacin
DA: Clindamycin SXT: Sulphamethoxazole/Trimethoprim

N.B. The diameters of all inhibition zones exclude the well

DISCUSSION

Absolute Ethanol (positive control)

Chemicals, belonging to the alcohol group, are widely used as disinfectants and antiseptics as they have a bactericidal and fungicidal effect (Prescott *et al.* 1996). Different microorganisms have variable degrees of tolerance to different concentrations of ethanol (Ingram 1990) thus absolute ethanol was used as a positive control in every test to ensure inhibition of all strains. Absolute ethanol was also chosen as it can easily be obtained for use in the laboratory.

Garlic

Garlic is a common, accessible, safe, inexpensive spice which has antibacterial, antifungal, antiprotozoal and antiviral properties (Bakri and Douglas 2005) and may have beneficial implications in infectious diarrhoeal cases (Dog 2006). Allicin, the primary biologically active antimicrobial compound (Saeed and Tariq 2006), has been shown *in vitro* to have a broad spectrum of inhibitory activity against Gram-negative and Gram-positive pathogenic microorganisms, yeasts, *Candida species*, *Cryptococcus neoformans*, *H. pylori* and even microorganisms which have gained resistance to antibiotics (Jezowa *et al.* 1966; Rode *et al.* 1989; Cellini *et al.* 1996; Whitemore and Naidu 2000), while supporting the development of natural flora in the host (reviewed by Irfan 2010). Several garlic preparations, available on the market, have been subjected to clinical trials and have been reported to be as effective as the natural spice (Scott 2006). However, during the extraction process important phytochemicals are often lost resulting in the final processed product of garlic providing less protection against various pathogens than whole garlic (Scott 2006).

In the current study, all of the strains tested were inhibited by all forms of garlic, albeit to different degrees. Crushed garlic displayed the greatest inhibitory effect on all strains tested followed by garlic paste, garlic juice and aqueous garlic solution. Potent antimicrobial elements in raw garlic are released upon crushing; chopping or cutting of the clove (Yamada and Azuma 1977). The sudden exposure to air activates the allinase enzyme converting alliin to allicin (Stoll and Seebeck 1948; Irfan 2010). Alliin and garlic powder have been shown to be potent scavengers of hydroxyl radicals (Kourounakis and Rekka 1991); while allicin interferes with bacterial DNA polymerase, required for DNA replication (reviewed by Irfan 2010). An *in vitro* study demonstrated that allicin mainly exhibits its antimicrobial activity through partial or total inhibition of RNA synthesis suggesting that it is a primary target for allicin (Feldberg *et al.* 1988).

Fresh garlic juice, a by-product of crushed garlic, also has high allicin levels but is ineffective after prolonged air exposure as illustrated by the current study. Allicin decreases to non-detectable levels within 1-6 days (Brodnitz *et al.* 1971; Yu and Wu 1989) reacting with other garlic components and transforming into diallyl sulfides (Lawson *et al.* 1991) and ajoene (Yamada and Azuma 1977). This explains the lower inhibitory activity of garlic paste in this study. In the current study, the inhibitory effect of aqueous garlic solution was also negligible suggesting that allicin may be poorly miscible in water (Block 1985). However, it could also indicate strain variability or that the technique used to prepare the aqueous garlic solution of this study was inadequate as indicated by others. Cellini *et al.* (1996) illustrated 90% inhibition against the 16 clinical strains of *H. pylori* with an aqueous extract of garlic at a concentration of 5 mg ml^{-1}. Using

a standardized aqueous garlic extract (40 mg of thiosulfinate per litre of water) Sivam *et al.* (1997) were able to demonstrate inhibition among all strains of *H. pylori*. De Wet *et al.* (1999) reported that all 38 clinical isolates of *Campylobacter* and all 32 of *Helicobacter* were susceptible to the aqueous garlic solution. The inhibitory concentration of garlic reported by Cellini *et al.* (1996) and Sivam *et al.* (1997) could be achieved in the stomach through the consumption of a medium sized garlic clove or the equivalent in garlic supplements (Sivam 2001). Dr. Scott reported that a daily dose of one to two medium cloves of garlic would be sufficient to exhibit an antimicrobial effect against a range of pathogens without adverse effects (Scott 2006).

Ginger

Ginger contains several bioactive compounds such as borneal, camphene, citral, eucalyptol, linalool, phenylandrene, zingiberine and zingiberol phenols which have been shown to have great therapeutic potential (Hirasa and Takemasa 1998; Ahmad *et al.* 2008 reviewed in Sunilson *et al.* 2009). In the current study, every strain tested was inhibited by all forms of ginger but the level of inhibition differed between the different forms of ginger. Crushed ginger generally demonstrated the greatest inhibitory effect against all strains and its activity was greater compared to that of the paste. Stabilizers in the paste may have enhanced the antimicrobial activity against some microorganisms (Khan *et al.* 2009). Pure ginger juice, obtained by crushing the ginger root, may have lost its activity due to long-term exposure to the air, as in the case of garlic juice.

The preparation technique of a sample could also influence the inhibitory effect against strains of bacteria as illustrated by the current and Sunilson *et al.*

(2009) studies. In the current study, the *A. butzleri*-like and *C. upsaliensis* strains were more susceptible to the aqueous ginger solution than to the ginger juice. It may be that these strains were tested last allowing more antimicrobial elements to diffuse into solution as the volume of liquid decreased. The inhibitory effect of the aqueous ginger solution against *C. jejuni* subsp. *jejuni* biotype II strains in the current study was lower (9 mm) to that of Sunilson *et al.* (2009) (11.4±0.6 mm) but similar to their petroleum ether extract (9.3±1.7 mm). In the current study, ginger was cut into pieces; water added in a 1:1 ratio; incubated at 85ºC for 35 min; and analyzed; whereas Sunilson *et al.* (2009) used a more complicated procedure. Their water extract was prepared by vacuum drying fresh ginger rhizome pieces; milling it to a coarse powder; extracting with water by Soxhlet extraction; and concentrating through vacuum evaporation. With this procedure, their water extract produced one of the higher inhibitory effects against *C. jejuni* which was only second to their methanol extract (12.3±0.8 mm). In the current study, the inhibitory effects against most *Helicobacter* strains were similar to the aqueous ginger solution and ginger juice suggesting that similar amounts of antimicrobial constituents were present in both samples. Mahady *et al.* (2003) reported that all 19 strains of *H. pylroi*, five of which were Cag A+ were killed by the direct effects of gingerols and root ginger. Based on current research, Dr. Scott reported that the regular ingestion of ginger could help in treating and/or preventing the proliferation of *H. pylori* in the stomachs of humans (Scott 2006). However, the exact dose of whole ginger required to obtain the desired antimicrobial effect has not been determined as yet. Ginger, as opposed to antibiotics, is the safer option as antibiotics destroy many valuable intestinal microorganisms along with the target microorganisms (Scott 2006). In

addition, many microorganisms gain resistance to important antibiotics with time (Morsi 2000; Alfredson and Korolik 2007; Oyedeji *et al.* 2009).

Dried Parsley

Although most of the strains tested were inhibited by aqueous dried parsley, the effect appeared to be strain dependant (table 2). *C. curvus* was the only species resistant to dried parsley (table 2), albeit only one strain was tested. The efficacy of dried parsley against *H. pylori* was also noted and in agreement with O'Mahony *et al.* (2005) and Weerasekera *et al.* (2008) where all six clinical strains of *H. pylori* were inhibited by boiled aqueous extracts of fresh parsley.

Dried Thyme

Thyme has potent antibacterial properties with a wide spectrum of activity against antibiotic-resistant strains (Hersch-Martinez *et al.* 2005). Its potency is supported by the large inhibition zones observed for *C. lari* and *H. pamatensis* but inhibition also appears to be strain dependant (table 2). Further work is required to confirm this as only one strain each of *C. lari*, *C fetus* subsp. *fetus*, *C. curvus* and *H. pamatensis* could be obtained for testing. The aqueous dried thyme solution of the current study displayed good inhibitory effects against both *H. pylori* strains. This was confirmed by the Tabak *et al.* (1996b) study where an aqueous extract was found to reduce the growth and potent urease activity of *H. pylori* strains. The inhibitory effect of thyme was greater for *A. butzleri* in the Cervenka *et al.* (2006) study than in the current study. In the Cervenka *et al.* (2006) study, methanol and chloroform extracts were used in comparison to the heated aqueous dried thyme solution of the current study.

Data from the current study suggests that the antimicrobial components in dried thyme are not easily extractable in water. Greater inhibition might have been obtained in the current study using fresh thyme; or by grinding dried thyme prior to adding the water to allow increased diffusion of the antimicrobial components into the water. However, these options were not explored in this study.

Cardamom

Generally, a low inhibitory effect was observed for cardamom but *C. lari* displayed the greatest sensitivity to cardamom (20 mm) (table 2). Using a heated aqueous solution a 5 mm inhibitory zone was observed for each of the two tested *H. pylori* strains (table 2). Preliminary evidence suggests that *Eletteria cardamomum* Maton (Green cardamom), also used in the current study, may exhibit greater antibacterial properties than *Amomum subulatum* Roxb (Black cardamom) (Mahady *et al.* 2005; Zaidi *et al.* 2009). However, Zaidi *et al.* (2009) reported that green cardamom was ineffective against seven clinical and one reference strain of *H. pylori* even at 500 μg ml^{-1}; whereas the black form displayed complete inhibition at a concentration of 125 μg ml^{-1}.

Fresh Lemon juice

Lemon oils have antibacterial and antifungal activities but literature pertaining to the antibacterial activity of its juice is lacking (Saeed and Tariq 2006). The active chemical components of lemon are limonene, citral, citronellal, α-terpineol, linalyl, geranylacetate, α-terpinene, β-bisabolene, trans-α-bergamotene, nerol and neral (Saeed and Tariq 2006). Fresh lemon juice displayed good inhibitory activity against most microorganisms (table 2)

suggesting that the microorganisms may either be sensitive to one or a combination of the active chemical components listed above (Fisher and Philips 2006; Fisher *et al.* 2007). These microorganisms may also be sensitive to the acidic environment created, in part, by citric acid in lemon juice.

Honey

Honey mainly consists of glucose and fructose (Molan 1992). It may also contain additional medicinal compounds such as essential oils, flavanoids, terpenes and polyphenols but the actual composition of honey depends on the plant from which pollen was obtained (Molan 1992). The antimicrobial mode of action of honey involves several pathways with hydrogen peroxide content; non-peroxide and osmotic effects postulated to aid in antimicrobial killing of honey (Allen *et al.* 1991; Al Somal *et al.* 1994). It is possible to identify other non-peroxide antimicrobial components in honey by performing a catalase test. However, this was not done in the current study. By adding catalase to Manuka honey samples, Dr. Peter Molan was able to remove hydrogen peroxide and identified a non-peroxide element UMF which is unique to some Manuka honey (Anonymous 2009).

In the current study, all of the strains tested were inhibited by fynbos honey (without dilution with water). The lowest inhibition was recorded for *C. curvus* (9 mm) and the highest for *H. cinaedi* (31 mm), followed by *H. fennelliae* (30 mm); while the two strains of *H. pylori* displayed an average inhibition of 20 mm. In 2008, Mavric *et al.* identified methylglyoxal (MGO) as the main antibacterial constituent of Manuka honey. However, the presence of MGO was not known or determined in the fynbos honey samples, tested in this study.

Ali *et al.* (1991) illustrated that all isolates of *H. pylori* were inhibited by 20% "natural honey". A similar trend was observed for *H. pylori* strains of the current study using fynbos honey. However, further work is required to confirm this as only two strains of *H. pylori* could be obtained for testing. Ali *et al.* (1991) further demonstrated the antimicrobial activity of 20% honey against antibiotic resistant isolates. Numerous *in vitro* studies have been conducted on honey reporting on its good inhibitory effect against various microorganisms. However, its antimicrobial effect against infections associated with *Campylobacter* and related microorganisms have been explored in very few *in vivo* cases. Haffejee and Moosa (1985) observed reduced symptoms in patients suffering from bacterial diarrhoea using honey.

Crushed onion

Onions and garlic belong to the *Allium* family (Benkeblia 2004) and possess antibacterial and antifungal activities due to the presence of allicin; powerful phenolic; and sulphur compounds (Griffiths *et al.* 2002). The current study revealed similar data to Lawson (1996) illustrating that garlic possess nearly 3 times the amount of sulphur-containing compounds as onions. Although good inhibitory effects were generally observed in the current study against the tested strains, strain variability was observed among several species of *Arcobacter*, *Campylobacter* and *Helicobacter*. The presence of crushed onion resulted in intermediate inhibition against *C. jejuni* subsp. *jejuni* biotype I (6 mm) and *C. jejuni* subsp. *jejuni* biotype II (8 mm); while *C. coli* (11 mm), *C. lari* (10 mm), *C. fetus* subsp. *fetus* (10 mm), *C. curvus* (10 mm) displayed good inhibition but the greatest inhibition was exhibited by one strain each of *C. upsaliensis* (20

mm) and *H. cinaedi* (20 mm). Interestingly, all strains of *H. fennelliae*, *H. pylori* and *H. pamatensis* were inhibited by crushed onion illustrating its susceptibility to the antibacterial properties of onion. Aqueous extracts of Chinese leek (soft leek) have also been shown to produce strong inhibitory activities against *C. jejuni* subsp. *jejuni* and *C. coli* strains (Lee *et al.* 2004). However, when the extracts were exposed to extreme temperatures (>75ºC) or alkaline environments a significant loss of inhibition was observed as it impedes the activity of allicin (Agarwal 1996).

Black cumin seeds

Black cumin seeds, its oil and extracts act as an immune stimulant (Salem 2005) and have antimicrobial activity against several Gram-negative and Gram-positive bacteria, including multidrug resistant microorganisms (Topozada *et al.* 1965; El-Fatatry 1975; Morsi 2000; Aljabre *et al.* 2005). Furthermore, the diethyl extract of black cumin seeds have been shown to have a synergistic or an additive effect with several antibiotics (Hanafi and Hatem 1991). In the current study, most of the species tested were susceptible to the inhibitory effect of heated aqueous black cumin seeds. However, in some cases inhibition was shown to be strain dependant, while all four *A. butzleri*-like strains displayed resistance to black cumin seeds (table 2). Zuridah *et al.* (2008) have shown that black cumin seeds were ineffective against extended-spectrum β-lactamase (ESBL) producers most of which belong to the *Enterobacteriaceae* family such as *K. pneumonia* and some strains of *E. coli*. The production of β-lactamase is the most common mechanism of resistance among Gram-negative microorganisms (Philippon *et al.* 1989). In contrast, resistance may be a

consequence of the preparation technique. Mashhadian and Rakhshandeh (2005) have shown that methanol and chloroform extracts of black cumin seeds exhibited high inhibitory effects against standard and hospital strains of *C. albicans*, coagulase-positive *S. aureus* and *P. aeroginosa*, while their aqueous extract was ineffective against all tested microorganisms.

Crushed chillies

Dorantes *et al.* (2000) indicated that the potent antimicromial activity of chillies are due to the presence of cinnamic and *m*-coumaric acids. Recently, investigations have shown that whole chilli or its active compound, capsaicin, can prevent ulcer formation (reviewed in Satyarayana 2006). *H. pylori* have often been associated with the formation of ulcers (Ramakrishnan and Salinas 2007, Jackson 2008). The bactericidal activity of chillies against *H. pylori* was been demonstrated in the current and O'Mahony *et al.* (2005) studies. Most of the species tested was susceptible to crushed chillies, however strain variability has been observed among the *A. butzleri*-like; *C. jejuni* subsp. *jejuni* biotype I; and *C. upsaliensis* strains, while all strains of *A. butzleri* and *C. curvus* were shown to be resistant to the antimicrobial activity of freshly crushed chillies (table 2). Resistance to some chillies has previously been reported (Dorantes *et al.* 2000).

Ground Black Pepper

Ground pepper contains potent antimicrobials such as piperine. Dr. Scott has reported that black pepper can increase the number and efficacy of white cells resulting in an increased defence against various pathogens (Scott 2006). Ground

pepper in combination with other spices have been shown to have highly potent inhibitory effects on various microorganisms as ground pepper is known to boost the activity of biochemical active compounds present in a variety of spices by up to several hundred percent (Lambert *et al.* 2004). The antimicrobial activity of ground pepper has also been demonstrated in the current study. All strains of most species of *Arcobacter*, *Campylobacter* and all species of *Helicobacter* were inhibited by the heated aqueous ground pepper preparation (table 3). Piperine is not the only antimicrobial present in black pepper thus Dr. Scott advises that the whole/fine product should be ingested to ensure that the full protective benefits of black pepper would be obtained (Scott 2006). However, it is also possible that some microorganisms could be resistant to black pepper. This has been noted in the current study where all four *A. butzleri* strains were resistant; while strain variability was noted among the *C. upsaliensis* strains (table 3) illustrating that piperine and other antimicrobials in ground pepper are ineffective against these strains.

Cinnamon

The bactericidal activities of cinnamic aldehyde and eugenol, primary antimicrobials, have been proven against various foodborne pathogens (Valero and Frances 2006). All strains tested were inhibited by cinnamon (stick and fine) (table 3) supporting previous studies where cinnamon was found to possess antimicrobial properties against a range of pathogenic microorganisms, including *Arcobacter*, *Campylobacter* and *Helicobacter* (Tabak *et al.* 1996a; Smith-Palmer *et al.* 1998; Cervenka *et al.* 2006). In this study, fine cinnamon displayed a two-fold or higher inhibitory effect in comparison to stick-cinnamon

suggesting that fine cinnamon may contain higher antimicrobial levels. *A. butzleri* strains were more sensitive to the fine cinnamon aqueous solution than to the stick cinnamon aqueous solution with inhibition zones of 40 mm and 10 mm, respectively. Inhibitory zones for *A. butzleri* were lower in the Cervenka *et al.* (2006) study, however, it should be noted that different extracts were used. They observed inhibition zones of 30.9 mm and 19.4 mm for the chloroform and methanol extracts, respectively. The current study thus suggests a non-toxic cinnamon extract that can have a greater inhibitory effect on the growth of microorganisms than an extract containing solvents such as methanol and chloroform which are toxic to humans. The greatest inhibitory effect was observed for *H. pamatensis*: 40 mm for the stick-cinnamon and 70 mm for the fine cinnamon water extracts. The observation regarding *H. pamatensis* is novel, however, further work is required using more strains to confirm this. High inhibitory activity was observed with the heated aqueous solution of cinnamon against *H. pylori* (table 3). *In vitro* water extracts have not been tested clinically but ethanol (Tabak *et al.* 1999) and methylene chloride (Tabak *et al.* 1999) extracts have been tested against *H. pylori*.

Allspice and cloves

Research has shown that allspice contain high concentrations of important antioxidants such as proanthocyanidins ensuring protection against a range of diseases (Young and Woodside 2001). Clove essential oil has also been shown to exhibit antibacterial activity against a range of methicillin-resistant microorganisms (Enzo and Susan 2002). All of the strains tested were inhibited by allspice and cloves but approximately a two-fold higher inhibitory effect was

recorded for the fine product in comparison to the whole product (table 3) suggesting that antimicrobials are at higher levels in the former form. The heated aqueous allspice preparations of the current study yielded similar inhibitory results for *A. butzleri* to those of Cervenka *et al.* (2006) who used methanol and chloroform extracts of allspice. The current study thus suggests a non-toxic form of allspice that could be of value in the treatment of *A. butleri* infections. However, further work is required to confirm its efficacy, especially in clinical trials. Lower inhibition was observed with the methanol and chloroform clove extracts of Cervenka *et al.* (2006) in comparison to the heated aqueous fine clove solution of the current study suggesting a safer and more effective option for human consumption. However, their methanol and chloroform allspice extracts were more effective than the heated aqueous mixtures of the current study suggesting that the active antimicrobial compounds of allspice is not easily extractable in water.

Turmeric

Curcumin, a primary component of turmeric, has antibacterial and antifungal properties (Lutomski *et al.* 1974). In the current study all strains of *Arcobacter* and *Helicobacter* and most strains of *Campylobacter* were inhibited but one strain of *C. coli* displayed resistance to the heated aqueous turmeric mixture (table 3). However, resistance of other *Campylobacter* strains have also been reported. Goel demonstrated resistance of the tested strain of *C. jejuni* against a hexane extract of turmeric (Goel 2007). Although the efficacy of turmeric may be directly dependent on the preparation method, the current and Goel (2007) studies further suggest that the antimicrobial effect of turmeric may be strain

dependant. It may also imply that some strains could evolve its cell membrane structure as a protective mechanism. However, further work is required to confirm this. The *H. pylori* results of the current study is in agreement with the O'Mahony *et al.* (2005) and Weerasekera *et al.* (2008) studies who demonstrated that all strains of *H. pylori* were killed within 15 min of exposure to the boiled aqueous turmeric extracts.

Inhibitory effects of antibiotics

Variable susceptibility, among the *A. butzleri*-like strains, was noted for cephalothin; penicillin G; and nalidixic acid (table 4). This, in part, is in agreement with Atabay and Aydin (2001) suggesting that strains may acquire resistance to antibiotics as a survival strategy. Variation in strain susceptibility to cephalothin was also noted by Kabeya *et al.* (2004). The data of this study indicated that one strain of *A. butzleri* and four *A. butzleri*-like strains were resistant to teicoplanin; oxacillin; clindamycin; and sulphamethoxazole/trimethoprim which are in agreement with Atabay and Aydin (2001) and Fera *et al.* (2003). *C. jejuni* strains are generally known to be sensitive to gentamicin; while ciprofloxacin-resistant strains are on the increase in most countries (Alfredson and Korolik 2007). Bester and Essack (2008) analyzed a total of 77 *C. jejuni* poultry isolates, 56 of which were isolated from chicken broiler and 21 from layer chickens. They reported that 1.8% of the broiler chickens and 19% of the layer chickens were resistant to gentamicin; while 8.9% of the broiler chickens and 23% of the layer chickens were resistant to ciprofloxacin. Although lower numbers of *C. jejuni* strains were tested in the current study, a similar trend was also noted with 10% and 50% of the *C. jejuni*

subsp. *jejuni* biotype I strains resistant to gentamicin and ciprofloxacin, respectively (table 4). None of the *C. jejuni* subsp. *jejuni* biotype II strains of the current study were resistant to gentamicin but 20% was resistant to ciprofloxacin (table 4). Modolo *et al.* (2003) reported 51 strains of *Campylobacter*, consisting of 29 *C. jejuni* subsp. *jejuni*; 14 *C. jejuni* subsp. *doylei*; five *C. upsaliensis*; two *C. coli* and one *C. lari*, isolated from 29 diarrheic and 22 diarrhoea-free dogs susceptible to gentamicin at concentrations ranging between 0.5-4 μg ml^{-1}. Oporto *et al.* (2009) reportedly tested 72 strains of *C. jejuni* isolated from free-range chicken (19), dairy sheep (25) and cattle (28) by broth microdilution. They found that all strains were susceptible to gentamicin; 52.8% were resistant to ciprofloxacin and nalidixic acid; while 47.8% were susceptible to both antibiotics. In the current study, the two *C. jejuni* subsp. *jejuni* biotype II isolates were both sensitive to gentamicin, naladixic acid and ciprofloxacin (data incorporated as an average of all *C. jejuni* subsp. *jejuni* biotype II strains in table 4). The high resistance rates for nalidixic acid in the Bester and Essack (2008) study raised a concern as 35.7% of the broiler chickens and 52.4% of the layer chickens were resistant to this antibiotic. A similar result was observed in the current study where 50% of the *C. jejuni* subsp. *jejuni* biotype I strains and 20% of the *C. jejuni* subsp. *jejuni* biotype II strains were resistant to nalidixic acid. The type strain of *C. lari* illustrated resistance to clindamycin in the Huysmans and Turnidge study (1997) whereas the *C. lari* strain of the current study displayed good susceptibility. In the Vandenberg *et al.* (2006) and current studies, *C. upsaliensis* displayed strain variability; while resistance was observed for teicoplanin elsewhere (Preston *et al.* 1990). The *H. cinaedi* data generated in the current study is in agreement with that of Jie-song *et al.* (1999), however

varied from the Melito *et al.* (2001) where *H. cinaedi*, *H. fennelliae* and *H. pamatensis* isolates were susceptible to cephalothin and nalidixic acid; while their *H. pylori* isolate was susceptible to cephalothin but resistant to nalidixic acid.

In conclusion, the inhibitory effects of dried parsley, dried thyme, cardamom, fresh lemon juice, crushed onion, black cumin seeds, crushed chillies, ground pepper and various antibiotics appeared to be species or strain dependant. Every strain, tested, was inhibited by garlic; ginger; fynbos honey; cinnamon; allspice; and cloves. The addition of one or a combination of these culinary products to food as a marinade or to processed foods would inhibit the growth of *Arcobacter*, *Campylobacter* and *Helicobacter* strains in food products and would presumably be safer to eat. The consumption of these culinary products in various dishes or cuisines may also serve as protection against infections caused by species of *Campylobacter*, *Arcobacter*, *Helicobacter* and other microorganisms. However, variable prevention rates of chronic degenerative diseases and pathogenic infections have been correlated to the quantities of spices used in cooking dishes, globally (Scott 2006). Similarly, the amount of antibiotic is correlated with the degree of protection against a particular microorganism. Spices; herbs; and other culinary products appear to be the better option compared to antibiotics due to several reasons. Over the years, the rate of antibiotic resistance of various microorganisms have increased at an alarming rate and the use of antibiotics often results in a range of side-effects; whereas culinary products are natural, contain a range of potent antimicrobial components operating via different pathways and do not have any side-effects (Scott 2006). Antibiotics normally target a specific microorganism,

while culinary products are able to provide antimicrobial benefits against a vast range of microorganisms. Many antibiotics have a narrow safety margin, i.e. their therapeutic and toxic doses are very close together; while spices and other culinary products have a wide safety margin, i.e. their beneficial and toxic doses are far apart (Scott 2006). Furthermore, antibiotic usage is generally more risky during pregnancy; with the aged; and when used concurrently with other medications, while the ingestion of culinary products are less risky. Spices are generally safe when consumed in normal dietary quantities, except in certain cases, i.e. any substance or product can become toxic in high enough quantities (Scott 2006). Thus in order to derive maximum benefits from the culinary product and to ensure that the quantities ingested are safe for human consumption, researches often focus their attention on societies that incorporate large amounts of a culinary product into their traditional cuisines (Scott 2006).

ACKNOWLEDGEMENTS

Sincere gratitude is extended to the Department of Biotechnology at the University of the Western Cape, National Research Fund (NRF) of South Africa, Muslim Hands (South Africa) and the South African National Zakah Fund (SANZAF) for financial support.

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CONCLUSION AND FUTURE PROSPECTS

BACKGROUND

Isolation and detection of the target microorganisms using the IAC

The general approach in determining whether *Campylobacter, Arcobacter* and/or *Helicobacter* are present in a test sample is to try to isolate these microorganisms on non-selective media such as tryptose blood agar (TBA). This is particularly important if a new species of the microorganism is suspected. The culturing method used in this thesis was based on methods by Ceelen *et al.* (2006) and Lastovica (2006). However, due to limitations in culturing such as contaminant overgrowth caused by the faster growing microorganisms (Pentimalli *et al.* 2009), it was decided to incorporate PCR to confirm the presence or absence of the microorganism(s) in the tested samples. PCR inhibitors naturally present in the sample such as haeme (Akane *et al.* 1994) and leukocyte DNA (Morata *et al.* 1998) in blood; and organic and phenolic compounds, fats, polysaccharides, proteins, glycogen and Ca^{2+} in food (Wilson 1997; Rossen *et al.* 1992) often lead to "false-negative" results suggesting the absence of target microorganisms. Various pre-processing strategies have been developed to remove or reduce the negative effects of PCR inhibitors from the tested samples (Rådström *et al.* 2004). The agarose-DNA approach developed by Moreira (1998) was used in this thesis. The use of an Internal Amplification Control (IAC) is imperative in PCR to confirm that optimal PCR conditions are present in

every tube (Lübeck *et al.* 2003; Josefsen *et al.* 2004). The IAC may be constructed using a competitive or non-competitive strategy (Hoorfar *et al.* 2004). The competitive IAC strategy was successfully used in multiplex PCR in this thesis.

Survival of *Campylobacteraceae* and *Helicobacter* strains in blood

Campylobacter form part of the normal enteric flora in animals such as poultry, cattle and pigs and may be transmitted to humans through the consumption or handling of contaminated foods (Stern *et al.* 2003; Gallay *et al.* 2007). *Campylobacter*, *Arcobacter* and *Helicobacter* have also been associated with, among others, septicemia and bacteraemia (Vandamme *et al.* 1990; Hsueh *et al.* 1997; Lastovica 2006). This emphasizes the ability of these microorganisms to cause disease in blood and using blood as a possible mode of transmission to other hosts. To date, no study has been done to determine the survival of *Campylobacter*, *Arcobacter* and *Helicobacter* in chicken blood. As a result, it was decided to incorporate this study in the thesis.

Treatment of *Campylobacteraceae* and *Helicobacter* strains

Species of *Campylobacteraceae* and *Helicobacter* have been detected in drinking water (Diergaardt *et al.* 2004); retail meats including: pork, beef and poultry (Stanley *et al.* 1994; Kabeya *et al.* 2004); poultry processing plants (Son *et al.* 2006); raw milk (Lovett *et al.* 1983; Dore *et al.* 2001;

Scullion *et al.* 2006), etc. Some strains of some species of *Campylobacteraceae* and *Helicobacter* have acquired resistance to a range of antimicrobial agents including those used in the treatment of diarrheal illnesses (Mansfield and Forsythe 2000; Ceelen *et al.* 2005; Gallay *et al.* 2007). As a result, alternative strategies are being sought to treat infections of *Campylobacter*, *Arcobacter* and *Helicobacter*. One of the strategies is the use of herbal medicines, tea, plant essential oils and plant extracts (Diker *et al.* 1991; Smith-Palmer *et al.* 1998; Cervenka *et al.* 2006). In this thesis, it was decided to determine the effect selected culinary products (garlic, ginger, fresh lemon juice, pure local honey, onion, dried parsley, dried thyme, cardamom, fresh lemon juice, crushed onion, crushed chilies, ground pepper and turmeric) have on the growth of strains of different species of *Campylobacter*, *Arcobacter* and *Helicobacter*.

ACHIEVEMENT OF AIMS

Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples

The aim of this study was to evaluate a new method, in comparison to two published methods (Moreira 1998; Monteiro *et al.* 2001), to remove PCR inhibitors from poultry DNA samples to detect *Campylobacter* by multiplex PCR in the presence of a competitive Internal Amplification Control. Both, spiked and unspiked poultry tissue and blood samples were analyzed. The aim of this study has been met as it illustrated that the new method was more

effective than the two published methods in removing PCR inhibitors from poultry samples thereby allowing amplification of target fragments by multiplex PCR in the presence of the competitive IAC. The IAC was amplified in the spiked and unspiked samples; while both the target (946 bp) and the IAC (580 bp) fragments were amplified in the spiked samples.

Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken

The aim of the study was to detect and isolate *Arcobacter* and *Campylobacter* in chicken products and blood. An attempt was made to isolate *Campylobacter* and related microorganisms, based on two publications (Ceelen *et al.* 2006; Lastovica 2006). After removal of PCR inhibitors, using the new method, multiplex PCR was used by incorporating the competitive IAC. This was followed by 16S rRNA gene sequencing, generation of 16S rRNA phylogenetic trees and Amplified Fragment Length Polymorphism (AFLP) profiling to confirm the absence of target microorganisms. The aim of the study was met as three *Arcobacter* species were detected in chicken: *A. cibarius* in liver; *A. cryaerophilus* in caeca; and *A. butzleri* in "mala" contents and "free-range"/organic chicken blood. In addition, *A. butzleri* was isolated from a "free-range" chlorine-free organic chicken liver; while two *C. jejuni* subsp. *jejuni* biotype II isolates were isolated from "free-range"/organic chicken blood. The isolation of *C. jejuni* subsp. *jejuni* from chicken blood is a novel finding. The detection of *A.*

butzleri and isolation of *C. jejuni* subsp. *jejuni* in "free-range"/organic chicken blood suggest a possible reservoir and transmission route. AFLP profiling was highly informative and discriminatory in identifying related strains of *Arcobacter* and *Campylobacter*, in comparison to the 16S rRNA phylogenetic trees.

In vitro Survival test of _Campylobacteraceae_ and _Helicobacter_ species in "free-range"/organic chicken blood at various temperatures

The aim of this novel *in vitro* study was to determine how long strains of *Campylobacter*, *Arcobacter* and *Helicobacter* could survive in "free-range"/organic chicken blood at the tested temperatures. Various strains of *Campylobacter*, *Arcobacter* and *Helicobacter* were inoculated in "free-range"/organic chicken blood and incubated at -20, 4, 37 and 42ºC as well as at ambient room temperature (±22ºC). The aim of this novel *in vitro* study was met. *Campylobacter*, *Arcobacter* and *Helicobacter* strains were able to survive at -20, 4, 37 and 42ºC as well as at ambient room temperature in chicken blood. However, it was found that *Campylobacter* and *Arcobacter* strains survived much longer than those of *Helicobacter*. Most notably, an *A. butzleri* strain was able to survive up to 297 days in blood stored at 4ºC. This study also illustrated that strain viability could be detected for a longer period with differential streaking than filtration.

Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

The aim of this study was to determine the effect of some culinary products (non-irradiated spices; herbs; fresh lemon juice; pure local honey; garlic, ginger and onion) on the growth of strains of *Campylobacter*, *Arcobacter* and *Helicobacter* species in comparison to ciprofloxacin and other antibiotics. This study used the well-diffusion method to determine the effect of various culinary products on the growth of the tested strains. The aim of this study was met. Garlic; ginger; pure local honey; cinnamon; allspice; and cloves proved to be potent growth inhibitors against all of the *Campylobacteraceae* and *Helicobacter* strains tested; while the inhibitory effects of dried parsley, dried thyme, cardamom, fresh lemon juice, crushed onion, crushed chilies, ground pepper and turmeric appeared to be species or strain dependant. It was also noted that neither antibiotic used in this study had the ability to inhibit all of the tested strains.

FUTURE PROSPECTS

Evaluation of a new method to efficiently remove PCR inhibitors from poultry DNA samples

The new method for the removal of PCR inhibitors could be used to ensure the safety of poultry by adequately detecting pathogens which may ultimately result in a reduced risk of contracting various foodborne illnesses and infections. Furthermore, by using a competitive and noncompetitive

IAC, the new method could be evaluated using other samples such as environmental, faecal, water as well as other tissue and food samples to determine its efficacy.

Detection and isolation of *Campylobacteraceae* in retail and "Free-range"/organic chicken

Further work could be done to determine the virulence potential of the positive samples and isolates by PCR and AFLP profiling. PCR could be used to determine whether key virulent genes are present; while AFLP profiling could determine genetic relatedness among virulent and non-virulent strains.

***In vitro* Survival test of *Campylobacteraceae* and *Helicobacter* species in "free-range"/organic chicken blood at various temperatures**

A larger study could be done using strains of various origins of every species of *Campylobacter*, *Arcobacter* and *Helicobacter* inoculated in "free-range"/organic and "battery" chicken blood, human blood as well as blood from other land and aquatic animals. This could be done to determine whether the survival of strains of different species of *Campylobacter*, *Arcobacter* and *Helicobacter* is host-dependant.

Inhibitory effect of selected culinary products versus antibiotics against the growth of *Campylobacteraceae* and *Helicobacter* species

This study could be repeated on a larger scale, using more strains of every species of *Campylobacter*, *Arcobacter* and *Helicobacter* from various origins. Studies could be conducted to determine the efficacy of using one or a combination of these culinary products as a food marinade or as an additive to processed foods to prevent the proliferation of *Campylobacter*, *Arcobacter* and *Helicobacter* in food products. It could also be used in shelf-life studies. Furthermore, these culinary products could used in various *in vivo* studies using mice or rats and later on humans to determine the efficacy of raw culinary products in treating infections of *Campylobacter*, *Arcobacter* and *Helicobacter*. This would be of interest to many people, especially those who cannot afford expensive treatments or who cannot afford regular transportation costs to collect the antibiotics and/or to be vaccinated.

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APPENDIX A

Gels and Buffers

1. 1.6% Low Melting Point Agarose

- Weigh out 3.2 g of low melting point agarose and make up to a final volume of 200 ml with 1x TBE buffer.
- Heat and boil in a microwave until the agarose has dissolved.

2. 2% Agarose Gel

- Weigh out 4 g of agarose and make up to a final volume of 200 ml with 1x TBE buffer.
- Heat and boil in a microwave until the agarose has dissolved.
- Once dissolved, add 25 µl of EtBr (100 µg ml^{-1}) to the agarose solution.
- Cool to 55ºC before pouring.
- Pour the agarose solution into the required gel tray containing the desired comb size and allow it to set for approximately 30 min.

3. EtBr (100 ug ml^{-1})

- Dispense 250 µl of 10 mg ml^{-1} molecular grade EtBr solution (Promega, U.S.A.) into a glass bottle wrapped in foil.
- Add 24.75 ml of Millique H_2O to make up to a final volume of 25 ml of 100 µg ml^{-1} EtBr solution.
- Mix thoroughly and store at room temperature.

4. 10x Tris-Borate EDTA (TBE) Buffer

- Weigh out 108 g of Tris base, 55 g of Boric acid and 9.3 g of EDTA and add to 800 ml of Millique H_2O, in a sterile 1 l bottle.
- Mix with a magnetic stirrer while adjusting the pH to 8.0 (EDTA will only dissolve above pH 8.0)
- Adjust the volume to 1 l with additional Millique H_2O.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.
- Store at room temperature.

5. 1x TBE Buffer

- Measure out 100 ml of 10 x TBE Buffer and pour it into a sterile 1 l bottle.
- Top up with Millique H_2O to 1 l.
- Mix thoroughly before using and store the remainder at room temperature.

6. 6x Loading Buffer (Gel Tracking Dye)

- Dissolve 62.5 mg of bromophenol blue, 10 g of sucrose in 15 ml of Millique H_2O and add 1 ml of 0.5 mol l^{-1} of EDTA (pH 8.0).
- Make up to a final volume of 25 ml with Millique H_2O.
- Mix thoroughly and store at room temperature.

7. Tris-EDTA (TE) Buffer

- Measure out 1 ml of 0.5 mol l^{-1} of EDTA (pH 8.0) and 5 ml of 1 mol l^{-1} of Tris-Cl (pH 7.6) and make up to a final volume of 500 ml with Millique H_2O.
- Mix thoroughly.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

8. Lysis Buffer per 1 ml

- Aliquot 10 µl of Tris-Cl (pH 8.0) into a sterile eppendorf tube.
- Add 500 µl of 1 mol l^{-1} EDTA (pH 9.2), 0.01 g of Lauroyl sarcosine and 2 mg of Proteinase K.
- Top up to 1 ml with Millique H_2O.

APPENDIX B

Solutions, Media and Media Additives

1. 10% Sodium dodecyl sulphate (SDS) (w/v)

- Weigh out 40 g of SDS (in a fumehood), using gloves and a mask, and dissolve thoroughly in 400 ml of Millique H_2O.
- Heat to ± 80ºC to dissolve while mixing with a magnetic stirrer.

2. 20 mg ml^{-1} Proteinase K

- Weigh out 20 mg of proteinase K and dissolve thoroughly in 1 ml of Millique H_2O.
- Sterilize using a filter.
- Store in 20 µl aliquots at -20ºC.

3. 5 mol l^{-1} Sodium Chloride (NaCl)

- Weigh out 14.61 g of NaCl and dissolve thoroughly in 50 ml of Millique H_2O.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

4. 1 mol l^{-1} NaCl

- Dispense 10 ml of 5 mol l^{-1} of NaCl in a sterile 50 ml bottle.
- Top up with Millique H_2O to 50 ml.
- Mix thoroughly.
- Store at room temperature.

5. Hexadecyltrimethyl ammonium bromide (CTAB)/NaCl

- Dissolve 4.1 g of NaCl in 80 ml of Millique H_2O.
- Slowly add 10 g of CTAB while heating and stirring using a magnetic stirrer.
- If necessary, heat to 65ºC to dissolve.
- Adjust to a final volume of 100 ml with Millique H_2O.

6. 24:1 Chloroform/isoamyl alcohol

- Measure out 4 ml of isoamyl alcohol and add 96 ml of chloroform to make a final volume of 100 ml.
- Mix thoroughly.
- Store at room temperature.

7. Phenol

- Weigh out 500 g of commercially crystallized phenol.
- Add 0.6 g of 8-hydroxyquinoline, 7.5 ml of 2 mol l^{-1} of NaOH, 130 ml of Millique H_2O and 6 ml of 1 mol l^{-1} of Tris-Cl (pH 7.6).
- Leave overnight to liquefy.
- Mix thoroughly.
- Dispense in 50 ml aliquots and store at -20ºC. (The result is a phenol solution in 10 mmol l^{-1} Tris (pH 7.6)

8. 25:24:1 Phenol/chloroform/isoamyl alcohol

- Measure out 50 ml of phenol (as prepared above), 48 ml of chloroform and 2 ml of isoamyl alcohol and pour into a sterile 100 ml bottle.
- Mix thoroughly.
- Cover the bottle with foil as it is light sensitive.
- Store in the fridge.

9. 70% EtOH

- Pour 70 ml of absolute EtOH into a 100 ml bottle.
- Add 30 ml of Millique H_2O.
- Mix thoroughly and store at room temperature.

10. 1 mol l^{-1} Tris-HCl (pH 8.0)

- Dissolve 121 g of Tris base in 800 ml of Millique H_2O.
- Adjust to pH 8.0 with HCl pellets.
- Mix thoroughly.
- Once the desired pH is obtained, make up to 1 l with Millique H_2O.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

11. 10 mmol l^{-1} Tris-HCl (pH 8.0)

- Aliquot 5 ml of Tris-HCl (pH 8.0) into a sterile 500 ml bottle and top up with Millique H_2O to 500 ml.
- Mix thoroughly.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

12. 0.5 mol l^{-1} EDTA (pH 8.0)

- Dissolve 93.06 g EDTA in 400 ml of Millique H_2O by stirring vigorously with magnetic stirrer while adding about 10 g of NaOH pellets until pH 8.0 is reached.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

13. 0.125 mol l^{-1} EDTA (pH 8.0)

- Aliquot 62.5 ml of 0.5 mol l^{-1} EDTA (pH 8.0) into a sterile 250 ml bottle.
- Top up to 250 ml with Millique H_2O and mix thoroughly.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

14. 1 mol l^{-1} EDTA (pH 9.2)

- Dissolve 186.12 g EDTA in 400 ml of Millique H_2O by stirring vigorously with magnetic stirrer while adding about NaOH pellets until pH 9.2 is reached.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

15. 2x Yeast Tryptone Broth

- Dissolve 16 g of Tryptone, 10 g of Yeast Extract and 5 g of NaCl in 1 l of Millique H_2O.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

16. *Arcobacter* Enrichment Broth (AEB)

- Dissolve 28 g of AEB in 1 l of Millique H_2O and mix thoroughly.
- Autoclave at 121ºC for 20 min at 15 pounds of pressure.

17. Tryptose Blood Agar (TBA)

- Dissolve 15 g of TBA powder in 500 ml of Millique H_2O.
- Mix thoroughly and autoclave at 121ºC for 20 min at 15 pounds of pressure.
- Cool to 50ºC (cool enough to hold)
- Add 50 ml of horse blood, mix and pour plates.
- Set at room temperature for approximately 30 min in a laminar flow.

18. 10x Phosphate Buffered Saline (PBS) (pH 7.2)

- Dissolve 80 g of NaCl, 2.0 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 in 800 ml of Millique H_2O.
- Adjust to pH 7.2.
- Top up with Millique H_2O to 1 l.
- Sterilize by autoclaving at 121ºC for 20 min at 15 pounds of pressure.

19. 1x PBS (pH 7.2)

- Aliquot 100 ml to of 10x PBS into a sterile 1 l bottle.
- Top up to 1 l with Millique H_2O and sterilize by autoclaving at 121ºC for 20 min at 15 pounds of pressure.

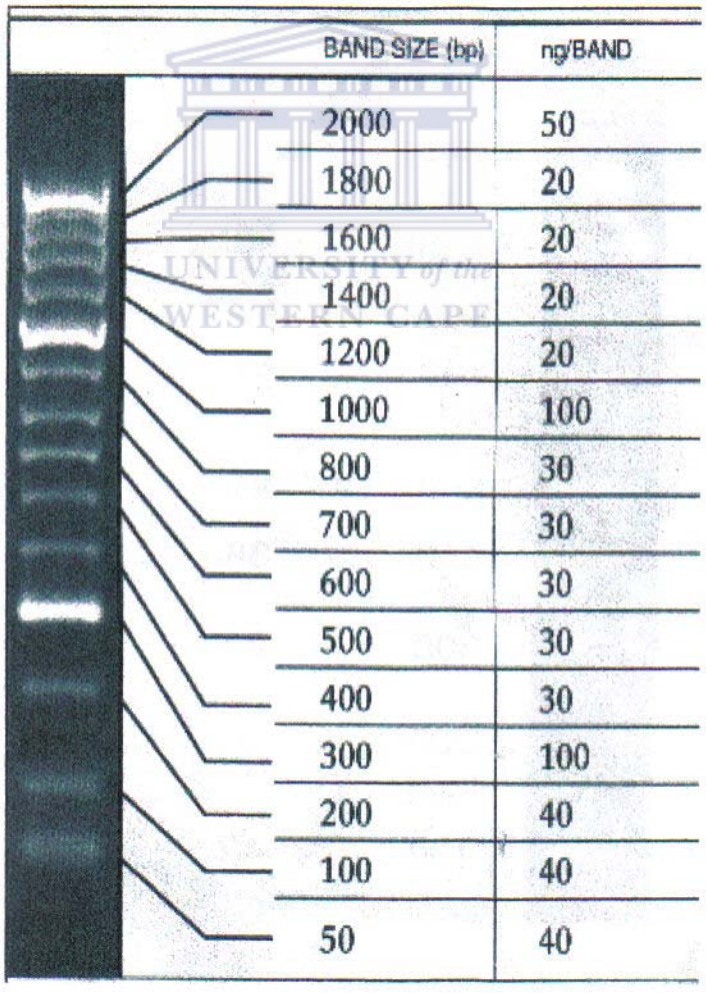
APPENDIX C

Molecular Weight Markers

and

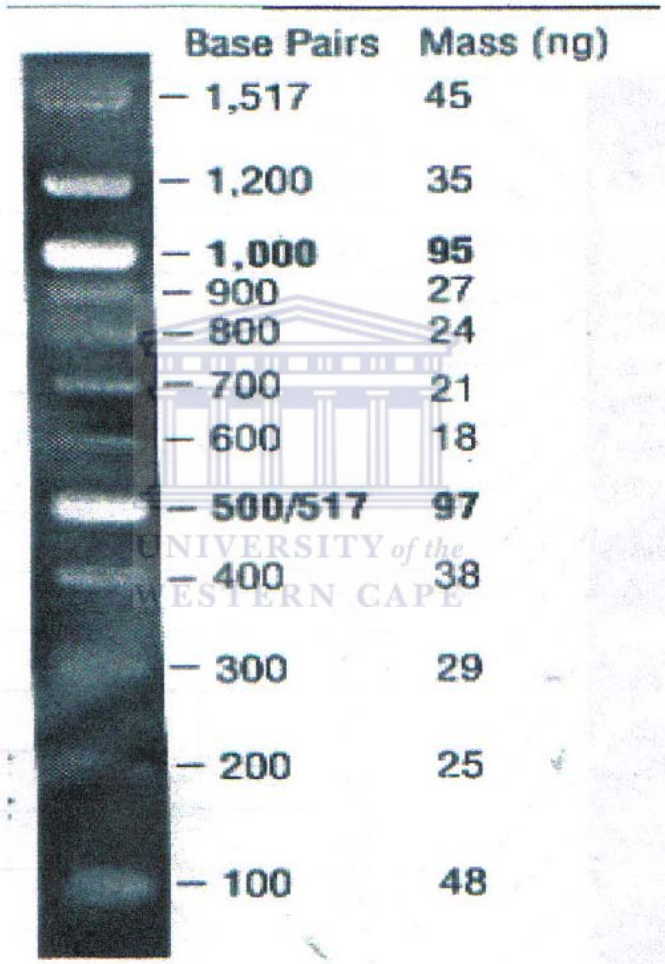
Cloning Vector

Hyperladder II (Bioline, U.S.A.)

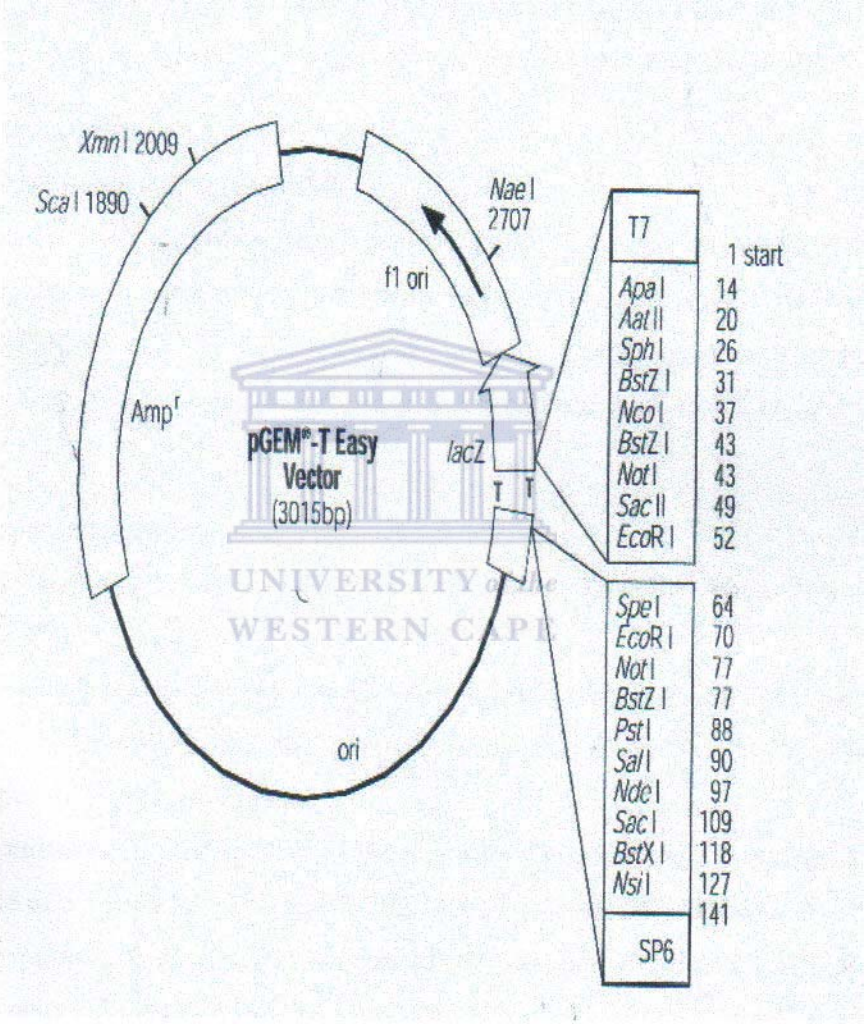


BAND SIZE (bp)	ng/BAND
2000	50
1800	20
1600	20
1400	20
1200	20
1000	100
800	30
700	30
600	30
500	30
400	30
300	100
200	40
100	40
50	40

100 bp DNA marker (New England Biolabs, U.K.)



pGEM-T Easy Cloning Vector (Promega, U.S.A.)



APPENDIX D

The Cape Town Protocol for the isolation and identification of *Campylobacter, Arcobacter* and *Helicobacter* (short version)

PRIMARY ISOLATION PROCEDURE

By using the **membrane filter technique** for processing stool samples, blood agar plates (no antibiotics added) and incubating in an **increased H_2-microaerophilic atmosphere,** virtually all known species of *Campylobacter, Helicobacter* and *Arcobacter.*

Required materials:

Tryptose Blood agar: Oxoid CM233

Membrane filter: 47 mm diameter, pore size 0.6 urn, Schleicher & Schuell ME26

Hydrogen enhanced atmosphere: Anaerobic sachets **NO CATALYST** (Oxoid BR38 or BBL 70304). One Sachet per small jar (~12 plates) or 4 sachets per large jar (~36plates) or Evacuate jar to 560 mm Ha and replace with 15% CO_2 and 85% H_2 gas mixture.

1. Specimen preparation

a) *Stools:* *Prepare a watery emulsion of stools in sterile saline. Mucoid samples should* be vortexed.

b) Intestinal scrapings (PM specimens) and sheath washings (bulls): shake up in saline.

c) *Gastric Biopsy Material:* *Roll gently over the surface of 2 or 3 TBA plates using a* swab dipped in Tryptic Soy Broth.

d) Blood cultures: Squirt ~0.2 ml of the mixture taken from the blood culture bottle over the surface of a TBA plate. **DO NOT USE THE FILTER METHOD.**

2. Place a 0.6 micron pore-size membrane filter (Schleicher & Schuell ME26) directly on to a TBA plate using sterile forceps.

3. Flood the central area of the filter with the emulsion using a transfer pipette. **Do not splash or spill beyond the filter margin.** Re-flood 2 or 3 times.

4. Remove and discard the filter **within 15 minutes.** Incubate the plate as soon as possible in a CO_2 incubator for the time being, before incubating in the H_2 enriched atmosphere generated by the BR 38 gaspak. Sterilize the forceps between specimens (heat- then cool in 70 % alcohol).

5. Incubate the plate in H_2 for 6 days, examining **every two days.** Do not discard or ignore the primary plate once growth has been obtained, as several *Campylobacter* species may be present with different growth rates. The H_2 atmosphere is obtained by the use of an Oxoid BR38 gaspak **without catalyst** or a 15 %CO_2 and an 85 % H_2 gas mixture.

6. Morphologically different colonies (shape, size, time needed for growth to appear, etc.) could indicate a mixed infection of two or more *Campylobacter* species.

IDENTIFICATION PROCEDURE

Campylobacter colonies are mostly buff coloured or dirty yellow. Exceptions are *C. helveticus. H. fennelliae, H. cinaedi,* and *H. rappini;* they have a thin flat film-like growth, which initially can even look like a swarming *Proteus* and can take up to six days to become visible to the naked eye on initial isolation, especially in a mixed culture. The gastric helicobacters form tiny, translucent colonies. Colonies of *Arcobacter* tend to be whiter than *Campylobacter.*

H. fennelliae is the only species that has an odour - hypochlorite without the "sting".

A gram stain will confirm any *Campylobacter*-like organism as either comma, or gull-winged shaped, or very thin, long spirals *(H. cinaedi, H. fennelliae),* short and stubby *(C. mucosalis)* big and slightly curved (*C. hyointestinalis* and *Arcobacter* spp.) tiny, hardly curved (*C. concisus, C. curvus, C. rectus, C. ureolyticus)* or gigantic *(Anaerobiospirillum)* gram negative bacilli. Campylobacter are easily "wiped off the plate" - do not plate out "heavy handedly". Two loops used alternatively are vital for ensuring complete cooling after flaming.

Subculturing onto two TBA plates should yield enough culture material to perform all the tests necessary for identification. The antibiogram can be done at the same time by putting one antibiotic disc on each isolation plate (on the streak lines). Incubate plates in H_2 for 48 hours. Prepare slides at the same time for motility and a Gram stain.

Do not leave the culture plates on the bench for a prolonged period of time, place in CO_2 until the H_2 jar is put up. Time is of the essence, so leave the staining of the slides until later.

This whole procedure might have to be repeated, if at a later stage, a mixed growth becomes evident on either the primary or subsequent plates.

ONCE PURE GROWTH HAS BEEN OBTAINED, PROCEED AS FOLLOWS:

Step	Procedure	Use growth from	Incubate in	Comments
1	Prepare 2 subculture plates		CO_2 and H_2	Keep CO_2 plate up to 4 days *before scoring" no growth".* Examine H_2 plate after 48 hr.
2	Indoxyl acetate		aerobic	10 minutes
3	Antibiogram[1]	CO_2	H_2	48 hours
4	Catalase		aerobic	immediate
5	Nitrate reductase	CO_2	anaerobically[2]	overnight
6	Aryl sulphatase	**only from H_2**	H_2	48 hr for In Ac +ve strains 96 hr for In Ac -ve strains
7	Rapid H_2S	**only from H_2**	H_2	overnight
8	Hippurate	CO_2	CO_2	overnight
9	Aerobic 1/4 plate	CO_2	aerobic	48 hours

1. Antibiogram: Nalidixic Acid, Cephalothin, Ciprofloxacin. Never use more than 2 or 3 antibiotic discs per plate, preferably 1 per plate. It is more practical to incubate sensitivity plates in H_2 unless dealing with a known CO_2 organism. Larger inhibition zones are observed on CO_2.
2. Anaerobically or in a H_2 enriched atmosphere *Incubate in H_2 ONLY

NOTE: *C. hyointestinalis* catalase +ve *C. concisus* catalase –ve.

Media and reagents required

ITEM
Acetone
Butanol
Hydrogen peroxide (20 vol)
Potassium nitrate
Indoxyl acetate Sigma I 3500
Sodium hippurate Sigma H9380
Ninhydrin Merck 6762/BDH 10132 4E
McConkey Oxoid CM7b
Brucella base Oxoid CM 169
Technical Agar #3 Oxoid LI 3
Phenopthalein disulphate Sigma P0251
Na_2CO_3 15 % solution
Nutrient Broth # 2 Oxoid CM67
TSI Oxoid CM 277
Ferrous Sulphate
Sodium metabisulphite
Sodium pyruvate

The Cape Town Protocol (short version) TESTS and REAGENTS

INDOXYL ACETATE HYDROLYSIS

Preparation:

A 10 % (w/vol) solution of Indoxyl acetate (Sigma 13500) in acetone. Saturate filter paper strips with the above mixture and air dry. Store in amber bottle in the fridge.

The test:

Rub a loopful of bacteria onto a small area of the strip. Wet strip thoroughly (keep moist for up to 10 minutes) with distilled water. A number of tests can be done on the same strip, store partly used strips in a petri dish in the refrigerator.

Dark blue colour e.g. *C. jejuni* = positive. No colour change e.g. *C. fetus* = negative.

NOTE: Some campylobacteria produce a bacterial esterase, which, in the presence of oxygen, will hydrolyse indoxyl (a breakdown product of tryptophan) to indigo and indigo white.

NITRATE REDUCTION

Preparation:

0.1 % (wt/vol) Potassium Nitrate in Tryptose Blood agar plates

0.5 g KNO_3 dissolved in 3 ml distilled water

Filter aseptically into 500 ml prepared TBA media

Seal plates into plastic bags, plates will keep for weeks at 4°C

The test:

Stab and spread a large loopful of bacteria over a small area (do no more than 4 tests per plate). Incubate overnight, preferably under anaerobic conditions (essential for some species) or under H_2 enhanced conditions for 48 hours. Aerate (30 minutes) on bench before scoring.

Dark green brown zone (e.g. *C. jejuni jejuni)* = positive. No colour change (e.g. *C. jejuni doylei)* = negative.

Note: The formation of a green-brown zone indicates that the KNO_3 has been reduced to KNO_2, resulting in the oxidation of haemoglobin to methaemoglobin. Inoculate a known positive control onto any nitrate plate containing less than 4 tests, particularly when nitrate negative *C. jejuni doylei* or *Helicobacter fennelliae* is suspected. It is essential to incubate anaerobically for 24 hours or in H_2 for 48 hours without delay. *C. mucosalis* and *C. concisus* give very weak reactions.

CATALASE

The test:

A loopful of bacteria (taken carefully from a TBA plate - do not stab to cool) is picked up with a capillary tube containing Hydrogen peroxide (20 vols). Oxygen bubbles are trapped in the tube.

C. jejuni jejuni will give a positive result. *C upsaliensis* will give a negative / weakly positive result.

Notes:

Catalase breaks down hydrogen peroxide into oxygen and water. A positive reaction can be so strong that large bubbles are formed - this can block the capillary tube and prevent

the ascent of any bubbles. On close scrutiny the trapped bubbles can be seen stuck to the surface of the bacteria or forced out at the bottom.

ARYLSULPHATASE ACTIVITY

Preparation:

Brucell broth (Difco 0495-17-3)	14.00 g
Bacto Agar or Technical No 3	1.75 g
Phenolphthalein Disulphate (Sigma P 0251)	0.325 g
Dist H_20	500 ml

Steam to dissolve, tube in 4 ml amounts. Autoclave 15 ib/15 min. Prepare small batches to prevent false positives

Sodium Carbonate solution

Na_2CO_3 *anhydrous* 15 g

Distilled H_2O 100 ml

Store on the bench, do not refrigerate, as it will solidify.

The test:

USE ONLY ON A CULTURE GROWN IN H_2

Emulsify in the top 1/3 of the substrate using a very heavy inoculum (turbid).

Incubate in H_2 48 hours for Indoxyl acetate POSITIVE cultures

96 hours for Indoxyl acetate NEGATIVE cultures

Add about 1 ml of 15 % sodium carbonate (Na_2CO_3) solution - can give the tube a shake. The breakdown product of phenylopthalein forms a pink colour in the presence of Na_2CO_3.

A positive result produces a bright pink colour, e.g. *C. jejuni jejuni* 2. A negative result produces no colour change, e.g. *C. jejuni jejuni* 1.

HIPPURATE HYDROLYSIS

Preparation:

Stock = 5 % Hippuric acid (Sodium salt Sigma H 9380, or Merck 820648) in dist H_2O.

Store frozen

Hippurate broth

Hippuric acid stock solution	25 ml
Dist H_2O	100ml

Filter ~1 ml amounts into **glass tubes. Do not autoclave.**

Ninhydrin (Merck 6762, BDH 10132 4E)	3.5 g
50:50 Butanol:Acetone	*100ml*

Store Ninhydrin solution in freezer

The test:

Only *C. jejuni jejuni* and *C. jejuni doylei* are positive. Culture: from CO_2 plate ONLY. Incubate test in CO_2 ONLY. Thaw hippurate broth and inoculate heavily. Incubate overnight (more practical than a 2 hour test). Gently add ~0.5 ml ninhydrin solution. Do not shake (aerate) the tube. Read within 10 minutes (the 2 hour test needs 30 minutes incubation).

C. jejuni jejuni and *C. jejuni doylei* will give a positive result which is a strong purple colour. Other *Campylobacter* species will give a negative result, either colourless or very light purple.

Notes:

Hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidising agent, ninhydrin. Ninhydrin becomes reduced in the process and a purple coloured dye is formed. Sometimes H_2 cultures may give false positive results - Essential to use a CO_2 grown culture and to incubate the test in a CO_2 environment.

AEROBIC GROWTH AT ROOM TEMPERATURE

Squiggle-inoculate a positive control and three tests onto a TBA plate divided in 4. Leave on the laboratory bench for 48 hours. Only *Arcobacter* species will grow.

GROWTH ON MACCONKEY

Preparation:

MacConkey without NaCl or Crystal Violet **(ONLY Oxoid CM 7b** gives consistent results)

The test:

Inoculate lightly and streak out for single colonies on 1/2 plates. Incubate in H_2 for 48 hours.

A positive result is the growth of visible **single** colonies. If unsure, and for slow growing organisms, incubate for a further 48 hours.

SEMI-SOLID IRON BROTH FOR RAPID H_2S PRODUCTION

Preparation:

Bacto or Technical Agar	1.75 g
Nutrient Broth No. 2 (Oxoid CM67)	12.50 g
Dist H_2O	400 ml

Steam above to dissolve

Sodium metabisulphite $Na_2S_2O_5$	0.5 g
Sodium pyruvate $CH_3COCOONa$	0.5 g
Ferrous sulphate $FeSO_4.7H_2O$	0.5 g
Dist H_2O	50 ml

Dissolve the above

Combine the 2 solutions and bring the volume up to 500 ml with dist H_2O. Autoclave 15 lb/15 min in 100 ml amounts. Store this stock in the dark at room temperature. Dispense 2 ml amounts in sterile small screw capped plastic tubes. Store in the dark and at room temperature.

GROWTH ON MACCONKEY

Preparation:

MacConkey without NaCl or Crystal Violet **(ONLY Oxoid CM 7b** gives consistent results)

The test:

Inoculate lightly and streak out for single colonies on 1/2 plates. Incubate in H_2 for 48 hours.

A positive result is the growth of visible single colonies. If unsure, and for slow growing organisms, incubate for a further 48 hours.

TROUBLE SHOOTING AND USEFUL HINTS

Incubate for 48 hour periods

Some of the more fastidious species may be lost if the jar is opened after only 24 hours of incubation, especially during initial isolation. All the enzymes are at their peak and best results are obtained from 48 hr cultures. Both arylsulphatase and indoxyl acetate tend to give false negative results with old cultures – even a 3 day old culture. Rapid H_2S can also give problems, especially for *C. lari.* Hippurate is an exception to the rule, and older cultures can be used for these tests.

All the enzymes are better developed in H_2 than in CO_2

Sometimes too well - e.g. excess H_2S production of CO_2 species on PbAc and TSI, resulting in false positive reactions if performed from an H2 culture. Occasionally, otherwise obvious *C. coli* or *C. upsaliensis* cultures seem to be hippurate positive. Repeat from a culture grown in CO_2.

False positive hippurate reactions can also occur if: plastic tubes are used, the medium is defrosted a third time (i.e. re-freeze unused hippurate solutions only once), the incubated tube is aerated either by shaking or if left standing for too long after the ninhydrin has been added.

Rapid H_2S and arylsulphatase tests should always only be done from cultures grown in H_2-very poor or inconsistent results are obtained from CO_2 plates. It is more practical to use H_2 grown cultures to do all the tests (except for TSI and PbAc) while establishing if the isolate is capable of growth in CO_2.

Mixed infections

Very little variation will occur in results for the tests chosen for this identification scheme. Usually discrepancies in the results are indicative of a mixed infection.

Fresh TBA plates

The isolation rate of *Campylobacteraceae* strains is very poor on TBA plates that are older than 10 days. *Helicobacter* species are even more fastidious. Fresh plates, poured

twice weekly, and using older plates for subculture only, is recommended. Even on 3 week-old plates established cultures are often lost.

Alternate jars

With 4 sachets in a 36 plate anaerobic jar, more H_2 is available for the organisms. (Two sachets in a small [3 liter] jar produce too much pressure for efficient growth of the bacteria). It is preferable to use the large jars, otherwise alternate the culture plates - 2 days in a small jar, 2 days in a large jar.

New specimens

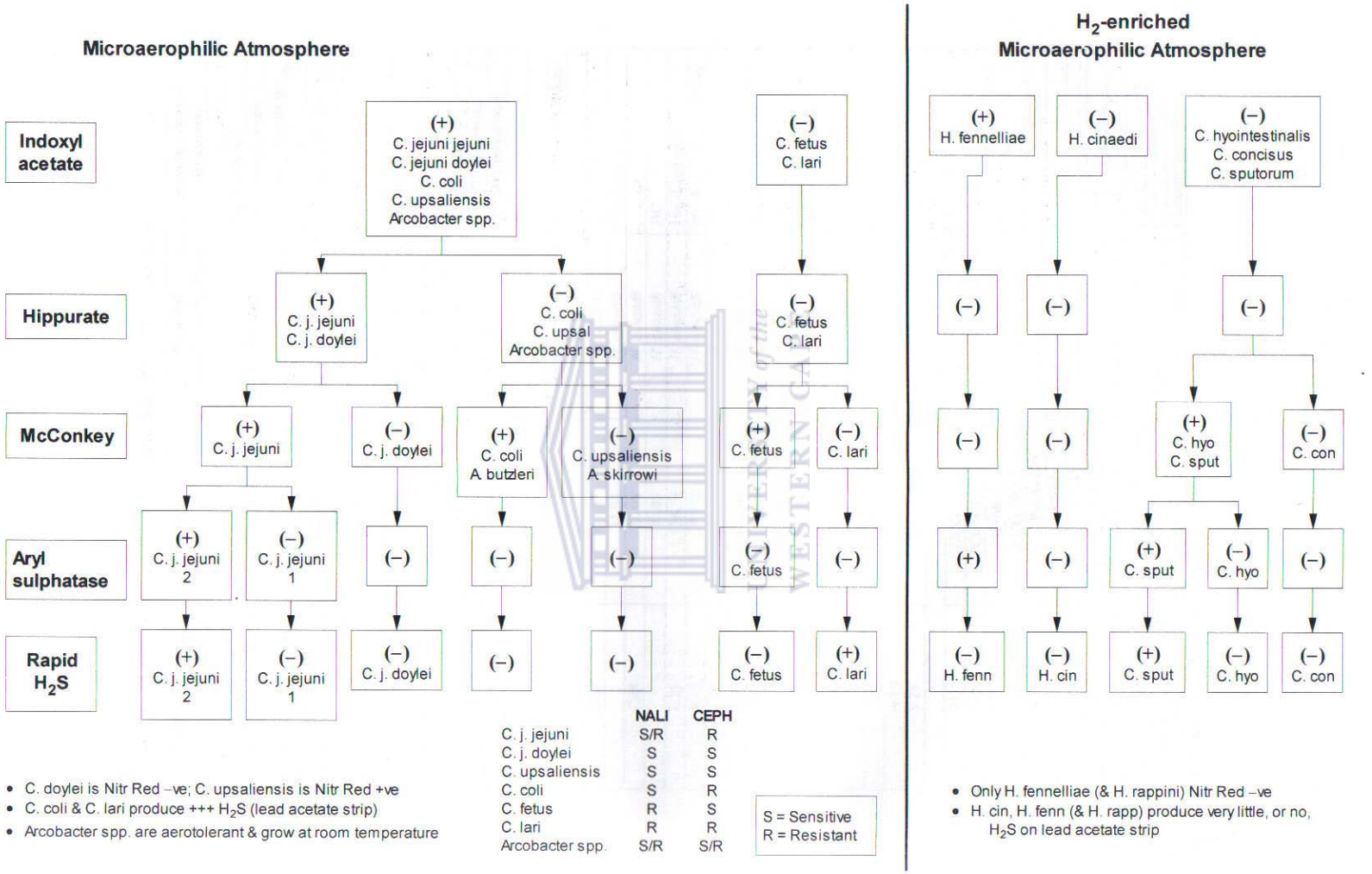
Culture plates must be incubated in H_2 as quickly as possible, e.g. the morning specimens by midday and the afternoon specimens by the end of the day. H_2-dependent organisms do not last too well in a CO_2 atmosphere over a prolonged period of time.

Distribution of *Campylobacter* and related species isolated from diarrhoetic stools at the Red Cross Children's Hospital, Cape Town, South Africa Oct. 1, 1990-Sept 30, 2005

Species	Number	%
C. jejuni subsp. *jejuni*	1794	32,37
C. concisus	1364	24.61
C. upsaliensis	1304	23.53
C. jejuni subsp. *doylei*	418	7.54
H. fennelliae	319	5.75
C. coli	170	3.06
C. hyointestinalis	53	0.96
H. cinaedi	48	0.87
CLO / HLO*	28	0.51
Arcobacter butzleri	20	0.37
C. fetus subsp. *fetus*	9	0.16
H. rappini / C. rectus / C. curvus	8	0.14
C. lari / C. sputorum bv *sputorum*	7	0.13
Total	5542	100.00

*CLO / HLO: *Campylobacter* or *Helicobacter* organisms that could not be fully characterized.

Cape Town Protocol for the Isolation of *Campylobacter* spp.



	NALI	CEPH
C. j. jejuni	S/R	R
C. j. doylei	S	S
C. upsaliensis	S	S
C. coli	S	R
C. fetus	R	S
C. lari	R	R
Arcobacter spp.	S/R	S/R

S = Sensitive
R = Resistant

- C. doylei is Nitr Red –ve; C. upsaliensis is Nitr Red +ve
- C. coli & C. lari produce +++ H_2S (lead acetate strip)
- Arcobacter spp. are aerotolerant & grow at room temperature

- Only H. fennelliae (& H. rappini) Nitr Red –ve
- H. cin, H. fenn (& H. rapp) produce very little, or no, H_2S on lead acetate strip

APPENDIX E: List of strains tested in the thesis

Microbank No./Label	Strain No.	Species	Source (if known)
Dsp 1520	Dsp 1520	*A. butzleri*	Clinical isolate
10.1	10.1	*A. butzleri*	Chicken liver
7	7	*A. butzleri*-like	Raw sewage/sludge
8	8	*A. butzleri*-like	Raw sewage/sludge
10	10	*A. butzleri*-like	Raw sewage/sludge
12	12	*A. butzleri*-like	Raw sewage/sludge
220.04	220.04	*C. jejuni*	Clinical isolate
219.04	219.04	*C. jejuni*	Clinical isolate
137.05	137.05	*C. jejuni*	Clinical isolate
216	216	*C. jejuni*	Blood culture
112.09	112.09	*C. jejuni*	Clinical isolate
N31 GB	356.95	*C. jejuni*	Clinical isolate
205.04	205.04	*C. jejuni*	Clinical isolate
204.04	204.04	*C. jejuni*	Clinical isolate
Ostrich	30500	*C. jejuni*	Ostrich: fatal enteritis
208.04	208.04	*C. jejuni*	Clinical isolate
B6	B6	*C. jejuni*	Healthy chicken stool
212.04	212.04	*C. jejuni*	Clinical isolate
222.04	222.04	*C. jejuni*	Clinical isolate
112.09	112.09	*C. jejuni*	Clinical isolate
7.2	7.2	*C. jejuni*	Chicken blood
7.4	7.4	*C. jejuni*	Chicken blood
115.09	115.09	*C. coli*	Clinical isolate
B3	B3	*C. coli*	Healthy chicken stool
B2	B2	*C. coli*	Healthy chicken stool
B4	B4	*C. coli*	Healthy chicken stool
B5	B5	*C. coli*	Healthy chicken stool
B7	B7	*C. coli*	Healthy chicken stool
Y2	Y2	*C. coli*	Healthy pig stool
B1	B1	*C. coli*	Healthy pig stool
Y1	Y1	*C. coli*	Healthy pig stool
191.01	191.01	*C. fetus fetus*	Clinical isolate
349.92	349.92	*C. lari*	Clinical isolate
88.05	88.05	*C. doylei*	Clinical isolate
213.09	213.09	*C. concisus*	Clinical isolate
271.05	271.05	*C. upsaliensis*	Blood culture
225.04	225.04	*C. upsaliensis*	Clinical isolate
G40	525.92	*C. curvus*	Clinical isolate
218.04	218.04	*H. cinaedi*	Clinical isolate
60.05	60.05	*H. cinaedi*	Blood culture
W34	170.04	*H. cinaedi*	Clinical isolate
P5	348.99	*H. cinaedi*	Clinical isolate
58.05	58.05	*H. fennelliae*	Clinical isolate
225.04	225.04	*H. fennelliae*	Clinical isolate
76.02	76.02	*H. fennelliae*	Clinical isolate
384.96	984.96	*H. fennelliae*	Clinical isolate
166.02	166.02	*H. fennelliae*	Clinical isolate
78.94	78.94	*H. fennelliae*	Clinical isolate
Q22	71.95	*H. fennelliae*	Clinical isolate
P27	981.94	*H. fennelliae*	Clinical isolate
Li 78.94	Li 78.94	*H. fennelliae*	Clinical isolate
Y36	Y36	*H. pylori*	Human gastric carcinoma
Y27	Y27	*H. pylori*	Human gastric carcinoma
WT9	WT9	*H. pylori*	Human gastric carcinoma
H3	CCUG 29255	*H. pamatensis*	