UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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DEVELOPMENT OF ISOLATION AND IDENTIFICATION METHODS FOR EMERGING SPECIES OF *CAMPYLOBACTERACEAE*

By

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ABSTRACT

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DEVELOPMENT OF ISOLATION AND IDENTIFICATION METHODS FOR EMERGING SPECIES OF *CAMPYLOBACTERACEAE*

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Recently, a number of emerging species of *Campylobacteraceae* have been isolated from clinical samples using antibiotic-free isolation media and a hydrogen-enriched incubation atmosphere, questioning their importance in human disease. Current, routinely used isolation and detection methods are designed primarily to detect thermophilic *C. jejuni* and *C. coli*, historically thought to be the most clinically relevant species. Emerging *Campylobacteraceae* species such as *C. upsaliensis*, *C. concisus* and *Arcobacter* species could also be clinically significant. It is thought these go undetected due to the use of current standard protocols which rely on isolation procedures using media that contain potentially inhibitory antibiotics and sub-optimal incubation conditions.

This PhD study was carried out as part of an EU collaborative project to investigate existing methods for isolation of emerging *Campylobacteraceae* species and to improve current isolation and identification procedures.

Evaluation of a range of currently used selective isolation media indeed showed that a number of selective media and antibiotics contained in these media inhibited the growth of a number of *Campylobacteraceae* species. Subsequently, a novel medium, ABA VAT, containing minimal levels of antibiotics was developed to allow the isolation of all *Campylobacteraceae* species. Commercially available incubation atmospheres were also shown to be sub-optimal for the growth of many *Campylobacteraceae* species. A novel, universal incubation atmosphere, containing low levels of oxygen and supplemented with hydrogen (3%, O_2, 10% CO_2, 7% H_2), permitted the growth of all *Campylobacteraceae* species. It was demonstrated that even the species thought to grow only under anaerobic conditions could grow in this atmosphere and that hydrogen was essential for growth of a number of species. A range of identification methods was also evaluated, including phenotypic tests, EDIC microscopy, latex agglutination, fluorescence *in situ* hybridisation and a typing method using a 2D gel electrophoresis approach.

The novel isolation methods developed were incorporated into protocols so that recovery of *Campylobacteraceae* species from sewage and salad vegetable samples could be investigated. Results showed that a number of non-*jejuni/coli Campylobacter* species could be isolated from sewage and salad vegetables. An important and previously unreported finding was the high isolation rate of the emerging pathogen, *A. butzleri*, from processed salad vegetables, which is potentially of public health concern.

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PUBLICATIONS AND CONFERENCE PRESENTATIONS

Reiman, L. E. and C.W. Keevil, 2006. An improved incubation atmosphere for growth of *Campylobacter* species on agar media in the presence or absence of selective supplements. *Applied and Environmental Microbiology*. Submitted.

Keevil, C.W., **Reiman, L.E.** and Loades, C.J., 2006. Optimal Culture Conditions for emerging *Campylobacter* spp., Extended abstract. *In* Duffy., G. and Cagney, C. (Eds), Proceedings of Emerging *Campylobacter* spp. in the food chain, CAMPYCHECK, Dublin, Ireland.

Reiman, L.E., Skipp, P.J. and Keevil, C.W., 2006, Identification of *Campylobacter* species through the use of Proteomic Analysis. Poster presentation. *In* Duffy., G. and Cagney, C. (Eds), Proceedings of Emerging *Campylobacter* spp. in the food chain, CAMPYCHECK, Dublin, Ireland.

Reiman, L.E., Skipp, P.J. and Keevil C.W., 2005, Comparative Analysis of Proteome Maps as a Tool for Identifying *Campylobacteraceae*, poster presentation. *In* Proceedings of ASM 105th General Meeting, Atlanta, Georgia, USA, 5-9th June.

Loades, C.J., **Reiman, L.E.** and Keevil, C.W., 2005, Development of an improved medium for the recovery of *Campylobacter* species avoiding common selective antibiotics, poster presentation. *In* Proceedings of ASM 105th General Meeting, Atlanta, Georgia, USA, 5-9th June.

Lastovica A.J., Le Roux, E., **Reiman, L.E.**, Keevil, C.W. and Clark, S.A., 2004, Evaluation of Latex Agglutination for the Rapid Identification of *Campylobacter* species, poster. ASM, *New Orleans, USA.*

Reiman, L. E., C.J. Loades and C.W. Keevil, 2004. Optimising Recovery of Campylobacter species Through Modification Of Gas Environments And Culture Media, poster presentation. *ASM, New Orleans, USA.*

Reiman, L. E., C.J. Loades and C.W. Keevil, 2003, Improved isolation of emerging *Campylobacter* species through optimisation of microaerophilic environments. Poster presentation. *Campylobacter*, *Helicobacter* and Related organisms, Aarhus, Denmark. *In* International Journal of *Medical Microbiology* (Abstracts of Scientific presentations), 293*:35, p. 87.*

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The CAMPYCHECK team (CAMPYCHECK 36-Month Review Meeting, National Food Centre, Teagasc, Ashtown, Dublin, February 2006).

ABBREVIATIONS

2D PAGE	Two dimensional polyacrylamide gel electrophoresis
ABA	Anaerobe basal agar
AFLP	Amplified fragment length polymorphism
BSA	Bovine serum albumin
CAT	Cefoperazone amphotericin teicoplanin
CCDA	Charcoal cefoperazone deoxycholate agar
CDSC	Communicable Disease Surveillance Centre
CDT	Cytolethal distending toxin
CEB	Campylobacter enrichment broth
cfu	Colony forming units
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary
CLM	*Campylobacter*-like morphology
CONT	Contaminated
DFVF	Danish Institute of Food and Veterinary Research
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
EC	European Commission
EDIC	Episcopic differential interference contrast
EU	European Union
FBP	Ferrous sulphate, sodium metabisulphate and sodium pyruvate
FISH	Fluorescence *in situ* hybridisation
GBS	Guillain Barré Syndrome
GLS	Glycosphingolipids
HPA	Health Protection Agency
IEF	Iso-electric focussing
IL-8	Interleukin-8
IMS	Immunomagnetic separation
L-ALA	L-alanine aminopeptidase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MACS	Modified atmosphere controlled system
MAD	Mesophilic anaerobic digestion

MALDI-TOF	Matrix-assisted laser desorption-ionization time of flight
MIC	Minimum inhibitory concentration
MRD	Maximum recovery diluent
MS	Mass spectrometry
NARTC	Nalidixic acid resistant thermophilic *Campylobacter*
NCCLS	National Committee for Clinical Laboratory Standards
OMP	Outer membrane protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pI	Isoelectric point
PNA	Peptide nucleic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribose nucleic acid
RNR	Ribonucleotide reductase
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T4SS	Type IV secretion system
TBA	Tryptose Blood Agar
TCA	Tricarboxylic acid
TCTC	Too contaminated to count
TNTC	Too numerous to count
UPTC	Urease positive thermophilic *Campylobacter*
VAT	Vancomycin amphotericin trimethoprim selective supplement
VBNC	Viable but non-culturable
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION TO *CAMPYLOBACTERACEAE*

1.1. INTRODUCTION TO *CAMPYLOBACTERACEAE*

Campylobacter is the most frequently isolated bacterial causative agent of diarrhoea in humans, particularly in very young children in both developed and developing countries. *Campylobacter* has been associated with other clinical conditions such as bacteraemia, Guillain Barré syndrome, haemolytic-uraemic syndrome, pancreatitis, and reactive arthritis (Nachamkin and Blaser 2000). The genus *Campylobacter* currently comprises 18 species, of which 14 have been isolated from humans. Historically, more than 95% of *Campylobacter* strains isolated and identified in cases of human disease have been *C. jejuni* subsp. *jejuni* or *C. coli*. However, the isolation techniques currently used in many diagnostic laboratories may not support the growth of other, potentially pathogenic non-*jejuni/coli Campylobacter* species. These species are thought to be fastidious, requiring special atmospheric and temperature conditions or prolonged incubation, or may be unable to tolerate the antibiotics commonly included in selective media. The disease potential of these non-*jejuni/coli* or emergent *Campylobacter* species is beginning to be appreciated, particularly where they have been looked for using alternative isolation techniques, but until further prevalence studies are carried out, the importance of these organisms will remain unknown.

The genus *Campylobacter* is found within the family *Campylobacteraceae* which includes organisms that are Gram-negative, oxidase-positive and curved or rod shaped. These bacteria are 0.2 to 0.5 μm wide and 5.0–8.0 μm long that may form spherical or coccoid bodies in old cultures or cultures exposed to air for prolonged periods (Figure 1.). They usually possess one polar flagellum (but sometimes two) which gives them a very characteristic 'cork-screw' motility (Corry *et al.* 1995a).

Campylobacter species are fastidious in their growth requirements, having a minimum growth temperature of about 30°C, which precludes growth in food, and are unable to grow or tolerate the normal atmospheric concentration of oxygen. Despite some being 'thermophilic' in their growth requirements, they are also sensitive to exposure to high temperatures and, consequently, the organisms will not survive in food that has been adequately cooked or pasteurised. The organisms are also sensitive to environmental stresses such as chilling, freezing, heating, acidification (readily killed at pH 2.3), drying and osmotic stress (will not grow in concentrations of 2% (w/v) NaCl) and are also unusually sensitive to superoxides and free radicals (Park 2002). Taken together these properties would appear to make them ill-suited to survival outside the host, yet they are widely dispersed in the environment and can be readily isolated from food, water and other sources, provided appropriate techniques are employed (Corry *et al.* 1995a).

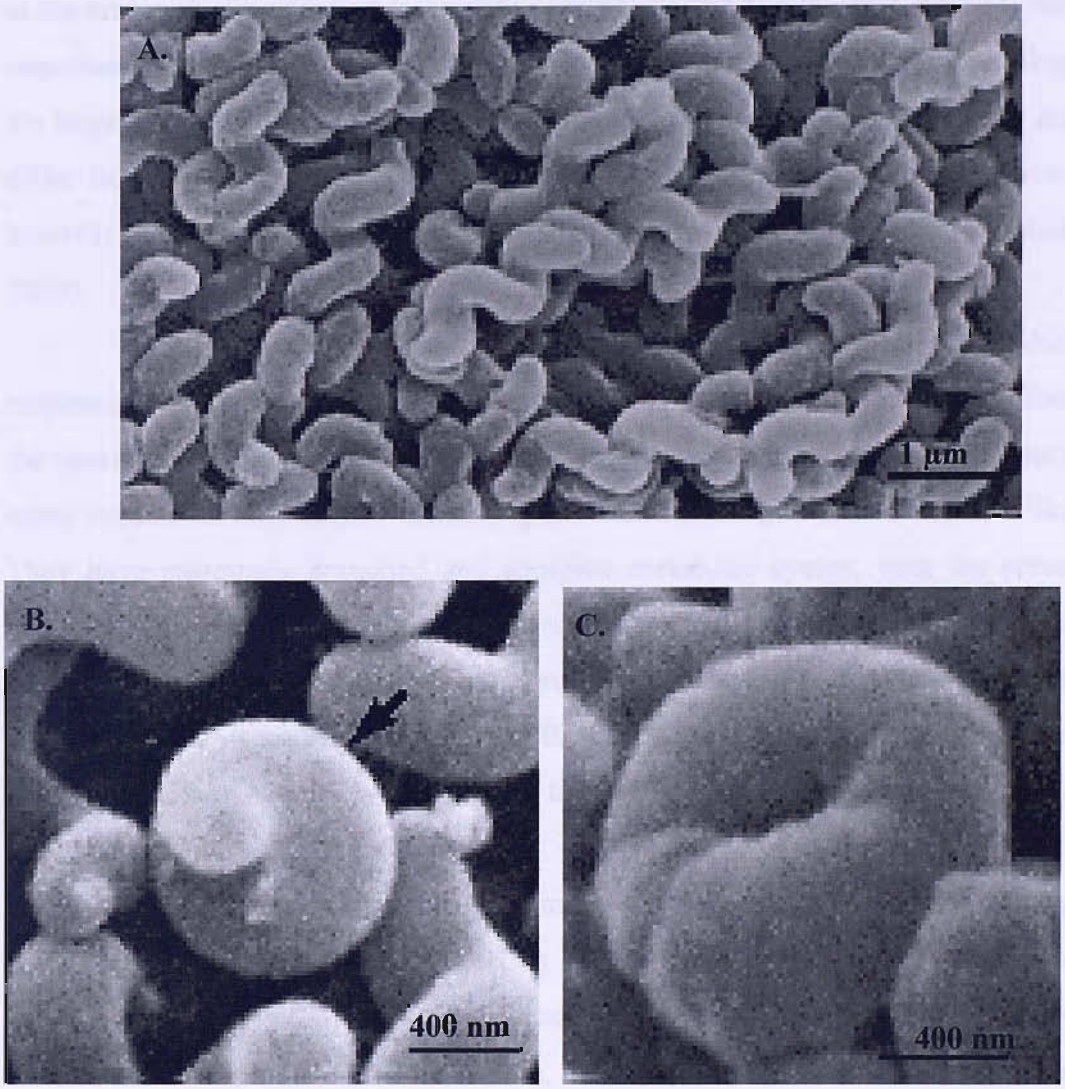


Figure 1. Electron Micrographs of Various Cellular Forms Observed in *C. jejuni.* a) typical, spiral morphology in healthy bacteria b) Subsequent electron micrographs of the *C. jejuni* clearly showing that older cells progressively degenerate to coccoid (spherical) bodies via unusual curled and c) even donut forms. From Ng *et al.* (1985a).

It is for these reasons that it is thought so astonishing that such a delicate organism is so successful at causing disease. The ability of this bacterium to remain as the most frequent cause of food-borne illness, despite these fastidious growth requirements, has been referred to as the *Campylobacter* conundrum (Jones 2001a). Enteric bacteria such as *Salmonella* and *Escherichia coli* have a range of interlinked genetic systems for adapting to adverse changes in the environment, adapting to oxidative, osmotic, acid and thermal stresses, but the genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.* 2000) has revealed that analogous systems are largely lacking in this organism. The survival strategy of *Campylobacter* must therefore differ from the conventional concept of globally regulated physiological responses to stress, however the basis of that strategy is largely unknown (Park 2002; Martinez-Rodriguez *et al.* 2004).

Campylobacters usually require strictly microaerophilic conditions with a respiratory type of metabolism. However, the required growth atmosphere does depend on the species investigated. Certain *Campylobacter* strains grow aerobically or anaerobically, or some require an atmosphere containing increased hydrogen (Lastovica and Skirrow 2000). They have extremely branched and complex metabolic cycles, with the presence of both anaerobic and aerobic metabolic pathways, which enables them to utilise alternative electron acceptors such as fumarate, and donors such as hydrogen and formate. *C. jejuni* and *C. coli* have genomes approximately 1.7 Mb in size, as determined by pulse-field gel electrophoresis, which is about one-third the size of the genomes of *Escherichia coli* serovars (Parkhill *et al.* 2000).

Campylobacter is the most common bacterial cause of food poisoning worldwide with ~600 million cases per year, costing countries hundreds of millions of Euros per annum in patient care, in addition to costs incurred due to decreased productivity as a result of worker absence (Post 1998). Therefore, the social and financial costs of *Campylobacter* infection are enormous. For example, health officials estimate that more than two million cases occur annually in the United States (Post 1998).

While there are 18 different species, of which 14 are thought to be pathogenic to humans, it is presumed that 95% of bacterial enteritis in humans is caused by *C. jejuni* and *C. coli* (Lastovica and Skirrow 2000) .

1.2. TAXONOMY

The family *Campylobacteraceae* comprises mainly *Campylobacter* and *Arcobacter* which are phylogenetic neighbours and share several other genotypic and phenotypic features. In addition, the family also contains the generically misclassified species

Bacteroides ureolyticus and strains originally described as free-living campylobacters, now known as *Sulfurospirillum* species (On 2001). The genus *Campylobacter* was first proposed in 1963 by Sebald and Veron (1963) when a new test was applied to detect fermentative metabolism and DNA base composition to distinguish them from the formally classified *Vibrio* species. Most of the *Campylobacteraceae* have been associated with disease in animals and man, with the exception of *Sulfurospirillum* species, which appear to be only found in the environment (Lastovica and Skirrow 2000). The taxonomy of *Campylobacter* and related bacteria has undergone significant changes during the past decade; a range of 'variants' of established species and a considerable number of novel species had to be inserted in to the existing taxonomic framework. Two new species, *C. insulaenigrae* and *C. lawrenceae*, isolated from marine mammals, have recently been described. *C. lawrenceae* was isolated from northern elephant seals on the California coast, and to date very little information exists on this organism. *C. insulaenigrae* has been isolated from a range of marine mammals from a number of locations and is genetically similar to *C. upsaliensis* and *C. helveticus* (Foster *et al.* 2004; Stoddard *et al.* 2007).

There has been much technological progress in molecular biology, biochemistry and several affiliated disciplines. Present day classifications are now primarily phylogeny-based since they are constructed around a backbone derived from similarity studies of highly conserved macromolecules such as 16S rRNA (Figure 2). Classifications may also be based on DNA hybridisation studies and those with around 70% homology are usually classed as the same species (On 2005). Other tests commonly used for the typing of *Campylobacter* species are summarised in Table 2. Generally, a combination of both molecular and phenotypical tests is required for accurate speciation and strain typing (On 1996).

1.3. INCIDENCE AND EPIDEMIOLOGY

In the last decade, while the incidence of a number of other leading causes of food poisoning such as *Salmonella* have declined, *Campylobacter* infections have continued to grow in Western nations (Figure 3). In England and Wales from 1991 to 2000 there had been a steady increase in reported cases per year by over 65% with 58,000 cases reported in 2000. However, since 2001 their has been a decline in incidence with approximately 46,000 cases reported in 2006 (Jorgensen *et al.* 2002; Evans 2003; Anonymous 2007). In comparison to this, from 1991 to 2000 numbers of reported *Salmonella* cases have decreased by 13% to 15,000, reaching the lowest number of reported cases for 10 years (Jorgensen *et al.* 2002). For every case reported to laboratory surveillance it is thought that another seven cases are estimated to occur in the community, suggesting that from 0.5% to 1.0% of the UK's

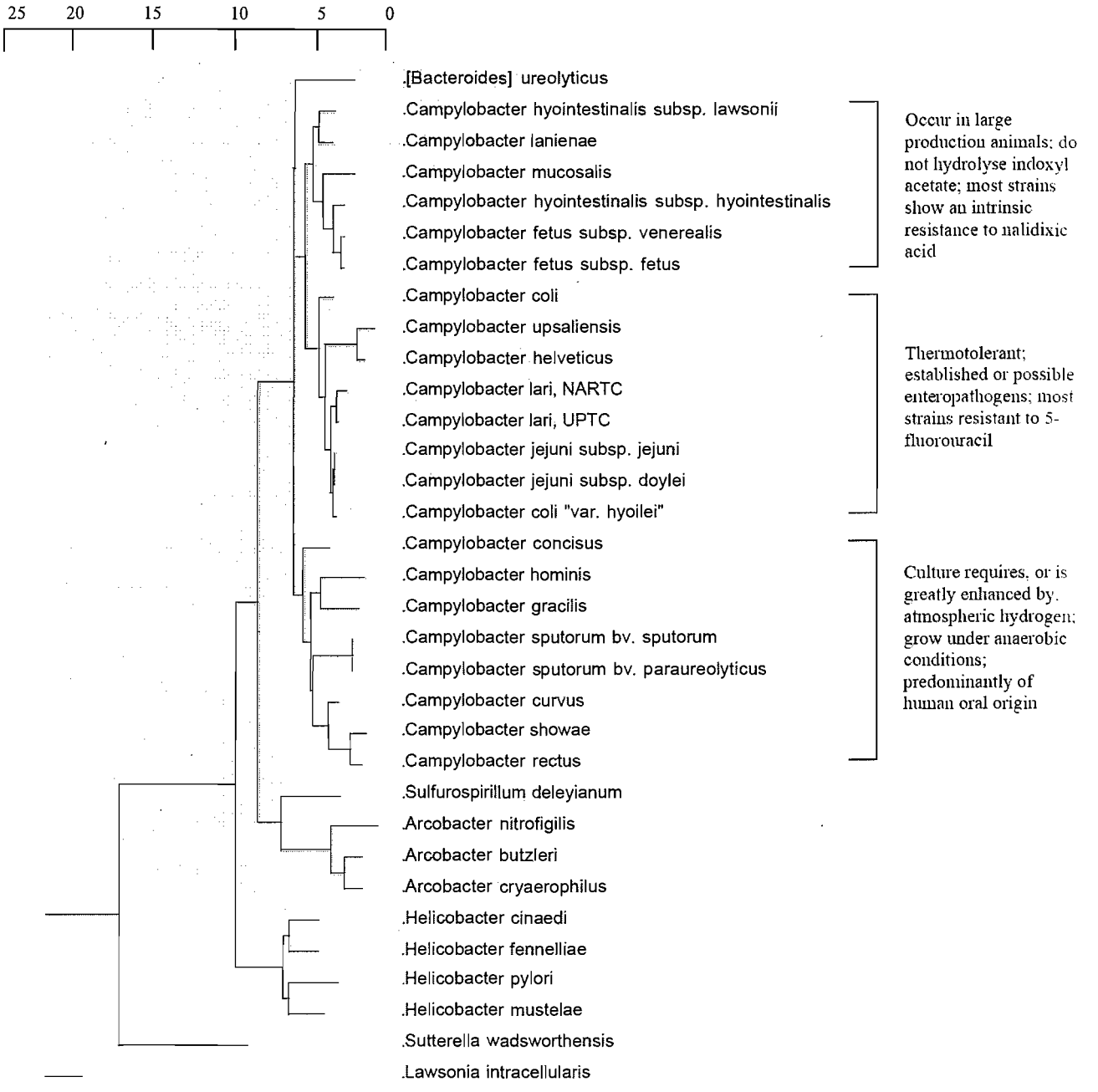


Figure 2. Phylogeny of *Campylobacter* species and Related Bacteria as Inferred by Comparison of 16S rRNA Gene Sequences. Important unifying traits of the major clades in *Campylobacter* are annotated on the right-hand side of the dendrogram. The scale bar indicates 25% sequence dissimilarity (On 2001). NARTC, Nalidixic acid-resistant thermophilic *Campylobacter*; UPTC, urease positive thermophilic *Campylobacter*. Two new species, *C. insulaenigrae* and *C. lawrenceae*, isolated from marine mammals, have recently been described which are not included in this phylogenetic tree (Stoddard *et al.* 2007).

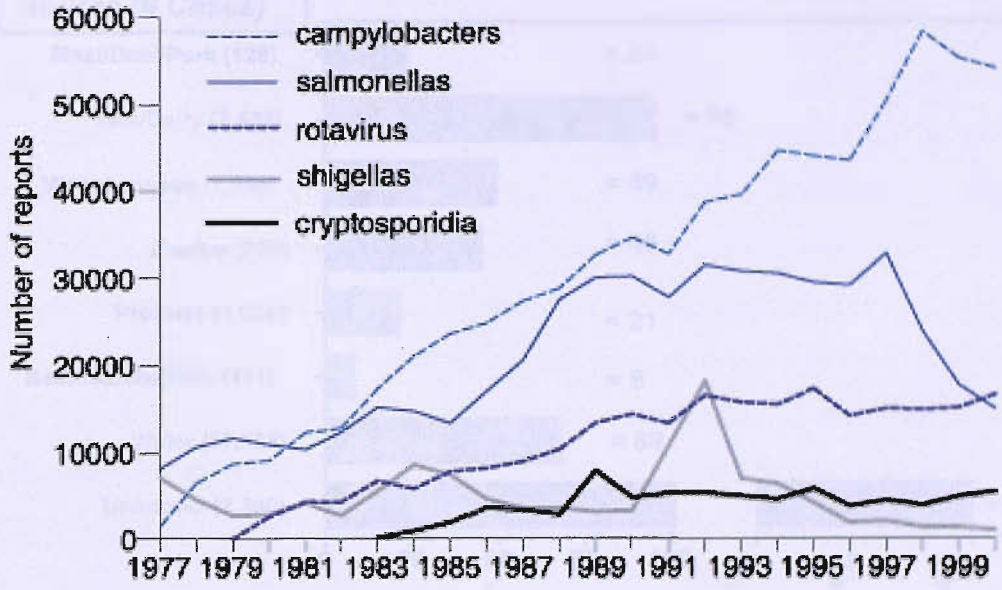


Figure 3. Numbers of Reported Gastrointestinal Infection in England and Wales. Source data Communicable Disease Surveillance Centre (CDSC) and reproduced in Keevil and Clark (2002).

population is infected annually (Adak *et al.* 2002; Evans 2003; Adak *et al.* 2005). Reasons for the underreporting include; the patient has *Campylobacter* but is asymptomatic and therefore does not seek medical attention; the symptomatic patient is not investigated for *Campylobacter* i.e. stool samples not taken; although isolated, *Campylobacter* is notreported; and laboratory may be unsuccessful in isolating *Campylobacter* species (most laboratories are unable to isolate non-*C. jejuni*/*C. coli* strains) (Lastovica and Skirrow 2000).

For many years it has been *C. coli* and *C. jejuni* that are thought to be the major causes of food-borne disease. However in recent years, since other species have become identified, it is thought that these are also major contributors, but due to lack of isolation techniques these other species have gone undetected and therefore causes are likely to be underestimated. It is thought that current routine culture methods are inadequate for growing a number of the emerging *Campylobacteraceae* (Lastovica and Skirrow 2000).

In spite of the frequency of *Campylobacter* infections, the source of infection has proved elusive. Recognised outbreaks are rare and usually caused by contaminated water, milk or poultry (Pebody *et al.* 1997; Miller and Mandrell 2005). However, these food products explain only a small proportion of sporadic cases, and the source of infection is not determined in more than 60% of UK *Campylobacter* cases (Figure 4) (Evans 2003). A recent large case/control study with more than 1000 patients involving questionnaires which covered topics such as food history, animal contact, medical history, travel and leisure

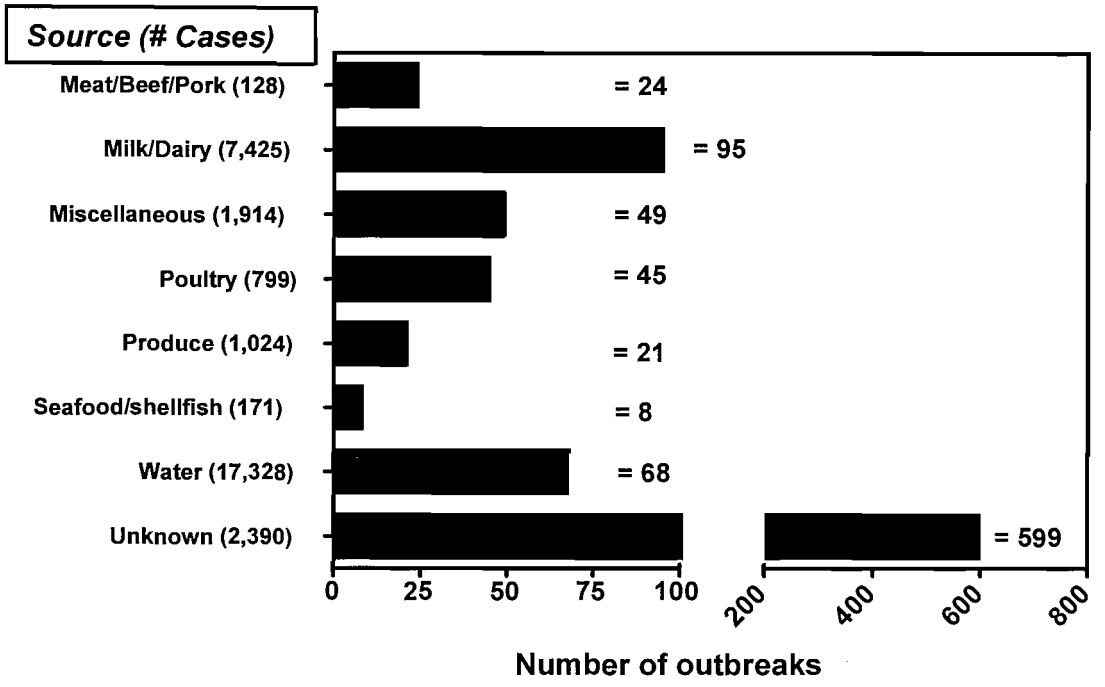


Figure 4. The Number of Outbreaks and Cases of Campylobacteriosis Associated With Different Food and Water Sources, 1978-2003. From Miller and Mandrell (2005).

activities etc showed that eating chicken, salad and vegetables, direct contact with farm animals and drinking water, or even drinking bottled mineral water were all associated with infection (Evans 2003). These were novel findings, since although eating chicken is a well established risk factor, consuming salad and bottled water are not. The association with salad may be explained by cross contamination of food within the home. However, the possibility that natural mineral water is a risk factor for *Campylobacter* infection could have wide public health implications since the bottled water industry is so huge worldwide (Evans 2003).

Although most sporadic cases of Campylobacteriosis are associated with preparation or consumption of poultry products, outbreaks have also been associated with consumption of unpasteurised milk or unchlorinated water. An estimated 20% of cases of illness are thought to be due to vehicles of infection other than food, including water (Figure 4) (Clark 2003; Miller and Mandrell 2005). *Campylobacter* species have been found to cause waterborne outbreaks worldwide; such outbreaks are a problem in Scandanavian countries where many people drink untreated water from streams and other sources. Untreated water has also been shown to be implicated in disease transmission in many other countries such as New Zealand, Finland, England, Wales, Australia, and the USA (Clark 2003).

Waterborne outbreaks tend to occur in spring or early autumn. It is also thought that the time of year has a large effect on whether an outbreak will occur. For example an

outbreak occurred in a farming community in Southern Ontario, Canada in 1985 which resulted from contamination of well water caused by heavy rains and spring run-off. A similar outbreak was recorded in a nearby village in 2000 due to well water contamination by surface water carrying livestock waste immediately after heavy rains (Clark 2003). Whether there are outbreaks or not will also depend on species and strains of *Campylobacter* and factors such as patient gender, age and state of immunity. Campylobacters occur in the gut of many species of bird and animal and appear to be particularly well adapted to this habitat. In poultry, for example, numbers can reach several millions per gram of caecal contents without causing any obvious ill effects in the host (Beery *et al.* 1988). Domesticated, farm and wild animals and birds are important reservoirs of campylobacters, and have all been implicated as sources of infection (Lightfoot *et al.* 1991; McElroy and Smyth 1993; Fernandez *et al.* 1996). Most *Campylobacter* infections occur as sporadic cases rather than outbreaks and this, coupled with the widespread occurrence of the organism and possibility of cross-contamination in the home, make it difficult to trace the sources of infection. The epidemiology of this disease thus remains poorly understood although it is anticipated that genotyping techniques now being applied will assist in identifying and characterising strains obtained from different sources (Wassenaar and Newell 2000).

It has been suggested that the distribution of sewage sludge to land may be one of the routes by which thermophilic campylobacters re-enter the human food chain (Jones 2001b). Both *Campylobacter* species and *Arcobacter* species have been commonly isolated in high numbers from both raw and treated sewage (Stampi *et al.* 1993; Moreno *et al.* 2003; Sahlstrom *et al.* 2004). In the EU, human faeces undergo a primary and secondary treatment, e.g. mesophilic anaerobic digestion (MAD), to reduce pathogen load before disposal. Recent research suggests that microaerophilic campylobacters are able to survive the MAD process (Horan *et al.* 2004) and may therefore represent a threat to the food chain if these wastes are recycled to land that is grazed by animals or used for growing crops including organic farming.

Despite the fact that salad vegetables are generally regarded as low risk foods they can harbour a range of pathogens and since these foods are not necessarily cooked before consumption they can pose a health risk to the consumer (Buck *et al.* 2003; Jones and Heaton 2006). Salad vegetables have been widely associated with *Campylobacter* outbreaks, however few studies have provided direct evidence for this, and isolation rates of *Campylobacter* species from vegetables are low (Sagoo *et al.* 2001; Evans *et al.* 2003; Jones and Heaton 2006). The consumption of salad vegetables has increased during recent years and a number of studies have shown that *C. jejuni* has been isolated from a range of both

processed and unprocessed salad vegetables (Park and Sanders 1992; Kumar *et al.* 2001). It is thought that microbial contamination could occur in the field from faecal contamination from urban and rural run-off, which may introduce enteric pathogens, or during processing (Kumar *et al.* 2001).

Reservoirs of newly described emerging *Campylobacter* species have been found in production animals, such as pigs, sheep, cattle, and poultry. Emergent campylobacteria have also been isolated from pets and wild animals, such as dogs, cats, hamsters, foxes, monkeys, rodents, and seals. However, non-mammalian species, such as wild birds and shellfish, have recently been implicated as reservoirs for these organisms (Miller and Mandrell 2005). Whether emerging *Campylobacter* species, if present in the food chain, are a potential health hazard must still be determined. Descriptions of each species including isolation, epidemiology and clinical associations are reviewed in section 1.4.

1.4. ISOLATION, EPIDEMIOLOGY AND CLINICAL SIGNIFICANCE OF *CAMPYLOBACTERACEAE* SPECIES

Campylobacter species are universally acknowledged as the most common bacterial cause of enteritis worldwide. Of the 18 recognised and named species of the genus *Campylobacter*, 14 species — *Campylobacter coli, Campylobacter concisus, Campylobacter curvus, Campylobacter fetus, Campylobacter hominis, Campylobacter hyointestinalis, Campylobacter jejuni, Campylobacter lanienae, Campylobacter lari, Campylobacter mucosalis, Campylobacter rectus, Campylobacter showae, Campylobacter sputorum*, and *Campylobacter upsaliensis* — have been isolated from symptomatic and asymptomatic humans. *C. jejuni* is by far the most commonly isolated *Campylobacter* species. However, a number of other species within the *Campylobacteraceae* have also been frequently isolated from food and clinical samples when alternative methods are used and the clinical significance of these emerging strains is beginning to be appreciated. In the following sections (section 1.4) the microbiology, epidemiology and clinical features of both *C. jejuni* and *C. coli* and other recognised *Campylobacteraceae* species are described in detail.

1.4.1. *C. JEJUNI* AND *C. COLI* SPECIES

C. jejuni and *C. coli* are by far the most commonly isolated *Campylobacter* species, and for this reason are currently perceived as the most important human enteropathogens among the campylobacters. *C. jejuni* has two subspecies, *C. jejuni* subsp. *jejuni* and *C. jejuni*

subsp. *doylei*. The clinical importance of the latter sub-species is less well characterised but it has been infrequently associated with gastroenteritis and septicaemia in paediatric patients (Lastovica 2006) (see section 1.5.2.6). *C. jejuni* subsp. *jejuni* is overwhelmingly the most frequently isolated *Campylobacter* worldwide and is associated with enteritis, particularly in young children, in both developed and developing countries. This bacterium is recognised as the most common antecedent pathogen associated with the Guillain-Barré syndrome and reactive arthritis. *C. jejuni* subsp. *jejuni* has also been associated with other clinical conditions, such as septicaemia, meningitis, haemolytic-uraemic syndrome, pancreatitis, and abortion. *C. coli* has been most frequently associated with pigs, although it is generally not thought to be a cause of disease in these animals (Payot *et al.* 2004). In humans, infection causes diarrhoea and *C. coli* is often regarded as the second most common *Campylobacter* species reported in human gastrointestinal illness (Skirrow 1994) . The separation *of C. jejuni* and *C. coli* remains an important taxonomic problem as the overall phenotype and genotype of both taxa are remarkably similar. There is some evidence to suggest that *C. coli* is a less diverse and more clonal species than *C. jejuni* (Duim *et al.* 2001).

1.4.2. EMERGING NON-*JEJUNI/COLI CAMPYLOBACTERACEAE*

1.4.2.1. *C. fetus*

C. fetus is separated into two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. This division stems from the realisation that two distinct disease entities could be attributed to two varieties of strains. DNA-DNA hybridisation studies showed that these subspecies are intimately related (Harvey and Greenwood 1983). *C. fetus* subsp. *fetus* colonises the intestine and causes sporadic abortion in sheep and cattle, usually late in gestation. C. *fetus* subsp. *venerealis* is adapted to the bovine genital tract and causes infertility by destroying the embryo in early gestation. This disease, known as bovine vibriosis or infectious infertility is the major concern to the cattle industry.

Campylobacter fetus was the first recognised *Campylobacter* species. It was isolated early this century as the causative agent of fetal infection and abortion in sheep and is now recognised as the major cause of septic abortion in domestic animals including cats and dogs (Garcia *et al.* 1983). However, since 1947, *C. fetus* has also been implicated as a causative agent of a range of human intestinal and extraintestinal illness. In contrast to *C. jejuni*, *C. fetus* is an uncommon and opportunistic pathogen of humans, primarily associated with bacteraemia and extraintestinal infections in patients with underlying diseases. *C. fetus* has been associated with septic abortions, septic arthritis, abscesses, meningitis, endocarditis and many other diseases. The clinical features of diarrheal disease due to *C. fetus* infection is

similar to that of *C. jejuni* (Rennie *et al.* 1994). *C. fetus* infection is common in immunocompramised patients, more than 75% of whom are men who suffer from serious medical conditions and where intestinal infections are associated with serious complications such as toxic megacolon (Kalkay et al. 1983). *C. fetus* accounts for more bacteraemia in adults than any other *Campylobacter* species except *C. jejuni* and *C. coli*. The fact that most systemic *C. fetus* illness occurs in debilitated hosts underscores the importance of normal host defences in resisting invasiveness by *C. fetus* beyond the gastrointestinal tract (Thompson and Blaser 2000).

Although gastroenteritis does occur with this species, it is thought that the incidence is probably underestimated due to insufficient culture conditions (Blaser 1998). It is likely that the susceptibility of *C. fetus* to cephalothin (often contained in isolation media) and the fact that many strains do not grow at 42°C may explain why it is not isolated from stools more frequently. Indeed filtration of stools through 0.45 or 0.65 μm pore-size filters onto the surface of agar plates allows for the isolation of *C. fetus* (Steele and McDermott 1984; Lastovica 2006). So far *C. fetus* subsp. *venerealis* has not been associated with human infection. However, owing to the difficulty of separating subsp. *venerealis* from subsp. *fetus* by phenotypic methods (mainly growth in the presence of 1% glycine), uncertaincy remains. Indeed, a study comparing a PCR method, developed for the differentiation and identification of these two subspecies, showed that when compared traditional phenotyping methods they were often misidentified (Hum *et al.* 1997). Therefore, it is likely that this is commonly occurring in diagnostic microbiology laboratories.

During the period from 1987 to 1989, 122 *C. fetus* isolates were reported to be mainly isolated from stool and blood samples (Lastovica and Skirrow 2000). It has also been found in poultry, swine and reptiles. Faeces from infected animals may contaminate soil, fresh water, and carcasses during abattoir processing. Therefore, human infection most probably results from consumption of contaminated food or water and several such incidences have been reported (Klein *et al.* 1986; Rao *et al.* 1990; Ichiyama *et al.* 1998).

A number of studies have been carried out to investigate the mechanisms of pathogenesis and virulence determinants of *C. fetus* (Thompson and Blaser 2000). Factors thought to be important in the pathogenesis include the unique structure of the lipopolysaccharide (LPS) (Blaser *et al.* 1994; Moran *et al.* 1996a; Moran *et al.* 1996b) and antigenic variation in proteins in the surface layer (S-layer proteins) (Blaser *et al.* 1994).

1.4.2.2. *C. upsaliensis*

C. upsaliensis is a recognised pathogen in both healthy and immunocomprimised patients, in whom it causes acute as well as chronic and recurrent diarrhoea (Bourke *et al.* 1998). It can also cause bacteraemia in debilitated and immunocomprimised patients and has been associated with haemolytic-uraemic syndrome, Guillain-Barré syndrome and spontaneous human abortion. *C. upsaliensis* was first isolated from the stools of healthy and diarrhetic dogs in 1983 and DNA-DNA hybridisation studies indicated this was a new species. *C. helveticus* is a closely related species found in dogs and cats, but has not been shown to cause human illness (Stanley *et al.* 1992). *C. upsaliensis* is a thermotolerant *Campylobacter* species that usually grows at 42°C, and growth of some strains is enhanced when hydrogen is present (Bourke *et al.* 1998; Lastovica and Skirrow 2000). It is catalase negative or weakly positive, hippurate and aryl sulfatase negative but nitrate and indoxyl acetate positive. A distinguishing feature of *C. upsaliensis* is that it is highly susceptible to both naladixic acid cephalothin with many strains being susceptible to cefaperazone (Goossens *et al.* 1990a; Byrne *et al.* 2001). A selective medium, CAT agar was developed with reduced levels of cefoperazone (compared to CCDA selective agar) to allow isolation of *C. upsaliensis*. A comparison of this medium to membrane filtration for the isolation of *Campylobacter* species from human, cat and dog stool samples revealed that CAT agar showed similar recovery of *C. upsaliensis* compared to a membrane filtration technique (Aspinall *et al.* 1996). In a study on paediatric stools in South Africa, *C. upsaliensis* (as well as a number of other emerging species) only began to be isolated when the isolation protocol was changed from antibiotic-containing media to the Cape Town protocol (membrane filtration onto antibiotic-free agar and subsequent incubation in a hydrogen enriched atmosphere (Lastovica 2006)).

C. upsaliensis has been predominantly associated with cats and dogs. One study showed that 64% of the strains identified from dog and cat stool samples were *C. upsaliensis*. It has also been isolated from a dog with chronic diarrhoea, from healthy puppies and kittens, asymptomatic cats, and asymptomatic vervet monkeys (Bourke *et al.* 1998). The exact source of *C. upsaliensis* in humans is unknown (Patton *et al.* 1989; Lastovica 2006). There is indirect evidence to suggest that person-to-person transmission of *C. upsaliensis* is possible (Goossens *et al.* 1995). The proportion of *Campylobacter* species that are isolated and identified as *C. upsaliensis* is relatively high when faecal samples are prospectively cultured for *C. upsaliensis*. For example, a study at a paediatric hospital in South Africa showed that 22.3% of the *Campylobacter* strains isolated were *C. upsaliensis*. This high isolation rate

could be attributed to the use of an improved isolation method (Lastovica 2006). In a Belgium study of 15,185 faecal samples, 802 yielded *Campylobacter* strains, 12% of which were identified as *C. upsaliensis* (Goossens *et al.* 1990a). Differences in prevalence and exposure to *C. upsaliensis* could possibly be due to differences in colonisation and host immune system and the nature of *C. upsaliensis* infection in different geographical areas.

C. upsaliensis has a plasmid carriage rate of about 90%, much higher than any other species of *Campylobacter* (Fouts *et al.* 2005). Although serotyping has been of limited value, SDS-PAGE protein profile analysis and pulsed-field gel electrophoresis has proved useful for the differentiation of individual isolates of *C. upsaliensis* and shown that there are high levels of molecular heterogeneity within strains of *C. upsaliensis* (Owen *et al.* 1989; Bourke *et al.* 1996). *C. upsaliensis* can be identified by PCR techniques based on the 16S rRNA gene or the GTPase gene (Linton *et al.* 1996). Restriction fragment length polymorphism and PCR assays have also been used successfully to identify clonal variants of *C. upsaliensis* (Goossens *et al.* 1995).

The usual symptoms associated with *C. upsaliensis* infection are gastrointestinal and include watery diarrhoea, abdominal cramps, vomiting and low-grade fever (Pickett *et al.* 1996). While most patients recover quickly, some are ill for several weeks. It is thought that in a high proportion of cases, bacteraemia is associated with gastrointestinal infection, particularly in patients with underlying medical conditions (Lastovica and Le Roux 2001). *C. upsaliensis* has also been isolated from the breast abscess of a patient with no reported animal contact or gastrointestinal symptoms, and has been linked to haemolytic-uraemic syndrome and Guillian Barré syndrome (Gaudreau and Lamothe 1992; Lastovica *et al.* 1997).

Although the mechanisms of pathogenicity of *C. upsaliensis* have not been fully characterised, a number of recent studies have given clues to the virulence factors and mechanisms that might be involved in pathogenesis. Sylvester *et al.* (1996) demonstrated that *C. upsaliensis* is capable of binding to Chinese hamster ovary (CHO) cells in cell culture. These authors also detected 50- to 90-kDa surface proteins on *C. upsaliensis* isolates that were capable of binding to phosphatidylethanolamine, a putative cell membrane receptor. *C. upsaliensis* has also been shown to bind to mucin, a protein found in the human intestine, indicating that *C. upsaliensis* expresses an adhesin capable of recognising a specific mucin epitope (cell membrane receptor) which is likely to aid in intestinal colonisation but also in the host immune response (Sylvester *et al.* 1996). Fouts *et al.* (2005) have discovered a novel putative *licABCD* virulence locus in *C. upsaliensis* with significant similarity to genes present in *Streptococcus, Haemophilus*, and *Neisseria* species. The presence of a cytolethal

distending toxin homologue in *C. upsaliensis* has been confirmed; however, the appropriate *cdt* genes have yet to be cloned (Pickett *et al.* 1996).

1.4.2.3. *C. helveticus*

C. helveticus has been recovered from the faeces of domestic cats and dogs after selection by filtration, or with the antibiotic cefoperazone (Stanley *et al.* 1992). It has been shown to be closely related to *C. upsaliensis* and although it has not been associated with human disease, it cannot be discounted that this pathogen is being underreported due to insufficient culture conditions or misidentification. The only phenotypic difference from *C. upsaliensis* is the inability of *C. helveticus* to grow on potato starch agar or reduce selenite, tests which are often not used in routine laboratories. A wider use of genetic methods could rectify this oversight. A PCR method has been developed which allows accurate species identification (Lawson *et al.* 1997) and recently a mass spectrometry (MALDI-TOF) method for accurate speciation (Mandrell *et al.* 2005) and multilocus sequence typing (MLST) system for accurate typing of strains have been described (Miller *et al.* 2005).

1.4.2.4. *C. hyointestinalis*

C. hyointestinalis was first identified and suggested as a possible cause of proliferative disease in pigs (Gebhart *et al.* 1983). The organism has subsequently been isolated from human stools and may be the cause of diarrhoea. A subspecies, *C. hyointestinalis* subsp. *lawsonii* has also been described based on differences in phenotypic and genotypic methods although its pathogenic role in animals and humans is unknown (Gebhart *et al.* 1983; On 1996).

C. hyointestinalis is closely related to *C. fetus* and, like *C. fetus*, is catalase and nitrate reductase positive, indoxyl acetate negative, susceptible to cephalothin and resistant to nalidixic acid. *C. hyointestinalis* has been reported to grow under microaerobic conditions, although some strains have been reported to require additional hydrogen (Lastovica and Skirrow 2000). All strains will grow at 37°C but only some strains will grow at 42°C. Generally, *C. hyointestinalis* can be differentiated from other hydrogen requiring species by the catalase test, lack of aryl sulphatase activity and intolerance to 3.5% NaCl (Burnens and Nicolet 1993). *C. hyointestinalis*, like many other cephalothin-sensitive campylobacters is thought to be under detected due to the use of selective media. Filtration onto antibiotic-free agar medium and incubation in a hydrogen enhanced microaerobic atmosphere at 37°C has

proved to be extremely efficient for the isolation of *C. hyointestinalis* (Lastovica and Le Roux 2000).

Pulsed-field gel electrophoresis and SDS-PAGE have been used to differentiate strains of *C. hyointestinalis* from each other as well as other *Campylobacter* species (Vandamme *et al.* 1990; Salama *et al.* 1992). DNA probes based on 16S rRNA have also been useful for discrimination of *C. hyointestinalis* from *C. fetus* and a PCR assay has been developed for the detection of *C. hyointestinalis* (Wesley *et al.* 1991; Linton *et al.* 1996).

C. hyointestinalis has been consistently isolated from the intestines of pigs with proliferative enteritis, but not from asymptomatic pigs or pigs with other enteric diseases. It has also been isolated from hamsters, cattle and non-human primates. Its role in disease still remains uncertain. A study has shown that 77% of *C. hyointestinalis* isolates identified from swine with proliferative enteropathy produced a cytotoxin, although the role of this cytotoxin in human infection is unknown (Ohya and Nakazawa 1992).

1.4.2.5. *C. lari*

C. lari is a nalidixic acid-resistant thermophilic *Campylobacter* (NARTC) which was first isolated in 1980 from sea gulls (of the genus *Larus*) and subsequently from other avian species, dogs, cats and chickens (Waldenstrom *et al.* 2002). A subgroup of *C. lari*, known as the UPTC (urease positive thermophilic campylobacter) has the unusual capacity (for a *Campylobacter* species) of hydrolysing urea; strains are usually sensitive to nalidixic acid, and have been frequently isolated from water and shellfish (Van Doorn *et al.* 1998). The species is ecologically, phenotypically and genetically diverse. *C. lari* has been isolated from a variety of environmental and animal sources. It has been frequently isolated from rivers and surface water in Europe, which is of significance since water is an established vehicle for the transmission of campylobacters to humans (Tauxe *et al.* 1985; Broczyk *et al.* 1987).

Generally *C. lari* strains are microaerophilic (although some isolates may also require hydrogen) and grow at 42°C. Studies have shown that they are resistant to nalidixic acid, cephalosporins, vancomycin and trimethoprim. Most are oxidase and nitrate reductase positive and do not hydrolyse urea (Tauxe *et al.* 1985). Correct identification of *C. lari* may not be made since many clinical microbiology laboratories may not routinely test for nalidixic acid resistance; one of the only features to differentiate it from *C. jejuni*. However, nalidixic acid-susceptible stains of *C. lari* have been reported as have nalidixic acid resistant *C. jejuni* strains, but *C. lari* is negative for hippurate hydrolysis and indoxyl acetate hydrolysis while *C. jejuni* is not (Popovic-Uroic *et al.* 1990).

A PCR assay specific for *C. lari* has been developed based on the nucleotide sequence of the 16S rRNA gene (Linton *et al.* 1996) and a novel putative GTPase gene (Van Doorn *et al.* 1997). A novel PCR reverse hybridisation probe assay has also been developed for the rapid detection of different *C. lari* variants revealing high levels of heterogeneity (Van Doorn *et al.* 1998). A recent study using amplified fragment length polymorphism and protein electrophoretic profiles showed that *C. lari* is genetically diverse and can be grouped into distinct genogroups. UPTC strains were shown to be genetically diverse and distinct from NARTC strains which encompass at least two genogroups (Duim *et al.* 2004) .

C. lari has been isolated from asymptomatic patients but can also produce acute diarrhoeal illness and bacteraemia in both immunocompetent and immunocompromised patients (Tauxe *et al.* 1985). A South African study showed that of 3,877 diarrhetic stool samples analysed, *C. lari* was only isolated twice (Lastovica and Skirrow 2000).

C. lari strains are capable of producing both cytotoxic and cytotonic factors (Johnson and Lior 1986). A recent study has identified a cytolethal distending toxin B (*cdtB*) gene locus among isolates of *C. lari* and shown that there was considerable genetic heterogeneity (Shigematsu *et al.* 2006). However the role of these factors in the disease process is largely unknown.

1.4.2.6. *C. jejuni* subsp. *doylei*

Based on DNA hybridisation studies, *C. jejuni* has been divided into two subspecies; *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. Although the pathogenic potential of *C. jejuni* is well established, the pathogenic potential of *C. doylei* is only just beginning to be appreciated (Lastovica and Skirrow 2000). Although infrequently isolated, *C. jejuni* subsp. *doylei* has been associated with gastroenteritis and septicaemia in paediatric patients (Lastovica 2006).

The inability to reduce nitrate to nitrite, is the determining phenotypic characteristic which distinguishes *C. jejuni* subsp. *doylei* from C. *jejuni* subsp. *jejuni* and all other campylobacters. *C. doylei* is microaerophilic, grows poorly at 42°C and is usually hippuricase and catalase positive. Strains are sensitive to nalidixic acid, but, unlike *C. jejuni* subsp. *jejuni*, are also susceptible to cephalothin. Filtration of stools onto antibiotic-free agar plates have successfully isolated these organisms and shown that *C. doylei* is often co-isolated with *C. jejuni* and *C. upsaliensis* (Lastovica 2006).

C. doylei has been largely associated with paediatric diarrhoea and isolation rates of up to 9.2% have been reported (Fernandez *et al.* 1997; Lastovica 2006). It is thought that systemic infection is often preceded by intestinal infection and *C. doylei* can often be isolated

from the blood of patients who have previously suffered from gastrointestinal disease. In a South African study, 24% of blood samples from paediatric patients were positive for *C. doylei* (Lastovica 2006), and in a study of aboriginal children in central Australia, *C. doylei* accounted for a staggering 85% of *Campylobacter* bacteraemia (Lastovica and Skirrow 2000). These observations suggest a pathogenic and invasive role for *C. doylei*, however, the mechanisms of pathogenesis and virulence determinants are unknown.

1.4.2.7. *C. lanienae*

C. lanienae was first isolated from asymptomatic abattoir workers (Logan *et al.* 2000) and strains have been subsequently found in pigs and cattle (Inglis *et al.* 2003; Sasaki *et al.* 2003). *C. lanienae* cannot be distinguished phenotypically from the closely related *C. hyointestinalis* and even using PCR tests based on 16S rRNA they cannot be adequately differentiated. However, AFLP (amplified fragment length polymorphism) and protein profiling affirm that *C. lanienae* is a genetically distinct species (On and Harrington 2000). The role of *C. lanienae* in human infection is not known due to its recent characterisation and lack of tests to adequately differentiate this species from the closely related *C. hyointestinalis.*

1.4.3. Hydrogen Requiring *Campylobacter* Species

There are six *Campylobacter* species which have an absolute essential growth requirement for hydrogen or formate. Five of them are found in the gingival flora of the human mouth, notably the periodontal pockets of diseased gums. These include *C. rectus*, *C. curvus*, *C. concisus* and *C. showae* of which are biochemically similar organisms. *C. gracilis* was formerly regarded as a *Bacteroides* species but was reclassified in 1995. *C. mucosalis* also has an absolute requirement of hydrogen for growth and has also been commonly isolated from oral cavities. Although all six species show a requirement of hydrogen for growth, in addition, species including *C. rectus*, *C. showae*, *C. curvus* and *C. gracilis* prefer anaerobic conditions for growth.

1.4.3.1. *C. rectus*

C. rectus was originally described as '*Wollinella recta*' prior to an extensive revision of the phylogenetic group in 1991 (Vandamme *et al.* 1991). It has mainly been found in the gingival flora of the human mouth, and is generally considered to be a periodontal pathogen in humans (Siqueira and Rocas 2003; Hayashi *et al.* 2005). One study has reported its

isolation from 80% of 1,654 adults and children with periodontitis during a 2-year study (Rams *et al.* 1993). Another study has reported the detection of two *C. rectus* isolates in the diarrhetic stools of South African children (Lastovica 2006). However, to date, this is the only study which has reported associations with gastrointestinal infection. It is possible that the low isolation rates could be due to use of sub-optimal culture conditions (Lastovica and Skirrow 2000). *C. rectus* has only been isolated when anaerobic or hydrogen-enhanced microaerobic atmospheres have been used with antibiotic-free blood agar plates (Rams *et al.* 1993; Lastovica 2006). Because of the fastidious nature of this organism, most studies have used PCR to detect this species. Immunoblot detection using monoclonal antibodies to surface structures has also been used (Ihara *et al.* 2003).

C. rectus is known to produce a variety of putative virulence factors, including lipopolysaccharide, crystalline surface layer (S-layer) (Kobayashi *et al.* 1993); (Miyamoto *et al.* 1998) and GroEL-like protein (Wang *et al.* 2000). Studies have shown that *C. rectus* secretes a 104 kDa cytotoxin capable of killing human HL-60 cells (Gillespie *et al.* 1992; 1993).

1.4.3.2. *C. concisus*

C. concisus was first isolated from the oral cavity of humans affected with periodontal disease (Tanner *et al.* 1987). The role of *C. concisus* in periodontal disorders is as yet unclear, since the organism can be found in both healthy (Macuch and Tanner 2000) and diseased (Kamma *et al.* 2000) sites, and in association with other oral bacteria (Socransky *et al.* 1998). Similarly, the potential of *C. concisus* as a gastrointestinal pathogen is uncertain. Although these bacteria have been identified from cases of diarrhoea in humans in frequencies equivalent to those of *C. jejuni* (Lindblom *et al.* 1995; Engberg *et al.* 2000; Lastovica 2006), they have also been isolated from healthy control patients at a similar rate (Van Etterijck *et al.* 1996). To date, no animal reservoir of *C. concisus* has been reported.

The role of *C. concisus* in human disease has been difficult to determine, since the species comprises of phenotypically indistinguishable but genetically distinct taxa (i.e., genomospecies) that may vary in pathogenicity. Studies using pulse field gel electrophoresis and AFLP profiling data and protein profiling analysis have shown that all *C. concisus* strains have unique AFLP and protein profiles, which can be grouped into four distinct clusters (Matsheka *et al.* 2002; Aabenhus *et al.* 2005a; 2005b). It is now thought that *C. concisus* is genetically and taxonomically diverse and contains at least four distinct genomospecies that may exhibit differences in their virulence potential (Aabenhus *et al.* 2005a; 2005b).

Cytotoxic activity has not been observed in *C. concisus* but a cytotonic effect on CHO (chinese hamster ovary) cells has been seen (Musmanno *et al.* 1998) and the detection of haemolytic phospholipase A activity in *C. concisus* indicates the presence of a potential virulence factor in this species and supports the hypothesis that *C. concisus* is a possible opportunistic pathogen (Istivan *et al.* 2004).

The fact that *C. concisus* requires a hydrogen enriched incubation atmosphere for growth, will not grow at 42°C and is susceptible to many antibiotics contained in selective media means that is likely to be severely underdetected in many routine laboratories. Indeed, when optimal isolation methods for *C. concisus* have been adopted, isolation rates from clinical faecal samples have been shown to be just as high as that of *C. jejuni* (Lastovica 2006). Considerable further work is needed with more discriminatory genotyping methods and suitable isolation methods before accurate assessment of the risk of *C. concisus* infection to human health can be determined (Newell 2005).

1.4.3.3. *C. curvus*

C. curvus was first described as '*Wollinella curva*' and included anaerobic, hydrogen-dependent strains from the oral cavity of humans, but was later found to be phylogenetically distinct from other *Wollinella* species (On 2001). *C. curvus* is a rarely encountered *Campylobacter* species in human, animal, and environmental samples. However, there have been a number of isolated cases. *C. curvus* has been recovered from patients with periodontal disease or septicaemia (Tanner *et al.* 1987). It has recently been isolated from diarrhetic stools from children in Belgium and South Africa (Lastovica 2006). A study investigating bacterial agents causing bloody gastroenteritis and a small outbreak of Brainerd's diarrhoea in northern California showed that 20 strains of *C. curvus* or *C. curvus*-like organisms were isolated by a microfiltration technique and prolonged anaerobic incubation. This suggests that *C. curvus* may be an underappreciated *Campylobacter* that may be involved in sporadic and outbreak cases of bloody or chronic diarrhoea in humans (Abbott *et al.* 2005), but due to sub-optimal isolation conditions it is unlikely to be detected and its clinical significance remains unknown.

1.4.3.4. *C. showae*

There is some evidence to suggest that *C. showae* is the primary pathogen in periodontal disease, since it has been found more frequently, and in higher levels, in diseased sites compared with healthy areas (Etoh *et al.* 1993; Macuch and Tanner 2000). However,

like the other hydrogen requiring species described, it is extremely difficult to culture and unlikely to be isolated if standard microaerophilic conditions and antibiotic containing media are used, therefore its role in human gastrointestinal disease remains unknown.

1.4.3.5. *C. gracilis*

Campylobacter gracilis was first isolated as *Bacteroides* species associated with periodontal disease. It has anaerobic growth requirements, a straight rod-shaped body and absence of flagella like *B. ureolyticus*, but phylogentic analysis clearly assigns it to *Campylobacter* (Vandamme *et al.* 1991). Like *C. concisus*, the role of *C. gracilis* in periodontal disease remains unclear (Macuch and Tanner 2000). *C. gracilis* has also been recovered from deep tissue and pleuropneumonia infections (Johnson *et al.* 1985).

1.4.3.6. *C. hominis*

C. hominis was originally detected in human faecal samples on the basis of its 16S rRNA gene sequence alone. Since the organism could not be cultured, it could not be subjected to closer scrutiny (Lawson *et al.* 1998). However, a close phylogenetic association with predominantly anaerobic campylobacters was noted and used to formulate an amended isolation protocol that involved screening of PCR positive stool samples for growth under anaerobic conditions, which was found to be successful for isolation of *C. hominis* (Lawson *et al.* 2001). A commensal role for *C. hominis* in the human gut has been proposed since it is found in both healthy and diarrhoetic stools (Lawson *et al.* 2001).

1.4.3.7. *C. sputorum*

C. sputorum has many features of the hydrogen requiring *Campylobacter* species even though it does not have a strict requirement for hydrogen. It is catalase-negative, and forms part of the human gingival flora. Until recently, *C. sputorum* was considered to have three biovars; biovar *sputorum*, living in the mouth and gastrointestinal tract, biovar *bubulus*, living in healthy genital tracts of cattle and sheep; and biovar *faecalis*, found mainly in the faeces of sheep and cattle. However, recent data have shown that biochemical differences between biovars *bubulus* and *faecalis* are unreliable and biovar *bubulus* is no longer recognised (On *et al.* 1998). Instead a group of catalase-negative, urease-positive *Campylobacter* strains isolated from cattle faeces formed a new biovar, *paraureolyticus,* which has also been isolated from a patient with enteritis in Canada (On *et al.* 1998; 1999). *C. sputorum* is generally considered a commensal, but cases of diarrhoea, abscess formation

and bacteraemia have been described (Tee *et al.* 1998). *C. sputorum* has been isolated from the human lung and abscesses of the groin and axillary areas and from the blood of a patient with a knee abscess (On *et al.* 1992). It has also been isolated from patients with gastroenteritis (Lindblom *et al.* 1995). As with the other hydrogen requiring species, it is likely that isolation rate of *C. sputorum* is underestimated due to sub-optimal culture conditions and, therefore, the true clinical relevance of this species remains unknown.

1.4.3.8. *C. mucosalis*

C. mucosalis is a hydrogen requiring species that has been associated with proliferative enteritis in pigs (Boosinger *et al.* 1985; Van der Walt *et al.* 1988). *C. mucosalis* is closely related to *C. concisus* at the genetic level using DNA-DNA hybridisation experiments (Roop *et al.* 1984) and AFLP cluster analysis (On and Harrington 2000). Studies have shown that differentiation of these two species using phenotypic analysis alone is prone to error due to variable characteristics of these species and therefore molecular analysis should be used (Lastovica *et al.* 1993; 1994; On 1994). Studies have shown that immunotyping, SDS-PAGE, DNA hybridization and RFLP studies all allow excellent discrimination between the two species. At present there is no known association of *C. mucosalis* and human disease as human isolates originally believed to be *C. mucosalis* were later identified as *C. concisus* (On 1994). However, due to the problems with misidentification using phenotypic techniques and inappropriate culture conditions for this organism, the true extent of its prevalence in humans, animals and the environment has not been elucidated.

1.4.4. Related Non-*Campylobacter* Bacteria

1.4.4.1. *Bacteroides ureolyticus*

B. ureolyticus is genetically related to the anaerobic *Campylobacter* species, requires an anaerobic or hydrogen enriched atmsophere for growth and like the other anaerobic *Campylobacter* species, is often found in the gingival flora of the human mouth and has been associated with periodontal disease. It has also been commonly linked to infection of both the male and female genital tract (Akhtar and Eley 1992; Woolley *et al.* 1992; Balmelli *et al.* 1994).

B. ureolyticus is presently considered to be a member of the *Campylobacteraceae*, however many complications exist with its taxonomic classification as a *Campylobacter* species. Phylogenetic analysis using 16S rRNA analysis along with protein profiles and

DNA-DNA hybridisation studies have clearly shown that *B. ureolyticus* is more closely related to *Campylobacter* than *Bacteroides* species (Taylor *et al.* 1987; Vandamme *et al.* 1995). Furthermore, numerical analysis of 67 phenotypic characters cluster *B. ureolyticus* with other 'anaerobic' *Campylobacter* species (Fontaine *et al.* 1984; 1986). *B. ureolyticus* can also be found in the intestine, along with many other *Campylobacter* species and it has been shown that other strains of *Campylobacter* also produce urease. However, the reclassification of *B. ureolyticus* as a *Campylobacter* species is complicated by the fact that the fatty acid content, disease associations, proteolytic metabolism and urease production are all atypical of *Campylobacter* species, therefore it has been suggested to wait until further data are generated before reclassifying *B. ureolyticus* as either a *Campylobacter* species or into a new genus (Vandamme *et al.* 1995).

1.4.4.2. *Arcobacter butzleri* and *A. cryaerophilus*

The genus *Arcobacter* was proposed in 1991 for bacteria previously identified as aerotolerant campylobacters or *Campylobacter*-like organisms (Vandamme *et al.* 1991; Meng and Doyle 1997). *Arcobacter* species primarily differ from campylobacters in their ability to grow under aerobic conditions and at temperatures between 15 and 30°C (Lastovica and Skirrow 2000). The genus *Arcobacter* comprises four species which were previously included in the genus *Campylobacter*: *A. nitrofigilis*, *A. cryaerophilus*, *A. butzleri*, and *A. skirrowii* (Vandamme and Goossens 1992). *A. nitrofigilis*, originally isolated from plant roots, has not been isolated in plants or animals. Another species of of autotrophic, sulphide-oxidising nitrogen-fixing *Arcobacter* (*Candidatus* Arcobacter sulfidicus) has recently been described (Wirsen *et al.* 2002). In addition, On *et al.* (2003) have identified another probable species, found in pig abortions and ducks.

Both *A. cryaerophilus* and *A. butzleri* have been associated with human disease and have been isolated from numerous different sources, including poultry, water, and humans exhibiting gastroenteritis and bacteraemia (On *et al.* 1995; Jacob *et al.* 1998; Johnson and Murano 1999; Wesley *et al.* 2000; Son *et al.* 2007). *A. skirrowii* has been commonly isolated from aborted cattle, sheep and pig foetuses but generally not humans. Although, recently, *A. skirrowii* was isolated from a person with chronic diarrhoea (Wybo *et al.* 2004). During an 8-year study period, *Arcobacter butzleri* was the fourth most common *Campylobacter*-like organism (after *C. jejuni*, *C. coli* and *C. upsaliensis*) isolated from 67,599 stool specimens using a variety of recovery methods (including selective isolation media and filtration onto antibiotic free agar) (Vandenberg *et al.* 2004). The study revealed that, generally, *A. butzleri* displayed microbiological and clinical features similar to those of *Campylobacter jejuni*;

however, *A. butzleri* was more frequently associated with a persistent, watery diarrhoea than bloody diarrhoea (Vandenberg *et al.* 2004).

A recent study showed that *Arcobacter* was shown to produce a substance that was toxic to some human cells in culture, but this substance was toxic in a manner different from that of *Campylobacter* CDT (cytolethal distending toxin) (Johnson and Murano 2002). However, information regarding its clinical significance, pathogenicity, and epidemiology is limited (Lehner *et al.* 2005). It is likely that the clinical importance and epidemiological prevalence of *Arcobacter* species is severely underestimated since although there are specific isolation media and molecular detection methods available for *Arcobacter* species, many routine laboratories tend to test only for *Campylobacter* species, as *Arcobacter* are not currently perceived as important pathogens. It is thought that a number of the antibiotics contained in *Campylobacter* selective isolation media are inhibitory to the growth of *Arcobacter* species (Houf *et al.* 2001b). Furthermore, both *A. butzleri* and *A. cryaerophilus* grow poorly or not at all at 42°C (Lastovica and Skirrow 2000), the temperature generally used for isolation of campylobacters i.e. *C. jejuni* and *C. coli*.

1.5. PATHOGENESIS, SYMPTOMS AND CLINICAL ASSOCIATION

The role of *C. jejuni* subsp. *jejuni* in human disease is well established, but the clinical relevance of other *Campylobacter* species (emerging campylobacteria) is far less well understood because relatively few strains of these organisms have been isolated from clinical material.

The paucity of isolated *Campylobacter* strains is undoubtedly due to a variety of reasons including current isolation protocols for *Campylobacter* species being biased in favour of the isolation of *C. jejuni* subsp. *jejuni* and *C. coli*. Very little information is available on the clinical aspects of infection from non-*jejuni/coli* isolates, however, the recent publication of the genome sequences of *C. lari* and *C. upsaliensis* have shown that they also contain similar virulence genes (Fouts *et al.* 2005). Some of the clinical associations and virulence determinants for individual *Campylobacteraceae* species have already been described in section 1.4 and are summarised in Table 1.

Campylobacter, specifically *C. jejuni*, is commonly found in the intestinal tracts of people or animals without causing any symptoms of illness (Pazzaglia *et al.* 1991; Hingley 1999). But eating contaminated or undercooked poultry or meat, or drinking raw milk or contaminated water, may cause *Campylobacter* infection, or campylobacteriosis (Hingley 1999). The infective dose of *Campylobacter* species is unknown, but it is believed to be low.

One experiment demonstrated that 500 organisms in 500 ml of milk was sufficient to cause acute symptoms in a healthy male (Robinson 1981).

Motility is perhaps the best-established virulence factor of *Campylobacter jejuni* and *C. coli* (Carrillo *et al.* 2004). It has also proved to be a vital weapon in the armoury of many other enteric bacterial pathogens, and new angles on the properties of bacterial flagellins are emerging such as glycosylation and variation in *fla* components (genes coding for flagellar subunits) (Korolik 2005). The pathogenesis of campylobacteriosis is not yet clearly understood, however it is thought that non-motile strains are avirulent. As well as being motile, chemotaxis is an important factor in colonisation of host tissues. Chemotaxis, the sensory control of motility to allow bias in swimming direction towards beneficial environments and away from unfavourable conditions, is also thought to be essential (Korolik 2005). The intestine is the natural habitat of campylobacters and therefore chemotactic motility is likely to be an important mechanism involved in colonisation. *C. jejuni* has a single flagellum at one or both poles and displays a darting-like motility. Chemoattractants of *C. jejuni* include components of mucin and bile, such as L-fucose and L-serine, as well as certain organic acids, for example pyruvate and succinate (Hugdahl *et al.* 1988). The mechanisms of the chemotactic signal transduction pathway have been well studied in *E. coli* and *Salmonella* Typhimurium and to a lesser extent in *Campylobacter* and other bacteria. The basis of the pathway appears to be highly conserved in motile bacteria, irrespective of type of flagella and the mode of locomotion they employ. The steps include detection of an environmental stimulus through ligand binding to clustered transmembrane MCP (methyl-accepting chemotaxis proteins) receptors, transduction of the signal to a histidine protein kinase (HK), CheA, which in turn passes the phosphate signal to a response regulatory protein, CheY that interacts with the flagella motor (Korolik 2005).

Bacterial diseases of the intestinal tract typically result from a complex set of interactions between the offending bacteria and the host (Hu 2005). Following passage through the stomach, *C. jejuni* adheres to and invades colonic epithelial cells, triggers signal transduction events that induce host cytoskeletal rearrangements and bacterial uptake, induce interleukin-8 production, and causes colitis (Hu 2005). The first step in invasion is bacterial adherence, most typically due to specific interaction between molecules on the bacterial surface (i.e. adhesins) and molecules on the host surface (i.e. receptors). It is also thought that motility and chemotaxis are essential for adherence (Hu 2005; Fauchere 1986). Common structures used to attach to host cells include pili (and fimbriae) as well as adhesins which include molecules such as flagella, outer membrane proteins (OMPs), and lipopolysaccharide (LPS) (Hu 2005). However the importance of these ligands is not defined and an

understanding of their specific involvement in disease pathogenesis is complicated by the presence of multiple adherence factors. Internalisation is the next step in pathogenesis and for this to occur, a bacterial protein secretion system is thought to exist where protein products are expressed which bind to host cell receptors (Hu 2005). Bacterial internalisation is also thought to require host cytoskeletal rearrangements resulting in endocytosis of the pathogen (Hu, 2005). Once internalised in the vacuole the bacteria move intracellularly to the basal lateral membrane before exocytosis into adjacent cells. All *C. jejuni* cells produce cytolethal-distending toxin (CDT), a nuclease that results in cell cycle arrest and host DNA damage and which also triggers IL-8 (interleukin-8) release from the epithelium causing inflammation and diarrhoea (Pickett *et al.* 1996).

The recent publication of the genomes of *Campylobacter* species has revealed many clues to potential virulence determinance of emerging species. Genes for Type IV Secretion Systems (T4SS), more similar to *A. tumefaciens* than to *C. jejuni* pVir T4SS, have been identified in *C. coli, C. lari* and *C. upsaliensis* megaplasmids. These could be potential virulence factors or may just be part of a conjugation system rather than for virulence (Fouts *et al.* 2005).

Studies on the glycobiology and glycomics of *C. jejuni* species have shown that glycosylation of structures including the flagella, and lipopolysccharide (LPS) and capsular proteins are essential in the virulence of *C. jejuni* (Karlyshev *et al.* 2005). Similarly, studies on other species have shown that glycosylated LPS structures have been demonstrated in *C. coli* and *C. fetus* species similar to ganglioseries glycosphingolipids (GSL) structures (Aspinall *et al.* 1993a; Senchenkova *et al.* 1997). Molecular mimicry of GLS has putatively been associated with GBS.

Symptoms of campylobacteriosis usually occur within two to ten days of ingesting the bacteria but severity will depend on strain or species type and the individual. Children, the elderly, and people with weakened immune systems or those with underlying disease (Post 1998) are particularly at risk. The most common symptoms include mild to severe diarrhoea, fever, nausea, vomiting, and abdominal pain (Hingley 1999). Typically the symptoms are unpleasant but not life threatening, however deaths do sometimes occur, most often amongst the elderly. Most people infected with *Campylobacter* can get well on their own without treatment, and those ill recover within a week to ten days. Occasionally, however, electrolyte replacement and rehydration is prescribed and severe cases are treated with antibiotics (e.g. erythromycin, tetracycline or quinolones). Occasionally complications can occur, such as urinary tract infections or meningitis. *C. jejuni* is also now recognised as a major contributing

factor to Guillain-Barré syndrome, the most common cause of acute paralysis in both children and adults (Hingley 1999; Hughes 2004).

1.6. CLINICAL ASSOCIATIONS

In contrast to the statement on the CDC website quoted in the Introduction, *C. jejuni* subsp. *jejuni* has not been the major *Campylobacter* species isolated from paediatric patients with enteritis and septicaemia in Cape Town, South Africa. During this study, over the last 15 years two-thirds of the *Campylobacter* isolates from the diarrheic stools of paediatric patients at the Red Cross hospital were identified as species other than *C. jejuni* subsp. *jejuni.* Furthermore, when directly compared to those of the recognised pathogen, *C. jejuni* subsp. *jejuni*, clinical associations of emerging campylobacteria were strikingly similar (Lastovica 2006). Among paediatric clinical isolates, the percentages of *C. concisus, C. upsaliensis*, and *C. jejuni* subsp. *doylei* were similar to those of *C. jejuni* subsp. *jejuni* for patients with diarrhoea (80 to 100%), vomiting (3 to 12%) and fever (4 to 9%) (Lastovica 2006). The emerging pathogen *C. upsaliensis* was isolated almost equally as often as the recognised pathogen *C. jejuni* subsp. *jejuni*. Another emerging *Campylobacter*, the infrequently isolated *C. jejuni* subsp. *doylei*, was three times more likely to be isolated from blood than stool culture.

Campylobacter species found in the oral cavity have rarely been reported to cause extraoral infections. However, Han *et al.* (2005) recently reported three cases of extraoral abscesses caused by oral *Campylobacter* species. The spread was most likely by means of the lympohaematogenous system. Significantly, these *Campylobacter* species were all isolated anaerobically and not by the conventional microaerobic procedures usually used for *Campylobacter* isolation. These organisms were identified by sequencing analysis of the 16S rRNA gene. The cases included a breast abscess caused by *Campylobacter rectus* in a lymphoma patient, a liver abscess caused by *C. curvus* in an ovarian cancer patient, and a post-obstructive bronchial abscess caused by *C. curvus* in a lung cancer patient. All three patients were treated with antibiotics with complete resolution of the lesions (Han *et al.* 2005).

Table 1. Summary of Disease Associations of *Campylobacteraceae* species. Adapted from Miller and Mandrell (2005) and Lastovica and Skirrow (2000).

Species or subspecies	Human Disease Association
C. coli	Enteritis
C. concisus	Enteritis, periodontal disease, bacteraemia
C. curvus	Enteritis
C. fetus subsp. *fetus*	Enteritis, bacteraemia, endocarditis, chorioamnionitis, thrombophlebitis, osteomyelitis, pleuropericarditis, meningitis/brain abscess, cellulitis, prosthetic hip joint infection, aneurism infection, abortion
C. fetus subsp. *venerealis*	Septicaemia (rare)
C. gracilis	Enteritis, periodontal disease, head/neck infections
C. helveticus	None/unknown
C. hominis	None/unknown
C. hyointestinalis	Enteritis, bacteraemia, proctitis
C. hyointestinalis subsp. lawsoni	None/unknown
C. jejuni	Enteritis, GBS, Miller Fisher syndrome, polyneuropathies, reactive arthritis, myocarditis
C. jejuni subsp. *doylei*	Enteritis, septicaemia
C. lanienae	None/unknown
C. lari	Enteritis, septicaemia/bacteraemia, septic shock, purulent pleurisy, urinary tract infection, reactive arthritis
C. mucosalis	Hemorrhagic colitis, septicaemia
C. rectus	Periodontal disease, hepatolithiasis
C. showae	Periodontal disease, hepatolithiasis
C. sputorum	Enteritis, septicaemia, axillary abcess
C. upsaliensis	Enteritis, bacteraemia, prosthetic knee infection
A. butzleri	Enteritis, bacteraemia
A. cryaerophilus	Enteritis, bacteraemia

1.7. PHYSIOLOGY, METABOLISM AND BIOCHEMISTRY OF *CAMPYLOBACTER*

Members of the genus *Campylobacter* are chemoorganotrophs, unable to ferment or oxidise carbohydrates which is likely to be due to the lack of phosphofructokinase that is necessary for glycolysis (Fouts *et al.* 2005). They obtain energy through the respiratory chain, from the metabolism of amino acids, and from the metabolism of intermediates of the tricarboxylic acid cycle (Kelly 2005). Menaquinone and its derivatives alkylated at position 6 are the quinones involved in the respiration process. Additionally, cytochromes *b*, *c* and *d* have been found in *C. jejuni* (Sellars *et al.* 2002). The biochemical signatures of campylobacters in many cases have been used to differentiate between species but this has proven problematic since many species contain atypical strains or have only one differentiating feature. Apart from certain atypical strains of *C. lari*, the thermotolerant campylobacters hydrolyse neither urea nor gelatine. They possess strong oxidase activity, but lack lipase (On *et al.* 1996). Several species —*C. jejuni, C. coli* and *C. lari*— produce catalase. They reduce nitrates but not nitrites. Hippurate hydrolysis has traditionally been the most reliable test to distinguish between *C. jejuni* (hippurate-positive) and *C. coli* (hippurate-negative), two of the species with high clinical incidence (Morris *et al.* 1985).

Recent publication of a number of *Campylobacter* species' genome sequences (Parkhill *et al.* 2000; Fouts *et al.* 2005) has revealed many clues as to metabolism and physiology of *Campylobacter* species, but many aspects of metabolism remain largely unknown since the sources of carbon and energy employed by the organism have not yet been identified fully (Mendz *et al.* 1997). The metabolic pathways are highly complex and branched with the ability to utilise a variety of alternative electron acceptors such as fumarate, and donors such as hydrogen and formate (Figure 6). It has been shown (Kelly 2001) that *C. jejuni* is emerging as a versatile and highly metabolically active pathogen, with a complete citric acid cycle and a complex and highly branched respiratory chain which allows both aerobic and anaerobic respiration. A study by Mendz *et al.* (1997) provided information about the energy metabolism of *Campylobacter* species, showing that conversion of pyruvate to acetate and formate suggested a mixed acid fermentation pathway is operational in these bacteria.

Most *Campylobacter* species are true microaerophiles which exhibit oxygen-dependent growth under microaerobic conditions, and fail to grow or grow poorly at the oxygen levels in air. One theory is that for microaerophiles and more specifically *C. jejuni*, oxygen is required for the enzyme ribonucleotide reductase (RNR), which is thought

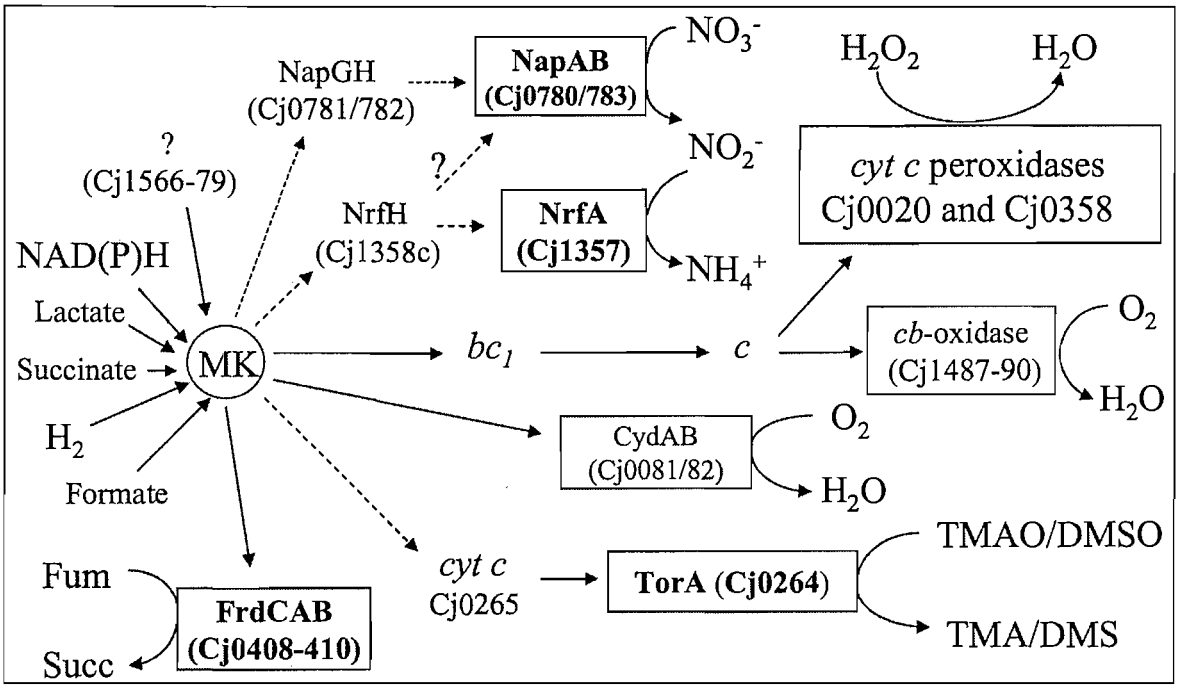


Figure 5. Predicted electron transport pathways in *C. jejuni*. A variety of potential electron donors (not all shown) feed electrons into the menaquinone pool (MK). Taken from Sellars *et al.* (2002).

occurs) (Kelly 2001). However this cannot be the case for the so called 'anaerobes', which can grow in the absence of oxygen, suggesting that alternative mechanisms must exist in these species.

The existence of a mixed-acid fermentation pathway in *Campylobacter* may be a manifestation of the metabolic adaptation of the bacterium to its ecological niche. It was postulated that *Campylobacter* has the metabolic machinery to utilise pyruvate effectively and is part of an active intermediary metabolism and under microaerophilic conditions is designed to optimise the use of energy, maintain proper redox balance and provide building blocks for biosynthesis (Mendz *et al.* 1997). Previous studies (Smith *et al.* 1999) have shown that many *Campylobacter* species show fumarase and fumarate reductase activity (two key enzymes in the TCA cycle). It is thought that the reduction of fumarate serves many biosynthetic and bioenergetic roles, for example succinate (reduced from fumarate) is required for tetrapyrrole synthesis and biosynthesis of uracil and protoporphyrin and the free energy change is used for phosphorylation via generation of proton gradients (Figure 5) (Smith *et al.* 1999). Fumarate respiration is thought to be the most widespread type of anaerobic respiration and fumarate reductase is a key component of this system (Kroger *et al.* 1992). The study by Smith *et al.* (1999) showed that fumarate reductase activity was present

in all *Campylobacter* species tested in microaerobic conditions and in *C. fetus* in anaerobic conditions; it was therefore concluded that fumarate reductase plays a fundamental role in these microaerophiles. The power required for reduction of fumarate to succinate can be supplied by a number of proton donors through their corresponding hydrogenases. The order of potential donors with increasing energy change upon fumarate reduction is:- hydrogen>formate>NADH>lactate>glycerol-3-phosphate>maltose. Electrons are passed from dehydrogenases to menaquinones, which in turn are oxidised by fumarate reductase, with fumarate as the terminal electron acceptor. The hydrogen donor depends on the species and the growth conditions. Hydrogen and formate act as electron donors for fumarate reductase in *Escherichia coli* and *Wollinella succinogenes* and the presence of hydrogen has been shown to be essential for the growth of many *Campylobacter* species (Lastovica and Skirrow 2000).

Campylobacter species and *Helicobacter pylori* have many metabolic similarities, requiring oxygen for growth and having terminal cytochrome oxidase activity in respiratory chains. Both also possess mixed acid fermentation pathways with pyruvate, oxidoreductase, hydrogenase, menaquinones and are able to utilise fumarate as a terminal electron acceptor via fumarate reductase. However the main difference is the presence of formate dehydrogenase in *Campylobacter*. Fumarate reduction seems to play an important role in these bacteria even under high oxygen tensions. Studies have suggested that *Campylobacter* species (unable to utilise sugars and glycolytic intermediates) may obtain energy through electron transport coupled phosphorylation with a chain including fumarate reductase and formate dehydrogenase, therefore providing more evidence for presence of both anaerobic and aerobic metabolic pathways (Smith *et al.* 1999).

1.7.1. *Campylobacter* Survival Mechanisms and Stress Responses

Previous findings (Park 2002) have shown that in comparison to other enteric pathogens, *Campylobacter* are extremely sensitive to many environmental factors, relating to there metabolism. For example they are sensitive to environmental levels of oxygen and it is thought that this is because although there are certain enzymes present to counteract oxygen derivatives e.g. superoxide dismutase, there is a lack of regulators of these oxidative defence enzymes that are found in other bacteria. However *Campylobacter* species are able to survive various different stresses which enable them to survive in the environment and colonise the gut, and recent studies are starting to reveal novel stress response mechanisms restricted to *Campylobacter* species (Murphy *et al.* 2003).

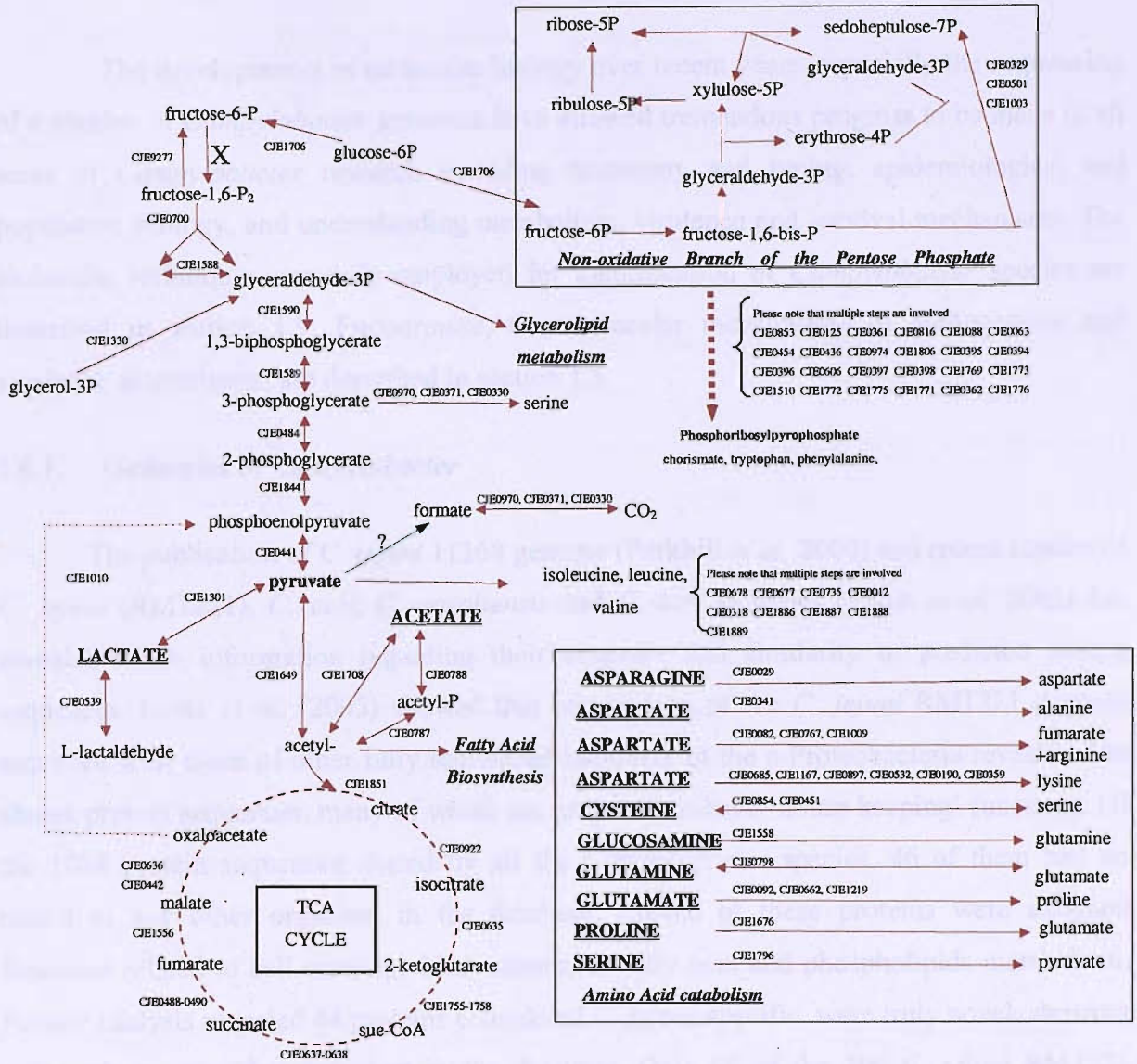


Figure 6. Main Pathways for Metabolism Derived from an Analysis of the Genome Sequences of Five *Campylobacter* Species. The tricarboxylic (TCA) cycle has major variations based on comparative analysis of five strains including *C. jejuni* (NCTC11168 and RM1221) *C. lari* RM2100, *C. upsaliensis* RM3195, and *C. coli* RM2228 (Adapted from Fouts *et al.* (2005). X = absence of phosphofructokinase required for glycolysis. Abbreviations denoted with CJ (*C. jejuni*) are the allocated names of genes identified for particular enzymes during the study.

1.8. MOLECULAR BIOLOGY OF *CAMPYLOBACTER* AND RELATED ORGANISMS

The developments in molecular biology over recent years, especially the sequencing of a number of *Campylobacter* genomes have allowed tremendous progress to be made in all areas of *Campylobacter* research including taxonomy and typing, epidemiological and population ecology, and understanding metabolism, virulence and survival mechanisms. The molecular techniques currently employed for identification of *Campylobacter* species are described in section 1.9. Furthermore, the molecular mechanisms of pathogenesis and virulence determinants are described in section 1.5.

1.8.1. Genomics of *Campylobacter*

The publication of *C. jejuni* 11168 genome (Parkhill *et al.* 2000) and recent closure of *C. jejuni* (RM1221), *C. coli*, *C. upsaliensis* and *C. lari* genomes (Fouts *et al.* 2005) has revealed much information regarding their structure and similarity of predicted coding sequences. Fouts *et al.* (2005) showed that comparison of the *C. jejuni* RM1221 genome sequence with those of other fully sequenced members of the ε-Proteobacteria revealed 540 shared protein sequences, many of which are proposed to have 'house keeping' functions. Of the 1084 protein sequences shared by all the *Campylobacter* species, 46 of them had no match to any other organism in the database. Eleven of these proteins were assigned functions related to cell envelope biosynthesis, or fatty acid and phospholipids metabolism. Further analysis revealed 44 proteins considered *C. jejuni*-specific, were truly novel, showing no match to any other organism in the database. Only 95 of the 300 *C. jejuni* RM1221 specific protein sequences were not in phage or genomic island regions. *C. jejuni* NCTC1168, not surprisingly, had the highest average protein percent identity (1,468 proteins averaging 98.41% identity) with *C. jejuni* RM1221 proteins. *C. coli* RM2228 was second, with 1,399 proteins averaging 85.81% identity. Surprisingly, *C. upsaliensis* RM3195 had the third highest average protein percent identity with *C. jejuni* RM1221 (1,261 proteins; 74.72% average identity), followed by *C. lari* RM2100 with 1,251 proteins having 68.91% average identity. Strains of *Wollinella succinogenes* and strains of *Helicobacter* had around 54 and 52% average protein identity with *C. jejuni* RM1221. A phylogenetic tree was created, based on these protein identities and compared to that of a tree based on 16S rRNA (Vandamme 2000). Findings showed that *C. jejuni* RM1221 was more closely related to *C. coli* RM2228 than to the other *C. jejuni* strain, NCTC11168 based on the 16S RNA tree. However, the tree based on protein identities showed that the two *C. jejuni* strains were more closely related to

each other than either is to *C. coli* RM2228. It was concluded that the protein tree was more accurate than the rRNA tree because the 16S rRNA does not have enough variation to resolve close relationships within this group of related organisms (Fouts *et al.* 2005).

Other important information revealed by the genome sequences of the five strains of *Campylobacter* in the study of Fouts *et al.* (2005) includes major structural differences associated with the insertion of phage and plasmid-like genomic islands, as well as major variations in the lipopolysaccharide complex. Poly G tracts are longer, greater in number and show greater variability in *C. upsaliensis* than in the other three species. It also showed many genes involved in host colonisation (*racR/S, cadF, cdt*, and *flagellin* genes) are conserved across species but variations that appear to be species specific are evident for a lipopolysaccharide locus, a capsular polysaccharide locus, and a novel *Campylobacter* putative *licABCD* virulence locus. The strains also vary in their metabolic profiles, as well as their resistance profiles to a range of antibiotics.

Genome mapping studies (Stoeva and Ward 2006) of *A. butzleri* have shown that the size of the genome was estimated at 2.57 Mb, 0.9 Mb larger than the genome of *C. jejuni*. A gene homologous to *pglF*, involved in bacterial glycosylation in *C. jejuni* was also located on the genome map. In *C. jejuni* and *Wollinella succinogenes*, most glycosylation genes are clustered together and the gene order is similar. However, no analogous organization was found in the region sequenced in *A. butzleri* (Stoeva and Ward 2006).

1.8.1.1. Phase Variation

Based on whole-genome sequence information from the *C. jejuni* genome (Parkhill *et al.* 2000), it was revealed that short nucleotide homopolymeric runs of variable lengths are commonly found in genes involved in the biosynthesis of *C. jejuni* carbohydrates (e.g. glycosyltransferase). Homopolymeric tracts are the simplest motifs of the short sequence DNA repeats that are proposed to be involved in a mechanism resulting in high frequency on-off switching of the associated genes in different strains. Gene inactivation by the deletion or insertion of a single base is also possible without phase variation. An example is glycosyltransferase which can be found as an inactive version, due to its ORF having 7 bases rather than 8, resulting in a truncated enzyme which is reflected in the LOS outer core structure. *Campylobacter* are thought to lack many of the adaptive responses common in other bacteria and it is possible that phase variation is an alternative strategy for survival. *C. jejuni* is an unusually diverse bacterial species as evidenced by variation in virulence, differences in the genetic content of strains and variability in tolerance to the environment (Park 2005). In a study by Park *et al.* (2005), when various strains of *C. jejuni* and *C. coli*

were exposed to a number of environmental stresses, only a small fraction of the original population were able to survive to grow and form colonies, and if these were sub-cultured and re-exposed to the same stress, a higher proportion survived. It is therefore thought that the increase in resistance was due to the survival of a subpopulation of cells having a particular genetic composition that increases their resistance to a particular stress (Park 2005). It is also thought that this may be the mechanism for virulence in some cases. Within any population of *C. jejuni* cells, only a small subset produces a phase variable capsule which promotes serum resistance, invasion and virulence (Bacon *et al.* 2001). The *C. jejuni* genome also contains numerous pseudogenes containing one frameshift mutation at potentially polymorphic regions (Parkhill *et al.* 2000).

1.9. CULTURE AND ISOLATION

A large range of media have evolved to isolate *Campylobacter* from various clinical, environmental and food samples. However these media have mainly been directed at isolation of species such a *C. coli* and *C. jejuni*. It is thought that other new emerging species including *C. upsaliensis* and *C. concisus* are sensitive to a number of antibiotics contained in these media. Corry *et al.* (1995a; 2002), in an extensive review of the literature, concluded that there were no generally accepted and optimised methods for isolation of *Campylobacter* species from faeces, food and water, which is also the case to date. Furthermore, a review by On (1996) similarly stated that significant improvements in the methods for detecting and identifying *Campylobacter* species are required.

The media used for isolation of campylobacters from food and water have largely derived from those developed for the isolation of campylobacters from faeces. The chosen medium must comply with the criteria that allow the isolation of campylobacters from complex samples, such as faeces or raw meat, where the presence of a high microbial load of other bacteria might also occur, as well as the recovery of injured *Campylobacter* cells from processed food. Many previous studies have clearly shown that use of different media will bias the isolation of *Campylobacter* species (Corry *et al.* 1995a). It must be noted that the approaches to isolation of *Campylobacter* species from man will be different to those from animal sources. In cases of diarrhoea in man, the numbers of *Campylobacter* species in the sample will be very high, whereas in veterinary laboratories it is likely that there will be a much higher ratio of non-*Campylobacter* isolates and so the isolation procedures must be altered accordingly. It is thought that in environmental and food samples campylobacters may be sub lethally injured with damage to their membranes, resulting in increased sensitivity to antibiotics contained in media, temperature stress and atmospheric oxygen

(Corry *et al.* 1995a). Studies have shown that growth of campylobacters is substantially reduced when plates of nutrient medium are stored in the presence of light and, in particular, air (Hoffman *et al.* 1979). It is known that species other than *C. jejuni* have different antibiotic susceptibilities and current media may not always be optimal for them (Lastovica and Skirrow 2000; Lastovica 2006). For this reason the true incidence of infection caused by different *Campylobacter* species may be understated. The results of the use of such suboptimal isolation methods are that the isolates which 'survive' the isolation procedure will be those which: (i) are able to 'out compete' the rest of the bacteria in the sample, i.e. able to grow faster; (ii) are resistant to the antibiotics in the isolation media; and (iii) are randomly selected by the laboratory technician as being a 'typical' *Campylobacter*. It is therefore clear that such a procedure is intrinsically biased and will mean that only species resistant to the antibiotics used in the media will be isolated. This introduces real doubt that the bacteria isolated are truly representative of those initially found in the sample. It must also be noted that samples, whether they are from poultry or man, are commonly known to support multiple species of *Campylobacter* and indeed multiple strains of the same species (Silley 2003). It is therefore not always possible to isolate a single strain from a human faecal sample and attribute it as the disease causing strain, as only the most elaborate identification techniques will be able to differentiate these.

1.9.1. Development of *Campylobacter* Isolation Media

A great number of culture media have evolved in response to the need to optimise performance. Because of the predominance of *C. jejuni* in enteric infection most developments in media have been directed towards detection of this pathogen. *C. jejuni* was first isolated in the early 1970's when J.P. Butzler and co-workers applied a filtration method, thereby using the small cellular size and vigorous motility of campylobacters to selectively isolate them from stools of humans with diarrhoea (Butzler *et al.* 1973). The main breakthrough, however, was provided a few years later by Skirrow, who described a selective supplement for the isolation of *C. jejuni* and *C. coli* strains from clinical samples. This comprised of a mixture of vancomycin (10 mg/l), polymyxin B (1250 IU per litre), and trimethoprim (5 mg/l), that was added to a basal medium with blood (Skirrow 1977). This simple isolation procedure thus enabled routine diagnostic microbiology laboratories to isolate campylobacters from faeces and to evaluate their clinical role. Blaser *et al. (Blaser et al. 1979)* developed the Campy-BAP medium, a selective medium now widely employed in clinical laboratories. Another selective medium is Campylosel (BioMérieux), which comprises cefoperazone, vancomycin and amphotericin B as selective agents.

Preston selective agar was specifically formulated for isolation of *Campylobacter* species from all types of specimens (environmental, avian, animal and faecal samples), in which these microorganisms may be outgrown by competing microorganisms (Bolton and Robertson 1982). Since blood is an expensive component and its quality is not homogeneous, Bolton *et al.* replaced it with serum (Bolton *et al.* 1983).

Karmali *et al.* (1986) developed a charcoal based selective medium (CSM) (including vancomycin, cycloheximide and cefoperazone) for the selective isolation of *Campylobacter*. Walmsley and Karmali (1989) succeeded in the direct isolation of *C. upsaliensis* on both CSM and Skirrow medium without preliminary enrichment or filtration steps.

mCCDA (modified charcoal cefoperazone deoxycholate agar) is a medium which has been routinely used for the isolation of *C. jejuni*, *C. coli* and *C. lari*. The selective supplement includes cefoperazone (32 mg per litre) and amphotericin B (10 mg per litre). Modified CCDA medium is based on the original formulation described by Bolton (Bolton *et al.* 1984b) which was developed to replace blood with charcoal, ferrous sulphate and sodium pyruvate. Improved selectivity was achieved when cephazolin in the original formulation was replaced by cefoperazone as the selective agent. Amphotericin B is included in the formula of the modified version to suppress the growth of yeast and fungal contaminants that may occur at 37°C. Aspinall *et al.* (1993b) developed a blood-free selective medium (CAT) as an alternative to CCDA with reduced levels of cefoperazone. This medium contains cefoperazone, amphotericin B and teicoplanin as selective agents, and has proved to be suitable for the recovery of a number of *Campylobacter* species including *C. upsaliensis*. To date, use of a filtration method using a 0.6 μm membrane onto antibiotic-free blood agar combined with incubation in a hydrogen enrciched microaerobic atmsophere has been shown to be the optimal method for the isolation of emerging *Campylobacteraceae* from clinical faecal samples (Lastovica 2006).

1.9.2. Evaluation of Media Currently Available for *Campylobacter* Isolation

In comparative studies of the selective media including Skirrow, Butzler, Blaser, Campy-BAP and Preston, the Preston medium was found to give the maximum isolation rate of *Campylobacter* species from all types of clinical specimens tested, and also to be the most selective (Post 1998).

In a study of healthy puppies and kittens for carriage of *Campylobacter* species, modified CCDA medium was found to be a suitable medium and more productive for *C. upsaliensis* in this application than CAT medium (Burnens 1992). Another study with isolates from Mexican paediatric patients concluded that overall, CCDA was the superior

medium compared with CampyBAP for isolating *Campylobacter* species (Arzate Barbosa *et al.* 1999) . The use of *Campylobacter* Blood-Free Medium (CCDA) is specified by the U.K. Ministry of Agriculture, Fisheries and Food (MAFF) (now DEFRA) in a validated method for isolation of *Campylobacter* from foods.

Another comparative study has shown that the least inhibitory media were Skirrow medium and CCDA. The study indicated that selective media used to detect *Campylobacter* species could select against the isolation of strains of *C. coli* (Ng *et al.* 1988). The effectiveness of Preston medium (Bolton and Robertson 1982) for the isolation of *Campylobacter* from faeces, water and from other ecosystems has been reported, however, several authors have described possible failures in the isolation of certain strains of *C. coli* due to sensitivity to polymyxin B, a component of the Preston medium (Lastovica 2000).

Tryptose Blood Agar (TBA) used in combination with selection by filtration is widely used for the isolation of fastidious *Campylobacter* species from paediatric stool samples (Lastovica 2006). It is this medium, supplemented with 7-10% defibrinated horse blood which is used as part of the Cape Town isolation protocol. To date the Cape Town protocol remains the only existing method which has isolated all types of *Campylobacter* species from blood and faecal samples. This method utilises selective filtration via a 0.6 μm membrane onto TBA supplemented with 7-10% blood and subsequent incubation in a hydrogen enriched atmosphere (Lastovica and Le Roux 2000; Lastovica and Le Roux 2003). Membrane-filtration techniques were initially designed for the isolation of *Vibrio fetus* (*C. fetus*) from cattle and later from human beings. Unlike many bacteria, campylobacters can usually pass through 0.45 μm and 0.65 μm filters. Several membrane pore sizes, culture media and isolation techniques have been tested (Bolton 1988; Aspinall 1996; Albert 1992).

1.9.3. Enrichment and Broth Media

It is sometimes advisable to consider an enrichment procedure for the isolation of *Campylobacter* species, especially if cells are suspected to be present in low number or are likely to be stressed. In keeping with techniques developed for the isolation of other pathogens, enrichment and pre-enrichment media have been used. The manner in which liquid enrichment media are used has been modified for food samples to avoid damage on sublethally damaged cells. This is done by a preliminary period of incubation at reduced temperature and sometimes by delayed addition of antibiotics.

Bolton *et al.* (1982) developed a "most probable number" method, based on Preston enrichment broth and capable of detecting *Campylobacter* at concentrations as low as 10 CFU/100 ml. More recently, a new blood-free enrichment broth (BFEB) has been used to

isolate *C. jejuni* strains under aerobic conditions (Tran 1998). A study using routine methods to recover *Campylobacter* species from turkeys in Denmark (Borck *et al.* 2002) concluded that there was a need for the development of specific enrichment protocols for specific samples as recoveries of campylobacters from turkey meat and neck skin differed depending on the growth medium used (Borck *et al.* 2002).

Campylobacter Enrichment broth (CEB) contains nutrients to aid resuscitation of sublethally damaged cells of *Campylobacter*. It contains FBP (ferrous sulphate, sodium metabislphate and sodium pyruvate) and is supplemented with blood to counteract the effects of toxic oxygen species. It is often supplemented with cefoperazone, vancomycin, trimethoprim and natamycin to inhibit growth of Gram positive and Gram negative organisms (Bridson 1998).

It must be noted that when enrichment techniques are used they will favour the isolation of faster growing strains (over slower-growing strains) and so may not be a true representation of the microbial population in the selected sample, whereas a direct plating procedure would provide a better representation of the population (Dickins *et al.* 2002).

1.9.4. Media Supplements

1.9.4.1. Antibiotic Selective Supplements

Antimicrobials in selective media developed for campylobacters have been chosen on the basis of those to which test strains were resistant and those most effective in inhibiting competitive flora. At least 17 different single antimicrobials have been used (cephalothin, cephazolin, cefsulodin, cephalexin, cefoperazone, trimethoprim, polymyxin B, colistin, vancomycin, teicoplanin, rifampicin, novobiocin, bacitracin, cycloheximide, actidione, amphotericin and nystatin) either singly or more often in combination (Corry *et al.* 1995a). As most reports in the literature do not state what methods were used, it is not possible to quantify any relationship between antibiotics used in the isolation media and susceptibility data (Silley 2003). Furthermore, it is likely that the strains used for susceptibility testing against antimicrobials used for inclusion in isolation media formulations would have been those isolated using antimicrobial-containing selective media in the first place and so the possibility of missing strains would have been perpetuated (Corry *et al.* 1995a). Although antibiotics have been included in isolation media, based on *C. jejuni* and *C. coli* being resistant, there is now evidence that some strains of *C. coli* and even a few strains of *C. jejuni* are likely to have been missed because of their sensitivity to cephalothin (although no longer used) and other antibiotics used in selective media (Brooks *et al.* 1986; Ng *et al.* 1988); (Walmsley and Karmali 1989; Goossens *et al.* 1990a; Bourke *et al.* 1998). Cephalothin,

colistin, and polymyxin B, which are present in some selective-medium formulations, may be inhibitory to some strains of *C. jejuni* and *C. coli* (Goossens *et al.* 1986; Ng *et al.* 1988) and are inhibitory to *C. fetus*, *C. doylei*, *C. upsaliensis* and *A butzleri*. Lawson *et al.* (1997) has also shown that that some strains of *C. upsaliensis* and *C. helveticus* were susceptible to levels of cefoperazone incorporated into selective media and consequently failed to grow or were detected with reduced sensitivity in the presence of this antibiotic. Emerging species including *C. upsaliensis*, *C. concisus*, *C. curvus*, *C. rectus* and *C. sputorum* biovar *sputorum* only began to be isolated from clinical faecal samples at a hospital in South Africa when isolation using the Cape Town protocol was introduced, which utilises selection by filtration rather than use of antibiotics (Lastovica and Le Roux 2000).

1.9.4.2. Blood and Other Media Supplements

Besides a variety of selective agents incorporated into the media, almost all of which are antimicrobials, media for campylobacters usually contain ingredients to neutralise the toxic effects of substances formed in the presence of oxygen and light (Corry *et al.* 1995a; 2002). The addition of certain agents able to inhibit the production of hydrogen peroxide and superoxide anions that might affect the growth of campylobacters has been considered (Barros-Velazquez *et al.* 1999). The incorporation of aerotolerant supplements such as FBP (ferrous sulphate, sodium metabisulphate and sodium pyruvate) or blood into media reduces this toxic effect but does not completely eliminate it (Corry *et al.* 1995a). Horse and sheep blood are the most widely used animal blood products in culture media. A study by Moran and Upton (1987) with *C. jejuni* demonstrated that the addition of blood or a supplement containing FBP minimised conversion of rods to coccoid forms in cultures and that it was the presence of hydrogen peroxide and its dissociation products in bacterial suspensions that increased conversion to coccoid forms and that a addition of active superoxide dismutase also a reduced this effect. Many *Campylobacter* species are capable of haemolytic activity (the lysis of haemoglobin to release iron), which can usually be readily seen as loss of red colour on blood agar plates (Tay *et al.* 1995).

1.9.5. Incubation Conditions

Species of the genus *Campylobacter* have traditionally been thought of as strictly microaerophilic; i.e. they exhibit oxygen-dependent growth, oxygen being the final electron acceptor in the respiration process, but they do not tolerate atmospheric oxygen concentrations (21 % v/v) (Barros-Velazquez *et al.* 1999). Recent work has shown that

optimal incubation atmospheres will vary according to species although no systematic investigation of the effect of atmosphere during incubation of media has been carried out to date (Silley 2003). To grow, *Campylobacter* species are thought to require oxygen concentrations ranging from 5%-10% and carbon dioxide ranging from 1–15% (Annable, 1998). Certain species are also thought to require an increased concentration of hydrogen for isolation. These include *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis* (Lastovica 2000). Although *C. jejuni* and *C. coli* do not require hydrogen for growth, a hydrogen-enriched atmosphere has been shown to strongly support growth of these species; it has been suggested that a gas mixture of 6% oxygen, 6% carbon dioxide and 3% hydrogen is sufficient for isolating these species (Nachamkin and Blaser 2000). Some species are also thought to strongly favour anaerobic conditions for growth, and therefore have been deemed the 'anaerobic' *Campylobacter* species. These include *C. gracilis*, *C. hominis*, *C. rectus* and *C. showae* and are often isolated from oral cavities. A number of these species, including *C. concisus, C. showae, C. rectus*, and *C. curvus*, have also been shown to have an essential growth requirement for hydrogen or formate. Hydrogen-dependent organisms are extremely difficult, if not impossible, to recover by the standard culture techniques currently employed in most routine diagnostic laboratories (Lastovica and Skirrow 2000).

The temperature of incubation is also critical. The thermotolerant campylobacters (*C. jejuni, C. coli, C. lari* and *C. upsaliensis*) grow well at 42–43°C. This temperature avoids the growth of a significantly broad spectrum of background microorganisms. Although the optimum temperature is 42–43°C, the above four species also grow at 37°C but not below 30°C or above 47°C (Barros-Velazquez *et al.* 1999). However, many non-thermophilic species will not grow at 42°C and so for isolation of all *Campylobacter* species a lower temperature of 37 °C is advisable (Lastovica and Skirrow 2000). Thermal treatment for more than 15 min at 60°C or for 30 min at 57.5°C destroys these microorganisms (Barros-Velazquez *et al.* 1999).

Several studies have reported that the composition of the medium and even of the commercial brand of the components used in the formulation of the media will affect the oxygen-tolerance of campylobacters and therefore have an effect on the type of atmosphere they can grow well in (Barros-Velazquez *et al.* 1999).

Campylobacter species are among many organisms (*Haemophilus*, *Neisseria*, *Brucella*) that are dependent on the fixation of carbon dioxide. These bacteria grow better if they are grown in an atmosphere with increased carbon dioxide, and require levels of around 5-10% for the initiation of growth, particularly on solid media (Moat 1995). Also, a variety of intermediates of glycolysis and the TCA cycle are siphoned-off these pathways to serve as

biosynthetic precursors. Consequently, carbon dioxide is used to replenish intermediates in the TCA cycle (anapleurotic function) through the action of pyruvate carboxylase, phosphoenolpyruvate carboxylase, or malic enzyme (Moat 1995).

Campylobacter species are known to require low levels of oxygen and the majority of species cannot grow anaerobically. One of the reasons they require oxygen is to fuel energy-yielding metabolic processes. It is thought that *Campylobacter* possess cytochromes and cytochrome oxidase, which are involved in the process of oxidative phosphorylation. Oxygen serves as the terminal electron acceptor in the sequence and water is one of the resultant products of respiration. The problem with this reaction is that some of the oxidation-reduction enzymes react with molecular oxygen to give rise to superoxide (O_2:), hydroxyl radicals (OH·), and hydrogen peroxide (H_2O_2), all of which are extremely toxic to many bacteria and other organisms (Moat 1995), and this is such the case for campylobacters where the oxygen toxicity threshold levels are extremely low. In many bacteria enzymes exist to dissipate these toxic molecules such as superoxide dismutase and catalase. It is thought that this is why blood is such a good component in culture media as it contains catalase and peroxidase activity.

A microaerobic atmosphere suitable for *Campylobacter* can be readily achieved by several methods including using commercial gas generating systems. The most widely used commercially available atmosphere generating kits are in the form of gas producing sachets (such as CampyGen (Oxoid Ltd) or AnaeroGen (Oxoid Ltd) used in conjunction with sealed plastic containers or anaerobic gas jars. However these packs are only directed at isolation of *C. coli* and *C. jejuni* species and these conditions are thought to be less then adequate for isolation of some emerging *Campylobacter* species. Another useful method is the evacuation-replacement technique, in which air is removed from the incubating jar and replaced by a gas mixture containing (v/v) 15% carbon dioxide and 80% nitrogen, the oxygen concentration being reduced to 5% (Barros-Velazquez *et al.* 1999). Candle jars have even been used to successfully isolate *Campylobacter* species; however the concentration of oxygen generated in candle jars is sub-optimal and is not recommended for routine use.

The gaseous environment used for incubation in the Cape Town protocol (using large, robust mayonnaise jars with the Oxoid BR38 anaerobic gas generating kit without catalyst) is un-defined, however dangerous levels of hydrogen and pressure are thought to be produced. Despite this, this method remains the gold standard for recovery of emerging *Campylobacter* species and is the only method to date in which all *Campylobacter* species have been isolated. Thus far, very little work has been carried out to investigate and quantify the optimal gaseous environment for incubation, especially for some of the emerging species.

1.9.6. Culture and Isolation of Non-*jejuni/coli* Species

To date, the majority of significant isolations of emerging campylobacters have used alternative techniques to highly selective media, such as filtration methods or non-cultural molecular based methods. The most highly documented example is the Cape Town isolation protocol which has been extensively used to isolate numerous types *Campylobacteraceae* from clinical samples of paediatric patients in South Africa (Lastovica 2006). The Cape Town isolation protocol was initially developed by Al Lastovica at the Red Cross Children's hospital in Cape Town for examination of stool samples from infected South African patients as a cheaper alternative to selective media. It involves filtration of liquid stools through a 0.60 μm pore-size mixed ester membrane onto an antibiotic-free blood (10%) agar plate. It is thought that the bacteria go through the membrane pores either by actively swimming through or by gravity. The filter is left on the plate for approximately 15 min, so long enough for the smaller *Campylobacter* to grow through but not so long as to let the larger competing micro organisms through. Plates are subsequently incubated at 37°C in a hydrogen-enhanced microaerobic atmosphere for 48-72 h, in mayonnaise jars with an anaerobic gas sachet without a catalyst, and subsequent speciation is carried out by extensive phenotypic and biochemical tests (Lastovica and Le Roux 2000; Lastovica and Le Roux 2001; Lastovica and Le Roux 2003). High numbers of *C. concisus* and *C. upsaliensis* have been recovered in from clinical samples in numbers comparable to *C. jejuni* and *C. coli* using the Cape Town isolation protocol. So far the Cape Town protocol has only been developed to yield isolates from clinical (blood and stool) samples where high numbers of *Campylobacter* will be present and the method is not quantitative.

A study by Goossens *et al.* (1990) for the isolation of emerging *Campylobacter* species from 15,185 stool specimens using filtration onto antibiotic-free blood agar showed that *C. upsaliensis* was isolated from 99 patients, *C. jejuni* subsp. *doylei* from 4, and *C. hyointestinalis* from 2 patients. *C. upsaliensis* was the only organism isolated from 83 patients and clinical information available for 77 out of these 83 patients showed that 92% of the patients had diarrhoea. This demonstrates that emerging species can be isolated if alternative methods are used. It is likely that *C. upsaliensis* is a severely undereported pathogen and may be an unrecognised and frequent cause of diarrhoea in man (Goossens *et al.* 1990b). Other studies applying filtration and less-selective media have shown that *A. butzleri* was the fourth most common *Campy*-like organism isolated from >67,000 stool specimens from patients with persistent, watery diarrhoea (Vandenberg *et al.* 2004) and that

Arcobacter species are inhibited by a number of antibiotics contained in *Campylobacter* selective isolation media (Houf *et al.* 2001b).

Very few data exist on the isolation of the 'anaerobic' *Campylobacter* species, but significant isolations have occurred if anaerobic incubation conditions together with filtration methods are used, although generally most significant detection has occurred when molecular methods have been applied (Johnson *et al.* 1985; Siqueira and Rocas 2003). *C. curvus* was isolated from more than 20 patients with Brainerd's diarrhoea when a filtration method and prolonged incubation were utilised (Abbott *et al.* 2005).

A number of studies (Albert 1992; Corry *et al.* 1995a; 2002; Modolo 2000) have reported the use of a combination of both a filtration method onto antibiotic-free blood agar and use of a selective isolation medium for optimal isolation.

1.9.7. The Current Dilemma and Problems with Under-reporting

Currently, many studies and clinical laboratories have used media and incubation regimes with the primary aim of isolating *C. jejuni* and *C. coli*, due to their perceived status as major pathogens. Unfortunately, antibiotic-containing selective agar is used for primary isolation. The WHO (World Health Organisation) has now recognised that this may be biased for *C. jejuni* and is known not to isolate these emerging species. Although the existance of new *Campylobacteraceae* was demonstrated over 10 years ago, very few substantial studies have been published to determine the true prevalence of these new species in the clinical environment. *Campylobacter* epidemiology is poorly understood, partly because there is a lack of generally accepted and widely applied typing schemes. However, recent studies in South Africa, Australia, Belgium and Denmark have shown that new emerging species such as *C. upsaliensis* and *C. concisus* can be isolated at high rates and are just as important as *C. jejuni* and *C. coli* due to alternative techniques being used (Vandenburg 2006; Lastovica 2006; Baker 1999). However, because of the poor isolation techniques used in most countries, it is not known whether other species are causing widespread disease and there is little or no epidemiological data on environmental or animal reservoirs that harbour these new species.

1.10. IDENTIFICATION TESTS

It is clear from the literature that the majority of identification tests available to date are generally limited to the identification of *C. jejuni* and *C. coli* species.

The most classical identification methods carried out in routine laboratories have included Gram staining, motility tests and biochemical tests. *Campylobacter* cells are Gram negative and have a spiral or S-shaped appearance and microscopic examination of the organisms in a wet film reveals their shape and characteristic darting, corkscrew-like motion due to a single polar flagellum. A number of biochemical tests can also be carried out on presumptive *Campylobacter* strains. For example tests for catalase, oxidase, hippurate hydrolysis, indoxyl acetate hydrolysis, H_2S production and sensitivities to cephalothin and nalidixic acid. Automated systems and biochemical panels that identify micro-organisms on the basis of their biochemical utilisation of various substrates are available allowing the user to identify an isolate after between 4 and 24 h.

The simplest rapid method in routine use for isolation of common *Campylobacter* species is by immunological confirmation in the form of the latex agglutination test. Latex particles are sensitised with specific antibodies which are then mixed with the sample and will visibly agglutinate in the presence of target antigen. Enzyme-Linked Immuno Sorptive Assays (ELISA) for *Campylobacter* species offer higher sensitivity than simple latex agglutination.

Molecular methods include nucleic acid probes (short sequences of nucleic acid which hybridise with the organisms' own nucleic acid (genomic or ribosomal). The hybridisation event must be detected and is usually measured by fluorescence. PCR is a technique that uses enzymes and thermal cycling to multiply the numbers of copies of a specific target DNA sequence to detectable levels. Detection is achieved by various means, traditionally by gel electrophoresis, however whilst providing exquisite theoretical sensitivity, in practice the technique requires traditional cultural sample enrichment to increase cell numbers if expected to be particularly low (Josefsen 2004).

Many recent studies have documented the development of methods for genotyping species and strains of *Campylobacter* including use of DNA profiling and sequencing techniques (Miller *et al.* 2005; Van Bergen *et al.* 2005). The adoption of these methods is essential for epidemiological tracking studies to be successful.

The most commonly used methods for identification of *Campylobacter* species to the species and strain level are summarised in Table 2.

1.10.1. Fluorescence *in situ* Hybridisation

In situ hybridisation (ISH) is one of the techniques available to identify the bacteria that compose heterotrophic microenvironments and determine where they are predominantly located. The advantage of this technique is that cell morphology can be observed, it is rapid

and sensitive, and the technique can be used directly to count the number of cells in a sample without the requirement for culture. It is based on the ability of RNA to form duplex strands by providing a RNA complementary sequence (probe) to one already existent in the cell (target), usually in the form of rRNA, and providing the necessary conditions for them to hybridise. A reporter molecule - fluorescent (FISH) or chromogenic (CISH) are the most common - can be attached to the probe so that it is possible to be tracked by fluorescence or brightfield microscopy. The FISH method is however a more widely established technique which has been used in diagnostic and other fields of microbiology (Pernthaler *et al.* 2001).

Peptide nucleic acid (PNA) probes are DNA probe mimics with an uncharged, neutral backbone, which provide the PNA probes with improved hybridisation characteristics such as high degrees of specificity, strong affinities, and rapid kinetics, as well as an improved ability to hybridise to highly structured targets such as rRNA (Egholm *et al.* 1993; Stender *et al.* 2002). In addition, the relatively hydrophobic character of PNA probes compared to the character of DNA enables PNA probes to penetrate the hydrophobic cell wall following preparation of a standard smear (Figure 7) (Egholm *et al.* 1993). This molecule has proved to be capable of forming PNA/DNA and PNA/RNA hybrids of complementary nucleic acid sequences, and its neutrally charged polyamide backbone has made FISH procedures easier and more efficient for different reasons: hybridisation can be performed efficiently under low salt concentrations, a condition that promotes the destabilisation of rRNA secondary structures and results in an improved access to target sequences that would be elusive using conventional FISH; hybridisation does not suffer from the electrostatic repulsions that occur when a DNA oligonucleotide is used. Because of the lower free energy of the hybrid a better specificity and more rapid hybridisation kinetics of the probe is achieved; it is not a substrate for the attack of proteases or endonucleases (Egholm *et al.* 1993; Moter and Gobel 2000; Stender *et al.* 2002). A number of DNA probes have been developed for the identification and direct visualisation of *Campylobacter* and

Table 2. Summary of Main Approaches Used to Identify *Campylobacteraceae*.

Method	Description	Species Identified	Culture dependant/ independent	Advantages	Disadvantages	Reference
Microscopy	Gram stain and wet mount to test for corkscrew motility and spiral cellular morphology.	Used to confirm the presence of *Campylobacter* genus, often as a pre-screen for presumptive ID.	Gram stain used successfully for presumptive identification of *Campylobacter* isolates directly in faecal specimens.	Often used in combination prior to subsequent molecular analysis, as is inexpensive and quick.	Cannot discriminate between *Campylobacter* species, easy to misidentify as *Arcobacter*, *Helicobacter* and other bacteria with spiral morphology.	(Thorson *et al.* 1985; Wang and Murdoch 2004)
Biochemical Characterisation	A range of biochemical tests testing for enzyme activity against a number of substrates i.e. catalase, hippuricase	Adequate for species identification of all *Campylobacteraceae* if a large range of biochemical tests evaluated (i.e. 70)	Isolated colonies are tested after culture isolation.	Could be easily be adopted by routine laboratories. Commercial tests have been developed but mainly limited to *C. jejuni/coli* species (i.e. API-Campy).	Hard to identify atypical stains. Highly labour intensive. Lack of standardised approaches. Campylobacters generally biochemically inert.	(On *et al.* 1996; Lastovica 2006) (Reina *et al.* 1995)
16S rRNA gene sequence comparisons	Unknown organisms identified by total 16S rRNA sequence analysis compared to sequences present in international databases (i.e. BLAST)	Partial discrimination between *Campylobacter* species	Isolated colonies are tested after culture isolation.	Sequence information can be used to develop species or genus specific primers and probes applied to PCR or hybridisation assays (see below).	Comparison of 16S rRNA sequences generally not adequate for identification of strains to species level as high strain-to strain differences and close similarities between species.	(Harrington and On 1999)
FISH using PNA/DNA probes	Fluorescence *in situ* hybridisation using 16 S targeted DNA or PNA oligonucleotide probes in conjunction with fluorescence microscopy	Generally limited to *C. jejuni* and *C. coli* species although one study also developed a *Campylobacter* genus probe Methods also developed to specifically detect *Arcobacter* spp.	*In situ* detection or in combination with initial culture isolation.	In situ technique which can be used to confirm isolates identification or directly on sample	Difficult to obtain required level of specificity required for speciation due to lack of discrimination between *Campylobacter* rRNA sequences	(Van den Berg *et al.* 1989; Lehtola *et al.* 2005; Schmid *et al.* 2005)
PCR (generally multiplex PCR is used)	Amplification of specific DNA fragments by annealing complementary primer sequences either side of target.	Genus and species specific protocols available for a range of *Campylobacter* spp.	Generally isolated colonies are tested after culture isolation. Can be used directly on enriched sample, but In situ analysis of samples inhibited by sample components.	Generally most widely method used for identification. Accurate, rapid and relatively inexpensive.	Problems with discrimination of *Campylobacter* species due to inadequate Rrna sequence divergence, especially between *C. jejuni* and *C. coli.* In situ PCR on samples is inhibited by presence of inhibitors to *Taq* polymerase.	(Linton *et al.* 1996; Harmon and Wesley 1997; Houf *et al.* 2000) (Vandamme *et al.* 1997)
Phage Typing	Identification based on a panel of bacteriophages with capacity to lyse specific *Campylobacter* species. Often used as an extension to serotyping.	Generally restricted to *C. jejuni* and *C. coli* species	Initial isolation by cultural methods required.	Inexpensive, no complex apparatus required.	Problems encountered due to high phase variation of surface structures. Lack of availability of broad range phages. High number of strains untypable.	(Grajewski *et al.* 1985; Frost *et al.* 1999)

Pulse field gel Electrophoresis (PFGE)	Bacterial cells embedded in agarose are lysed *in situ* and restriction enzymes used to cut large DNA fragments which are separated by a special electrophoretic method followed by analysis of DNA-restriction fragments.	Most studies focussed on *C. jejuni* and *C. coli* species, also limited studies on other species (*C. lari*, C. fetus, *C. hyointestinalis*, *C. upsaliensis* and *C. sputorum*).	Initial isolation by cultural methods required	Accepted as one of most powerful tools available. High levels of discrimination	Preparation of agarose blocks is tedious and labour intensive Apparatus used is specialised and expensive. Use of toxic formaldehyde is required during procedure.	(Chang and Taylor 1990; Lorenz *et al.* 1997; On and Harrington 2001; Broman *et al.* 2004)
DNA-DNA Hybridisation	Generally species with >70% whole genome sequence homology are considered the same species. The generally accepted method for speciation of *Campylobacter* species to date.	All species have been typed using this method.	Initial isolation by cultural methods required	High levels of discrimination allowing accurate speciation of Campylobacters.	Low throughput, not practical to implement in routine laboratories.	(Vandamme and Goossens 1992); On *et al.* 1996)
RAPD	Random amplification of polymorphic DNA, primers used are of arbitrary sequence using entire sequence of target organism.	Generally studies limited to *C. jejuni, C. coli* and *C. lari*.	Initial isolation by cultural methods required	Can provide level of discrimination comparable to PFGE. Quicker and cheaper than PFGE and does not require complex apparatus	Lack of reproducibility, method requires standardisation	(Mazurier *et al.* 1992; Hilton *et al.* 1997)
PCR-RFLP (mainly of *fla* A genes)	Restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products of the *flaA* and *flaB* genes followed by gel electrophoresis.	Most evaluations restricted to *C. jejuni* and *C. coli* spp.	Initial isolation by cultural methods required	Method is quick, simple, generally available and inexpensive, while allowing high level of discrimination.	Fla typing cannot be considered a stable long term method due to genetic instability of *fla* genes by intergenomic recombination between *fla* A genes of different strains.	(Giacoboni *et al.* 2005) (Alm *et al.* 1993) (Ayling *et al.* 1996)
AFLP	Amplified fragment length polymorphism fingerprinting. Resolves complex DNA polymorphisms using restriction enzymes to digest whole-cell DNA, followed by PCR of product fragments.	All 16 *Campylobacter* species.	Initial isolation by cultural methods required	High genotypic resolution to strain level can usually be obtained, allowing molecular epidemiological applications Rapid and easily stanardised	Laborious, highly skilled workers, specialised expensive apparatus required Further optimisation required as only a limited number of strains analysed to date and occasionally some strains cannot be characterised	(Wagenaar *et al.* 2001) (On and Harrington 2000; Duim *et al.* 2001)
MALDI-TOF MS	Whole-cell lysates used to observe protein biomarker ions in matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-TOF MS).	Previous studies using five thermophilic species including *C. jejuni*, *C. coli, C. lari, C. upsaliensis*, and *C. helveticus* and *C. concisus*	Initial isolation by cultural methods required.	Once implemented is rapid with high throughput.	Expenisve and specialised equipment required. Rigid standardisation required	(Fagerquist *et al.* 2005; Mandrell *et al.* 2005)
Whole-cell fatty acid methyl ester (FAME) analysis	Comparative analysis of whole-cell fatty acid composition using gas-liquid chromatography.	Mainly for *C. jejuni* isolates and some *C. coli* and *C. lari* species	Requires culture in highly standardised conditions.	Rapid, cheap and simple method, especially if automated system used.	Highly standardised conditions required. Occasionally more than one species can be clustered into one 'gas chromatography' group. Difficult to differentiate *C. jejuni* and *C. coli* spp.	(Coloe *et al.* 1986; Lambert *et al.* 1987; Vandamme *et al.* 1992)

Protein Electrophoresis	Comparison of whole-cell protein patterns obtained by highly standardised SDS PAGE	Teehnique has been applied to speciation of all *Campylobacter* species.	Requires culture in highly standardised conditions.	Extremely reliable for screening and identifying large numbers of strains. Generally excellent discrimination of *Campylobacter* species	Problems with differentiating *C. coli* and *C. jejuni* Not appropriate for routine identification as is laborious, time consuming and is difficult to achieve complete standardisation.	(Vandamme *et al.* 1990)
Ribotyping	Restriction fragment length polymorphisms of ribosomal DNA analysed by database comparisons.	Generally limited to *C. jejuni*.	Initial isolation by cultural methods required.	Using the the Qualicon Riboprinter® is fully automated and reproducible.	*Campylobacter* only have 3 ribosomal gene copies, reducing discriminatory power. High cost, small sample throughput	(Kiehlbauch *et al.* 1994; Manfreda *et al.* 2003)
DNA Microarray	Based on DNA hybridisation of isolate DNA to several target oligonucleotide probes arrayed onto microchips revealing species specific arrays.	Accurate identification of *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* demonstrated	Initial isolation by cultural methods required. Recent studies demonstrated detection directly from sample.	Relatively quick and accurate method to differentiate genomes of species, often to the strains level.	Expensive and specialised equipment required. Occasionally problems with reproducibility.	(Pearson *et al.* 2003; Volokhov *et al.* 2003; Keramas *et al.* 2004; On *et al.* 2006; Zhang *et al.* 2006)
MLST	Nucleotide sequencing of internal regions of housekeeping loci (with minimal nucleotide polymorohisms). Simple PCR based technique, which makes use of automated DNA sequencers.	Most studies on *C. jejuni* although recent studies also allow identification of *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*.	Initial isolation by cultural methods required.	High levels of discrimination can be achieved as loci are found at a number of different sites within the genome. High throughput.	Requirement for specialised equipment, expensive reagents, and highly trained personnel.	(Fearnhead *et al.* 2005; Miller *et al.* 2005)
Latex agglutination Test	Latex particles are sensitized with specific antibodies whieh are then mixed with the sample and will visibly agglutinate in the presence of target antigen.	Generally have only been applied to the detection of thermophilic species.	Generally used for confirmation of cultured isolates. Detection of isolates used directly in faecal samples attempted with limited success.	Generally only used for genus confirmation. Lacks sensitivity to many emerging species, lack of discrimination as cross reactions between closely related strains.	Rapid method, easy to use, many tests are commercially available (i.e.Microscreen *Campylobacter*, Microgen Bioprducts).	(Nachamkin and Barbagallo 1990; Yamazaki *et al.* 1990; Sutcliffe *et al.* 1991; Siragusa *et al.* 2004)
ELISA (Enzyme-Linked Immuno Sorptive Assay)	Cells coated to microtitre plate screened by primary anti-*Campylobacter* antibody, secondary antibody (specific for primary antibody) coupled to an enzyme or fluorophore used for detection	Generally have only been applied to the detection of thermophilic species.	Initial isolation by cultural methods generally used.	Offers higher sensitivity than simple latex agglutination.	Lack of accurate discrimination between species due to cross reactivity of different strains and phase variation of surface structures.	(Hochel *et al.* 2004)
Serotyping	Two schemes used: 1. Penner scheme based on Heat Soluble (HS) antigens; 2. Lior scheme detects variation in Heat Liable (HL) antigens.	Generally only used to type serotypes of *C. jejuni* and *C. coli*.	Initial isolation by cultural methods required.	New antisera being developed against recently isolated nontypeable strains so sensitivity to a wider range of species can be achieved.	High levels of nontypeable isolates, especially from human and veterinary strains. Generally classifies into broad groups.	(Patton *et al.* 1985; Rosef *et al.* 1985; Woodward and Rodgers 2002)

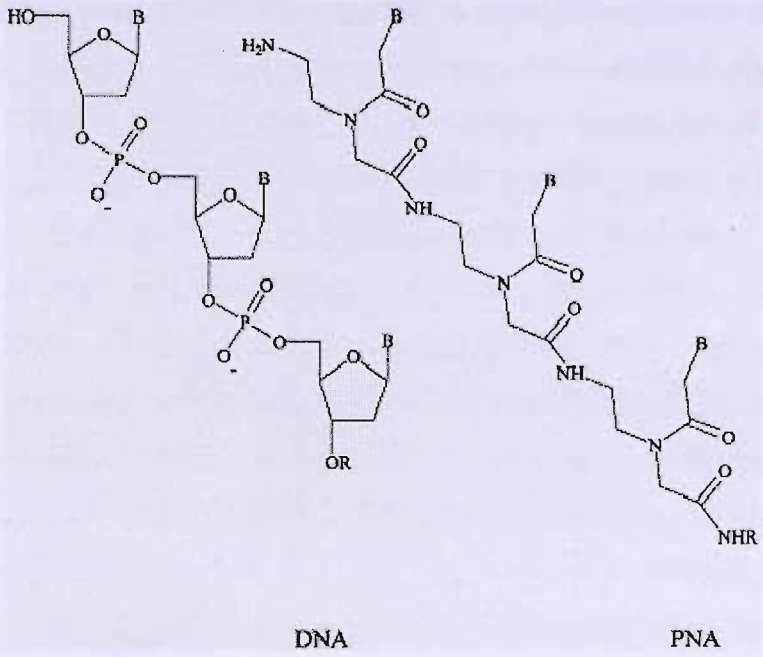


Figure 7. Comparison Between the DNA and PNA Chemical Structure. (Egholm *et al.* 1993). The phosphate backbone in DNA is replaced by a peptide backbone in PNA.

Arcobacter species from various sample types (Lecuit *et al.* 2004; Schmid *et al.* 2005; Lehtola *et al.* 2006a). A PNA probe has also been developed for detection of thermophilic *Campylobacter* species (Lehtola *et al.* 2005).

1.10.2. Use of Proteomics for Identification

Proteomics, the global analysis of proteins, describes a rapidly growing and maturing scientific discipline. It seeks to achieve what other large-scale enterprises in the life sciences cannot: a complete description of living cells in terms of their functional components, brought about by direct analysis of those components rather than the genes that encode them. The explosive progress over the past decade in the fields of genomics, bioinformatics and mass spectrometry has resulted in an increased capability to investigate and compare the global expression of cells, tissues and organisms. The term 'proteomics' was first used in 1995 and was defined as the large scale characterisation of the entire protein complement of a cell, tissue or organism (Andersen 1996). However the first protein studies that could be referred to as proteomics were reported 30 years earlier with the attempted characterisation of proteins expressed by *E. coli*. Although these proteins could be separated and visualised, they could not be identified (O'Farrel 1975). Today the growth of proteomics is a direct result of advances made in large scale nucleotide

sequencing and the sensitivity of protein methods. The majority of the DNA and protein sequence information has accumulated in the last 15 years (Broder 2000) and since the first complete genome sequence of *Haemophilus influenzae* was published (Fleischmann *et al.* 1995) there has been a rapid increase in the number of bacterial genome sequences available. Currently 211 genome sequences of microorganisms have been determined and many others are in progress. It is very difficult to predict the function of genes accurately from genetic data using only bioinformatics (Eisenstein *et al.* 2000). Transcriptonomics studies have become increasingly popular, including RNA arrays and serial analysis of gene expression. However mRNA is not a direct reflection of the protein content of the cell and studies have shown that there is in fact poor correlation between mRNA levels and protein expression levels. The reason for this is that the formation of mRNA is only the first step in a series of events leading to the synthesis of a protein. Firstly, many forms of a particular protein can be generated from a single mRNA and, secondly mRNA can be subjected to regulation at the protein translation level.

1.10.2.1. Separation Using 2D Gel Electrophoresis

By far the most common technique for the analysis of either a subset of proteins or all resolvable proteins in a cell is gel electrophoresis using either one- or two-dimensional separation (O'Connor *et al.* 2000). When using SDS-PAGE the proteins are both solubilised and given a charge by the detergent and further separated according to their molecular weight. SDS-PAGE is simple to perform, reproducible and can separate proteins with molecular weight between 10 and 300 kDa. For many applications SDS-PAGE is the method of choice but due to its limited resolving power it is only suitable for separation of mixtures after some form of purification (Graves 2002). When separation of more complex samples is required (such as total cell lysates), two dimensional electrophoresis (2D-PAGE) is the method of choice. Here, the proteins are separated on the basis of two distinct properties. In the first dimension isoelectric focussing (IEF) is performed and proteins are separated according to their net charge in the pH gradient. The second dimension is essentially SDS-PAGE where the proteins are further resolved according to molecular weight. The combination of these two methods achieves resolution far exceeding that of SDS-PAGE alone.

The primary application of 2D PAGE is expression profiling when the composition of the resulting images of two samples gives both quantitative and qualitative information. Another application of 2D-PAGE is monitoring post-translational modifications, which is possible because most of the modifications would alter the charge or mass of the protein.

However, the technique has its limitations specifically in reproducibility, automation as well as the number and types of proteins that it can resolve. A number of improvements over the years including introduction of immobilized pH gradients, the use of fluorescent dyes and the development of various detergents to aid the solubility of the proteins have improved the capabilities of the technique. Despite these advances there are still a number of protein classes that are not well represented on 2D gels including low abundance proteins, hydrophobic proteins and very basic proteins as well as very small or large proteins (Harry 2000).

Another obstacle for obtaining good quality and reproducible 2D PAGE profiles is maintaining the proteins in a soluble state during the IEF. Solubilisation can be defined as the process of disrupting the non-covalent or covalent interactions of the proteins with other substances which may or may not be proteinaceous. Such non-covalent interactions are the hydrogen bonding, ionic interactions, dipole moments, hydrophobic interactions and Van der Waals forces (Rabilloud 1996). For this reason special attention has to be paid to the cell lysis conditions, the choice of adequate detergents, chaotropes and the amount of reducing agents used. The role of chaotropes is to disrupt hydrogen bonding, leading to protein unfolding and denaturation. Urea has become the universally accepted component included in the rehydration solution to aid the solubility of proteins during the process of IEF.

1.10.2.2. Proteomics and Microbiology

Before the general availability of methods for protein identification, 2D-PAGE had been widely used to discriminate between related isolates of several bacterial species e.g. *Neisseria* species (Jackson *et al.* 1989) or *Haemophillus* species (Cash *et al.* 1995). Early application of 2D-PAGE used the method simply as a sensitive technique for the differentiation of closely related microbial isolates on the basis of protein charge and molecular weight. Global analysis of the bacterial proteins is now a feature of many investigations.

1.10.2.3. *Campylobacter* and Proteomics

Early proteomic studies on *Campylobacter* carried out over 20 years ago consisted of separation of outer membrane protein and flagella proteins by 1D- and 2D PAGE gels and identification by immunoblotting (Wenman *et al.* 1985; Dunn *et al.* 1987; Dunn *et al.* 1989; Wren *et al.* 2001) . Unfortunately 2D and other proteomic technology

was limited and studies were restricted to *C. jejuni* and other thermophilic species isolated at the time.

More recently, comparative protein profiling, using highly standardised one-dimensional SDS-PAGE of whole-cell proteins has been extensively used for the successful identification of a number of *Campylobacteraceae* species and shows a strong correlation with DNA-DNA hybridisation. It has also been successfully used for the identification of phenotypically aberrant campylobacters (Vandamme *et al.* 1996). However, the method is not appropriate for routine identification studies because it is very laborious, time consuming, and technically demanding to run the patterns in a consistently standardised way. Furthermore, problems remain with lack of resolving power and the differentiation of closely related species such as strains of *C. jejuni* and *C. coli* (On 1996; On and Harrington 2000; On 2001).

Other recent proteomic identification methods have used mass spectrometry to differentiate *Campylobacter* species by distinct protein biomarkers, which can be detected in MALDI-TOF spectra (Fagerquist *et al.* 2005).

Two dimensional electrophoresis is a highly resolving technique for arraying proteins by isoelectric point and molecular mass. It is a fundamental separation technique for proteomics which has previously been used to successfully determine phylogenetic relationships (Govorun *et al.* 2003; Dopson *et al.* 2004). Due to the high resolving power of 2D-PAGE it is thought this method would overcome problems with distinguishing closely related species due to the high level of resolving power.

1.11. THE CAMPYCHECK PROJECT

The CAMPYCHECK project is a shared cost project titled "Improved physiological, immunological and molecular tools for the recovery and identification of emerging *Campylobacteraceae* in the food and water chain" (QLK1 CT 2002 02201) within the EU Fifth framework "Quality of Life and Management and living recourses" programme and running for 3 years (March 2003-2006). The main objective of this project was to develop improved isolation, detection and identification procedures for all *Campylobacteraceae* from a range of typical samples tested throughout the human food chain. It is then hoped that these new isolation and detection methods can be used to assess the prevalence of a wide range of *Campylobacteraceae* species throughout the food chain.

The research carried out focussed on a number of areas, in particular on the development of routine isolation and detection methods which will allow effective screening of samples in outbreak situations and the generation of epidemiological data on

the microorganisms. The data generated on the prevalence of these microorganisms in various sample types will be essential to the establishment of control measures for water and food.

1.12. AIMS OF PHD PROJECT

This study, as part of the EC-funded CAMPYCHECK project aimed to assist towards developing routine methods for the isolation, detection and typing of all *Campylobacteraceae* species. These methods will have an application both in routine clinical and food laboratories, epidemiological studies (including outbreak investigation) and contribute establishing the prevalence and clinical importance of emerging *Campylobacteraceae* species. The aims of this PhD project are summarised as follows:-

1. Evaluate currently used isolation media and develop an isolation medium suitable for the growth of all *Campylobacteraceae* species.
2. Assess current incubation conditions and develop a universal incubation atmosphere suitable for growth of all *Campylobacteraceae* species.
3. Evaluate a range of antibiotic supplements currently used in selective isolation media and assess their inhibitory effects on *Campylobacteraceae* species. Further to this, select a suitable antibiotic supplement to include in a novel isolation medium that will successfully inhibit competitive flora but will have no inhibitory effects on *Campylobacteraceae* species.
4. Evaluate existing identification tests used for the routine identification of *Campylobacteraceae* species and develop novel tests for the confirmation and identification of *Campylobacteraceae* species, some in collaboration with industrial collaborators.
5. To incorporate the novel culture and identification methods into optimised protocols for the recovery of *Campylobacteraceae* species from sewage and salad vegetables.
6. To assess the prevalence of *Campylobacteraceae* species in sewage and salad vegetable samples using optimised protocols.

CHAPTER 2

MATERIALS AND METHODS

2.1. INTRODUCTION

The isolation procedures currently available are predominantly used for the isolation of thermotolerant *Campylobacter* species and no universal method exists to recover a wide range of *Campylobacteraceae.*

During this study, following initial assessment of existing methods, novel culture and identification methods were developed for the isolation of all *Campylobacteraceae* using pure cultures, which were then applied to sewage sludge and salad vegetables as examples of important points in the food chain. As the objective of the study was to improve current methods used to grow *Campylobacteraceae*, some of the standard methods initially described in this section were modified as the study progressed; variables modified included media type and incubation atmosphere.

2.1.1. Rationale

In this chapter methods that were used as standard to grow the CAMPYCHECK reference strain collection are detailed; subsequently procedures used to assess growth are described, of which the streak dilution method was used for comparative culture evaluation. This semi-quantitative streak dilution method was used to assess growth on a wide range of media and incubation atmospheres. Following these comparisons an optimised medium was chosen, namely Anaerobe Basal Agar (ABA) (Oxoid Ltd, Basingstoke, UK) supplemented with 5% laked horse blood (Oxoid) and an optimised incubation atmosphere, containing 3% oxygen, 7% hydrogen and 10% carbon dioxide. The Streak Dilution method was then used to assess the suitability of a range of current commercially available selective supplements. Antibiotic disc susceptibility tests were then used to determine the susceptibility to antibiotics commonly included in selective media. From these findings, a selective supplement, VAT (10 mg/l vancomycin, 10 mg/l amphotericin B and 5 mg/l trimethoprim) was developed, which was incorporated into the optimised recovery methods for recovering *Campylobacteraceae* from sewage and salad vegetable samples, described at the end of this section. Methods are also described for the optimisation of identification procedures for a range of *Campylobacteraceae* including evaluation of a range of biochemical tests, novel immunological tests and microscopy procedures, including the optimisation of a Fluorescence *in situ* Hybridisation (FISH) protocol, some tests of which were used to confirm the identify of presumptive *Campylobacteraceae* isolates recovered from samples.

2.2. CAMPYCHECK REFERENCE STRAINS, GROWTH AND MAINTENANCE

2.2.1. The CAMPYCHECK Reference Strain Collection

The *Campylobacter* reference strains used during this study were chosen carefully to represent emerging species that have been recently isolated (On 2001; Lastovica 2006). This was important if the new isolation and identification methods being developed were to be successful. The CAMPYCHECK reference strain set consisted of 59 strains, representing all currently described species and subspecies belonging to the *Campylobacteraceae* family, recognised as having human and veterinary importance. The CAMPYCHECK strain set included 16 *Campylobacter* species (including subspecies and biovars) and all 3 pathogenic *Arcobacter* species, with emphasis on human and clinical strains isolated from Europe, South Africa and the USA (chosen and compiled by Dr Stephen On, Danish Institute of Food and Veterinary Research (DFVF), Copenhagen, Denmark). The collection also incorporated 20 reference strains from the Culture Collection of the University of Göteborg (CCUG). Many strains were originally isolated using either the Cape Town protocol or using filtration methods with limited antibiotic selection (Lastovica 2006).

The CAMPYCHECK number (1-59) relates to each isolate shown in Table 3. For ease of explanations and descriptions in the results sections, the CAMPYCHECK isolate will be referred to by its species name and CAMPYCHECK number rather than referring to the strain code.

2.2.2. Growth, Maintenance and Storage of Reference Strains

CAMPYCHECK reference strains were received from the Danish Food and Veterinary Institute, Copenhagen, Denmark, as frozen stock cultures. These cultures had been stored in nutrient broth (Oxoid) with 10% (v/v) glycerol (Sigma) and upon arrival were stored at -80°C. Initially, to resuscitate the cultures, 50 µl of the glycerol broth culture was inoculated onto Tryptose Blood Agar (TBA) (CM233, Oxoid) supplemented with 10% (v/v) defibrinated horse blood (SR50, Oxoid). The inoculated plates were then incubated at 37°C for 48-72 h in the recommended conditions (Dr Stephen On, DFVF pers. comm.): species including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus*, *C. showae* and *Bacteriodes ureolyticus* were incubated in an anaerobic atmosphere and all other species were incubated in a microaerobic atmosphere supplemented with hydrogen (7% H_2, 4% O_2, 10% CO_2,

Table 3. CAMPYCHECK Reference Strain Set for Study. Table shows species and reference strain number, source of isolate i.e. the clinical source and geographical area where isolated from. CAMPYCHECK no. refers to the CAMPYCHECK number of which each strain has been allocated (1-59). Abbreviations: Lastovica, Professor A.J. Lastovica, University of Cape Town, Republic of South Africa (SA); Lab 33, Dr S.L. On, Danish Food and Veterinary Institute, Denmark; RM, Dr R. Mandrell, USDA, ARS, Western Regional Research Center, Albany CA, USA; CCUG, Culture Collection University of Göteborg, SE; NCTC, National Collection of Type Cultures, UK; Rigshospitalet, National Hospital, Copenhagen, Denmark.

Taxon	Strain no.	Source (clinical/geographic)	CAMPYCHECK no.
C. coli	RM 2228 (genome sequenced)	Chicken/ USA	1
	Lab 33 (hyoilei genotype)	Pig/DK	2
	300.97	Human, diarrhoea/SA	3
C. concisus	CCUG 13144 (type strain)	Human, oral/USA	4
	CCUG 19995 (genospecies 2)	Human, diarrhoea/Belgium	5
	Lastovica 396/96 (large genome size, ca. 2.186 mB)	Human, diarrhoea & vomiting/SA	6
C. curvus	SSI 19296	Human, diarrhoea/DK	7
	Lastovica 525.92	Human, oral/SA	8
	Lastovica 13A	Human, oral /SA	9
C. fetus subsp. fetus	CCUG 32114	Human abscess, Sweden	10
	Abdn 1076	Sheep abortion, Scotland	11
	241.99	Human, loose stools/SA	12
C. fetus subsp. venerealis	CCUG 11287	Human/France	13
	Armi 4402	Bovine/Australia	14
	Abdn SM5	Aborted bovine fetus/N. Ireland, UK	15
C. gracilis	CCUG 27720	Human, periodontitis (USA)	16
	CCUG 13143	Human, periodontitis (USA)	17
C. helveticus	CCUG 30566	Cat diarrhoea/Switzerland	18
	CCUG 34016	Cat faeces/Sweden	19
C. hominis	NCTC CH001 (type strain)	Human faeces/UK	20
	NCTC CH003	Human faeces/UK	21
C. hyointestinalis subsp. hyointestinalis	LMG 7538	Human diarrhoea, Sweden	22
	LMG 9260	Human diarrhoea, Belgium	23
	Lastovica 176.96	Human neonate, diarrhoea/SA	24
	Lastovica 234.95	Human infant, loose stools/SA	25

Table 3 Continued.

C. hyointestinalis subsp. lawsonii	CHY 5 (type strain)	Pig stomach/UK	26
	CCUG 27631	Pig stomach/Sweden	27
C. jejuni subsp. doylei	SSI 5384	Human/DK	28
	CCUG 18266	Human gastric biopsy/Germany	29
	269.97 (serotype 0:17)	Human blood, Kwashiorkor/SA	30
C. jejuni subsp. jejuni	NCTC 11168 (genome sequenced)	Human diarrhoea/UK	31
	RM 1221 (genome sequenced)	Chicken/USA	32
	SVS 4039	Cattle/DK	33
	RM 1864 (=81-176)	Human diarrhoea/USA	34
	47.97	Infant, blood/SA	35
C. lanienae	NCTC 13004	Human faeces/UK	36
	DARDNI G718D	Pig faeces/N. Ireland, UK	37
C. lari	RM 2100 (NARTC), genome sequenced	Human faeces/USA	38
	CCUG 22395 (UPTC)	Human diarrhoea/France	39
C. mucosalis	CCUG 21559 (serotype A)	Pig necrotic colitis/Scotland, UK	40
	CCUG 23201 (serotype B)	Pig intestine/Scotland, UK	41
C. rectus	CCUG 20446 (type strain)	Human, oral/USA	42
	CCUG 11645	Human periodontitis/Sweden	43
C. showae	CCUG 30254 (type strain)	Human, oral/Japan	44
	CCUG 11641	Human periodontitis/Sweden	45
C. sputorum	Lastovica 86.92 (bv. sputorum)	Infant diarrhoea/SA	46
	LMG 11764 (bv. paraureolyticus)	Human faeces/Canada	47
	CCUG 20703 (bv. fecalis)	Ovine faeces/UK	48
C. upsaliensis	RM 3195 (genome sequenced)	Human diarrhoea/SA	49
	CCUG 19559	Human diarrhoea/UK	50
	CCUG 19607	Canine faeces/Sweden	51
B. ureolyticus	CCUG 18470	Human urine/Sweden	52
	Rigshospitalet 9880	Human faeces/DK	53
A. butzleri	SSI 71032	Human faeces/DK	54
	Lastovica 29.97	Infant, loose stools /SA	55
	Lastovica 167.97	SA	56
A. cryaerophilus	CCUG 17801 (type strain: subgroup 1)	Bovine abortion/N. Ireland, UK	57
	SSI 70952	Human faeces/DK	58
A. skirrowii	BU 30CC 8B1	Poultry/UK	59

79% N_2). The anaerobic atmosphere was achieved using an Oxoid Anaerobic Gas Generating Kit (BR38, Oxoid) in conjunction with a palladium catalyst (Oxoid) in an Anaerobic Gas Jar (HP11, Oxoid) according to manufacturers' instructions. To confirm that anaerobic conditions had been achieved, an Anaerobic Indicator (BR55, Oxoid) was placed in the jar and monitored throughout incubation (a colour change from pink to white was an indicator that anaerobic conditions had been achieved). The microaerobic atmosphere supplemented with hydrogen was provided using a MACS VA500 Microaerophilic Workstation (Don Whitley Scientific Limited, UK).

2.2.3. Resuscitation of Strains for Experimentation

When required for experimentation strains were resuscitated directly from bead stock cultures stored at -80°C (PROTECT™, Technical Service Consultants, UK). On some occasions beads were stored at -20°C for short term storage (1-2 days), otherwise stocks were stored at -80°C. Long term storage at -20°C has been shown to reduce the viability of cultures when compared to -80°C (Gorman and Adley 2004) and so this was avoided during the study. One bead was removed from the vial using a 10 µl loop which was then directly inoculated onto TBA (10% defibrinated horse blood) or ABA (5% laked horse blood) by spreading across the surface of the plate. The plates were inverted and incubated in the recommended atmosphere (described in section 2.2.2) for 48-72 h. Solid agar media was used as opposed to a liquid broth culture to ensure that pure colonies were obtained. Once reference culture bead vials had been removed from the -80°C they were held on ice (4°C) and left out on the bench for as little time as possible to minimise the contents of the vials becoming completely defrosted, as the freeze-thaw process is thought to decrease viability of the cultures due to damage incurred on the cell membrane (Chang *et al.* 2003).

Once initiated, cultures were maintained on TBA medium supplemented with 10% (v/v) defibrinated horse blood (or ABA with 5% (v/v) laked horse blood) under the appropriate atmosphere (depending on species) at 37°C (see section 2.2.2). If the cultures were required for prolonged experimental use, they were sub-cultured onto fresh agar weekly for a maximum of five weeks (5 sub-cultures) before they were disposed of and newly resuscitated reference cultures were used. If cultures were incubated for more than a week before sub-culturing they lost viability and would die off on the next sub-culture. When working with the

cultures, the amount of time that the culture plates were left out on the bench was kept to a minimum due to the high sensitivity of campylobacters to an aerobic environment.

After resuscitation, cultures were checked for purity using the prototype Oxoid Biochemical Identification System (O.B.I.S.) for *Campylobacteraceae* (Smith *et al.* 2006a), which consisted of the KOH Gram test and test for absence of L-alanine aminopeptidase (L-ALA) (described in section 2.5).

Resuscitation of species other than those included in the CAMPYCHECK strain set i.e. for negative and positive control tests or to compare antibiotic susceptibility of competitor organisms was relatively simple since *Enterobacteriaceae* are much less fastidious than *Campylobacteraceae* in their growth requirements. These strains were obtained from Microgen Bioproducts (Camberley, UK) or were in-house Environmental Healthcare Unit strains (Southampton University, UK). To resuscitate these strains, PROTECT™ beads were inoculated directly onto TBA (10% blood) (or ABA, 5% blood) and incubated at 37°C for 24 h in an aerobic atmosphere.

As the study progressed and new methods for growth of *Campylobacteraceae* were developed, the conditions used for growth and maintenance of cultures changed. Eventually, stock cultures were resuscitated under an incubation atmosphere containing 3% O_2, 10% CO_2, 7% H_2 and 80% N_2. In addition, TBA medium was replaced with Anaerobe Basal Agar (ABA) supplemented with 5% laked horse blood rather than 10% defibrinated horse blood.

2.3. DEVELOPMENT OF CULTURE MEDIA FOR THE RECOVERY OF *CAMPYLOBACTERACEAE*

2.3.1. Introduction

Experiments described in this section include assessment and development of isolation media (including selective supplements). For the majority of experiments carried out, the entire CAMPYCHECK reference strain collection was evaluated. However, in some circumstances all strains were not included due to time constraints, or if growth of certain strains was problematic. For example, if certain strains failed to grow (during recovery from bead stocks or subculture) or were shown to be contaminated following subsequent confirmatory identification tests. The confirmatory tests used as standard to check for purity included the prototype Oxoid Biochemical Identification System (O.B.I.S.) for *Campylobacteraceae*, oxidase test (Oxoid) and test for aerobic growth.

2.3.2. Evaluation of Growth Assessment Methods

During this PhD study, a large number of culture comparisons with a wide variety of strains needed to be carried out. In view of this fact, along with considerations of strict project deadlines and time constraints, a growth assessment method needed to be used which would allow accurate yet quick comparisons without having to use an excessive number of plates. Although ideally a quantitative method such as the Miles-Misra serial dilution method would be the preferred method for growth evaluations, a disadvantage is that it would be time consuming and costly in terms of plates required. Therefore alternative semi-quantitative methods to this were evaluated. Three methods for assessing the growth productivity of *C. jejuni* CCUG 11168, *C. concisus* CCUG 13144 and *C. hominis* CH001 were compared on various media. Methods evaluated included the quantitative Miles-Misra method and semi-quantitative methods including the Ecometric and Streak Dilution method (Figure 8).

2.3.2.1. Growth of Strains and Preparation of Inocula

Organisms were resuscitated from bead stocks stored at -80°C (PROTECT™ Fisher Scientific) directly onto Tryptose Blood Agar (TBA) (CM233, Oxoid Limited, Basingstoke, UK) supplemented with 7% (v/v) laked horse blood (SR48, Oxoid). The plates were incubated in a MACS VA500 Microaerophilic Workstation (Don Whitley Scientific Limited, UK) (*C. hominis* was incubated in an anaerobic environment described previously) at 37°C in a microaerophilic atmosphere supplemented with hydrogen (7% H_2, 4% O_2, 10% CO_2, 79% N_2) for 48-72 h. The bacteria were subsequently sub-cultured onto fresh plates and incubated for a further 48 h. To standardise the inoculum, a dense suspension of each organism was prepared corresponding to an OD_{492nm} of 1.0 (Absorbance Microplate Reader, Tecan) (~$\log_{10}$ 10 cfu/ml) by taking sweeps of culture from the agar surface and suspending in 2 ml of maximum recovery diluent (MRD; Oxoid). Unless otherwise stated inoculated plates were incubated in a microaerobic atmosphere supplemented with hydrogen (7% H_2, 4% O_2, 10% CO_2, 79% N_2) at 37°C for 48 h.

2.3.2.2. Modified Miles-Misra Method

Growth was measured quantitatively using the Miles-Misra method (Corry *et al.* 1995b). Ten-fold dilutions of the prepared culture suspensions were diluted in MRD of which

50 µl of each dilution was inoculated and spread over one-quarter of the agar plate (i.e. four dilutions on each plate (Figure 8.A). Duplicate plates were incubated at each dilution and the mean number of colony forming units (cfu) was calculated. Productivity ratios (P_r) were calculated using the formula: $P_r = N_S/N_O$ (where N_S is the count (colony forming units per ml of original suspension) on the test medium and N_O is the count on Typtose Blood Agar (control medium).

2.3.2.3. Ecometry

The Ecometric technique is a semi-quantitative method based on streaking an inoculum of bacteria to extinction. Each suspension was streaked onto the surface of two of each type of agar plate using a modification of the Ecometric method (Corry *et al.* 1995b). A standard sterile disposable 1 µl loop was used to streak the inoculum using 5 parallel streak lines across quadrants of the plate to give 20 streaks in total (Figure 8.A). Growth was assessed by noting the end streak at which growth still occurred. This endpoint was used to allocate the absolute growth index (AGI) score. Growth on each streak was allocated an AGI of 5 and if there was growth on all 20 streaks an AGI of 100 was allocated. The final AGI was calculated as a mean of duplicate plates and from the AGI scores productivity ratios were calculated as for the Miles-Misra method but using the growth score instead of cfu counts.

2.3.2.4. Streak Dilution Method

The Streak Dilution method is similar to the Ecometric method in that the bacterial inoculum is streaked across the agar plate and sequentially diluted. It is similar to the method described by Corry et al. (1995b). A version of this has also been described for estimating the number of bacteria in urinary samples (Urquhart and Gould 1965) and is often used in laboratories for quality control of media. Each bacterial suspension was inoculated using a standard 10 µl loop onto the surface of two of each type of agar plate allowing sequential dilution of the culture suspension across successive quadrants (Figure 8.C). The culture suspension (described in section 2.3.1.1) was inoculated onto the first quadrant of the agar plate (1st quarter) using a back and forth motion until all the suspension had been transferred from the loop to the agar plate. A sterile loop was then used to streak out from the edge of the first quadrant (using five back and forth streaks) into the second quarter of the plate (2nd quadrant area). A further sterile loop was then used to streak out into the third quarter of the

Table 4. Representation of Growth Assessment Scores Used for the Streak Dilution Method.

Mean Growth Score	Rounded Growth Score	Growth Symbol	Growth Description
0	0	-	No growth
0.25-0.5	0.5	-/+	Scarce growth
0.75- 1.25	1	+	Low growth
1.5-2.25	2	++	Intermediate growth
2.5-3.25	3	+++	Good growth
3.5-4.0	4	++++	High growth

plate (quadrant area 3) in the same way. Finally a sterile loop was used to streak out (from quadrant 3) into the fourth quarter, extending into the middle of the plate. Following incubation the plates were assessed for growth using a semi-quantitative four point growth score according to areas of growth on the quadrant streaks. Mean scores were calculated and then for presentation of results, these scores were allocated a representative growth symbol (Table 4). Productivity ratios were calculated as for the Ecometric method.

The streak dilution method was only semi-quantitative; therefore no statistical analysis on this data was carried out.

2.3.3. Initial Evaluation of Basal Media and Selective Supplements

Previous literature has demonstrated that many commonly used *Campylobacter* selective isolation media are potentially inhibitory to a number of emerging *Campylobacteraceae* (Engberg *et al.* 2000; Lastovica and Skirrow 2000; Lastovica 2006). To verify this, a number of commonly used selective isolation media were evaluated by comparison of growth, with and without their selective supplements included in the basal medium. The non-selective TBA medium was included in the evaluation and was considered as the method of choice, since it is used as part of the Cape Town protocol, which has been shown to recover all *Campylobacteraceae* (Lastovica 2006). Thirteen *Campylobacteraceae* strains were selected for study from the CAMPYCHECK strain collection that included two

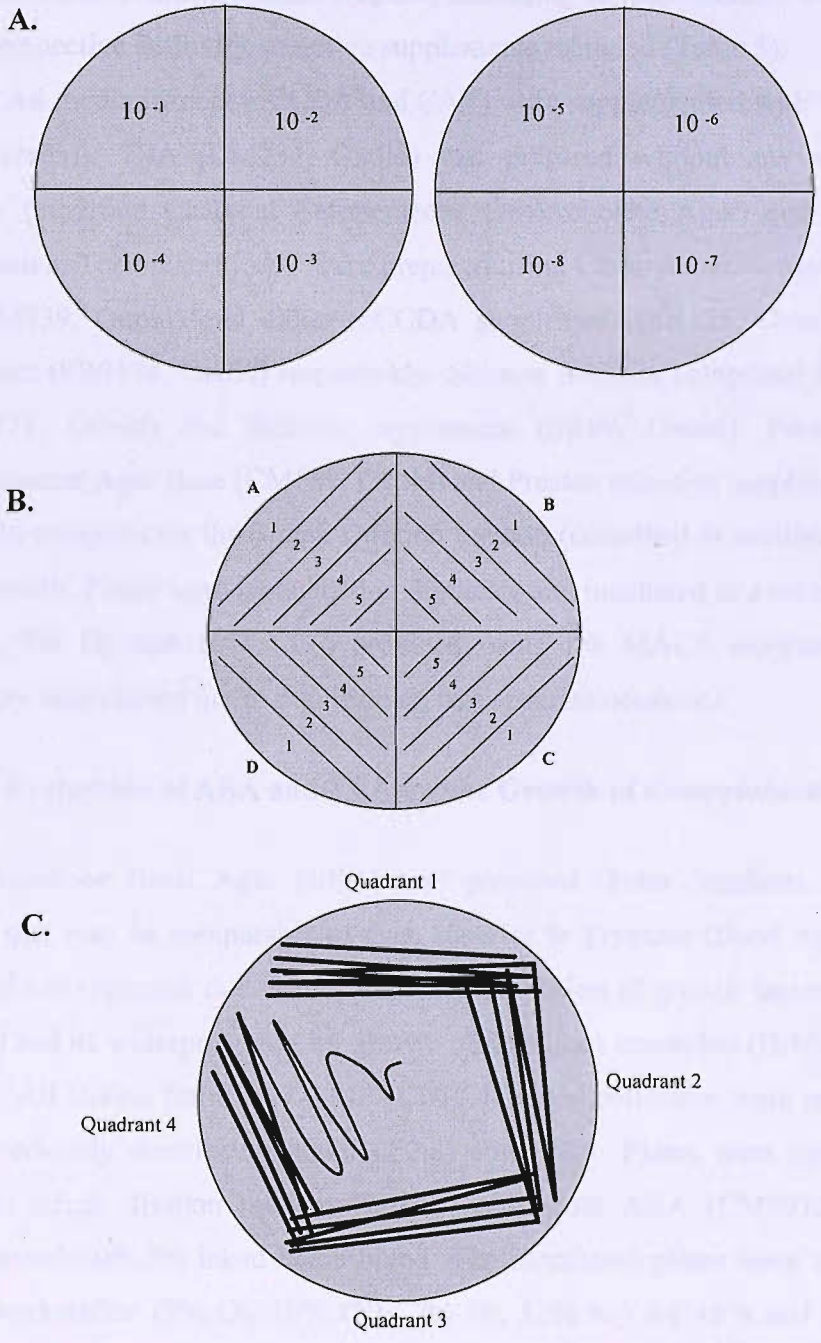


Figure 8. Schematic Diagram of the Three Growth Assessment Methods Evaluated. A. Schematic diagram of the Miles-Misra method; B. Schematic diagram of the Ecometric method; C. Schematic diagram of the streak Dilution method.

isolates each of *C. coli*, *C. jejuni*, *C. lari*, *C. upsaliensis*, three isolates of *C. concisus* and one *A. cryaerophilus* and *A. butzleri* strain. Media, including TBA (7% blood), mCCDA, CAT, Preston and Skirrow, were prepared according to manufacturer's instructions with and without respective antibiotic selective supplements included (Table 5).

All media (except mCCDA and CAT) were supplemented with 7% laked horse blood (SR48, Oxoid). TBA (CM233, Oxoid) was prepared without any selective supplement. mCCDA (modified Charcoal Cefoperazone Deoxycholate Agar) and CAT (Cefoperazone Amphotericin Teicoplanin) agar were prepared using Campylobacter blood-free selective agar base (CM739, Oxoid) and either mCCDA supplement (SR155, Oxoid) or CAT selective supplement (SR0174, Oxoid) respectively. Skirrow medium comprised Blood Agar Base No. 2 (CM271, Oxoid) and Skirrow supplement (SR69, Oxoid). Preston agar comprised Campylobacter Agar Base (CM689, Oxoid) and Preston selective supplement (SR117, Oxoid). For media comparisons the Streak Dilution method (described in section 2.2.1.4) was used to assess growth. Plates were inoculated in duplicate and incubated in a microaerobic atmosphere (4% O_2, 7% H_2 and 10% CO_2) provided using the MACS workstation for 48 h. The experiment was carried out in duplicate on two separate occasions.

2.3.4. Evaluation of ABA and TBA for the Growth of *Campylobacteraceae*

Anaerobe Basal Agar (ABA) was proposed (Peter Stephens, Pers. Comm.) as a medium that may be comparable or even superior to Tryptose Blood Agar for the growth of *Campylobacter* species, due to its complex composition of growth factors and other nutrients (Table 6) and its widespread use for growth of fastidious anaerobes (Bridson 1998).

All strains from the CAMPYCHECK strain collection were resuscitated according to the previously described method (2.2.3) onto TBA. Plates were inoculated in duplicate using the streak dilution method (2.3.1.4) onto both ABA (CM0972, Oxoid) and TBA supplemented with 5% laked horse blood. The inoculated plates were then incubated in the MACS workstation (3% O_2, 10% CO_2, 7% H_2, 82% N_2) for 48 h and assessed for growth. After carrying out this experiment, because ABA performed better than TBA, it was decided that this medium would be used (for maintenance of all *Campylobacteraceae* strains and as the isolation medium) in place of TBA from this point onwards.

Table 5. Composition of *Campylobacter* Selective Isolation Agar Media including Basal Media and Selective Supplements. *No selective supplement was included in TBA as it is usually used in conjunction with membrane filtration (Lastovica and Le Roux 2001). †Components thought to act as blood replacements include charcoal, and FBP supplement which are included in the base.

Medium	Basal medium	Blood supplement	Antibiotic Selective supplement
TBA	Tryptose Blood Agar Tryptose (10g/l), 'Lab-Lemco' powder (3g/l), Sodium chloride (5g/l), Agar (12g/l), pH 7.2 +/- 0.2	7% Laked	None*
Preston	Campylobacter Agar Base 'Lab-Lemco' powder (10g/l), Peptone (10g/l), Sodium chloride (5g/l), Agar (12g/l), pH 7.5 +/- 0.2	7% Laked	Polymyxin B (5,000 IU), Rifampicin (10mg/ml), Trimethoprim (10mg/l), Cycloheximide (100mg/l)
Skirrow	Blood Agar Base no. 2 Proteose peptone (15g/l), Liver digest (2.5g/l), Yeast extract (5g/l), Sodium chloride (5g/l), Agar (12g/l), pH 7.4 +/- 0.2	7% Laked	Vancomycin (10mg/l) Trimethoprim (5 mg/l) Polymyxin B (2500 IU)
CCDA	Campylobacter Blood-free Agar Base Nutrrient broth no.2 (25g/l), Bacteriological charcoal (4g/l), casein hydrolysate (3g/l), Sodium deoxycholate (1g/l), ferrous sulphate (0.25g/l), sodium pyruvate (0.25g/l), sodium pyruvate (0.25g/l), agar (12g/l), deionized water (1l), pH 7.4 + /- 0.2	None†	Cefoperazone (32 mg/l), Amphotericin B (10mg)
CAT	Campylobacter Blood-free Agar Base (see above)	None†	Cefoperazone (8 mg/l) Teicoplanin (4 mg/l) Amphotericin B (10 mg/l)

2.3.5. Evaluation of Blood Type and Composition

Both defibrinated and laked horse blood are commonly included in media at concentrations of 5% to 10% to counteract the toxic effects of oxygen derivatives and to provide an iron source for growth (Corry *et al.* 1995b). In this experiment growth on ABA supplemented with laked and defibrinated horse blood at different concentrations was compared. Blood is an expensive supplement and therefore if there were no differences between 5% and 10% it would be more economical to use the 5% concentration.

All strains from the CAMPYCHECK strain collection were resuscitated according to the previously described method onto ABA (section 2.3.1.4). Plates were inoculated in duplicate using the streak dilution method (described in section 2.2.1.4) onto ABA supplemented with either 5% (v/v) or 10% (v/v) laked (SR0048, Oxoid) or defibrinated horse blood (SR0050, Oxoid). The inoculated plates were then incubated in the MACS workstation (3% O_2, 10% CO_2, 7% H_2, 82% N_2) for 48 h and assessed for growth.

2.3.6. Confirmation of Inhibition of Selective Supplements

Previously (section 2.3.2) it was shown that selective supplements included in some selective media decreased the growth of a number of *Campylobacteraceae* strains. In the following experiments growth was compared when CCDA, CAT, Preston and Skirrow selective supplements were supplemented to TBA only, to cancel out any effects the basal media may have had on the action of the selective supplements when used in combination.
Oxoid *Campylobacter* selective supplements (2 x 2 ml vials) including Skirrow selective supplement (SR0069), Preston selective supplement (SR0117), CCDA selective supplement (CM0739) and CAT selective supplement (SR0174) were aseptically added to TBA (5% laked horse blood) (1 litre) and then 25 ml agar plates poured. All 59 strains from the CAMPYCHECK reference strain set were evaluated plus control non-*Campylobacteraceae* species including methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella* and *E. coli*. Strains were resuscitated onto TBA (5% laked horse blood) (described in section 2.3.1.4). The streak dilution method (section 2.2.1.4) was used to inoculate plates in duplicate. The plating media assessed included TBA (no supplement) and TBA supplemented with mCCDA, CAT, Preston, and Skirrow selective supplements. After plates were inoculated using the Streak Dilution method, they were incubated for 48 h at 37°C in the MACS

Table 6. Composition of Tryptose Blood Agar (TBA) and Anaerobe Basal Agar (ABA). ABA has a much more complex composition being mainly peptone based whereas TBA is tryptose based and has a slightly higher pH.

ABA		TBA	
Formula	**g/litre**	**Formula**	**g/litre**
Peptone	16.0	Tryptose	10.0
Yeast extract	7.0	Lab-Lemco powder	3.0
Sodium chloride	5.0	Sodium Chloride	5.0
Starch	1.0	Agar	12.0
Dextrose	1.0	pH 7.2 +/- 0.2	
Sodium pyruvate	1.0		
Arginine	1.0		
Sodium succinate	0.5		
L-cysteine HCl	0.25		
Sodium bicarbonate	0.4		
Ferric pyrophosphate	0.5		
Haemin	0.005		
Vitamin K	0.0005		
Dithiothreitol	0.25		
Agar	12.0		
pH 6.8 ± 0.2			

workstation (3% O_2, 7% H_2, 10% CO_2, 82% N_2). The antibiotic compositions of the selective supplements according to the manufacturer are shown in Table 5. These experiments were carried out in duplicate on separate occasions and an average of the growth score taken.

2.3.7. Antibiotic Susceptibility Testing of Antibiotics Commonly Included in Selective Isolation Media

Assessment of the antibiotic susceptibilities of *Campylobacteraceae* to a range of compounds commonly included in *Campylobacter* selective isolation media was carried out using antibiotic disc diffusion tests. It was thought that results would confirm which antibiotics were inhibitory to *Campylobacteraceae* species and therefore which ones to avoid or to include in a new selective medium. The antibiotic susceptibility disc diffusion method was chosen over other methods because it is a previously validated method proving to be quick, simple, accurate and inexpensive when compared to alternative methods such as agar dilution and E-tests (Gaudreau and Gilbert 1997). Strains from the CAMPYCHECK reference strain set and a range of other bacteria likely to be potential contaminants when isolating *Campylobacter* from various matrices were evaluated.

Strains were resuscitated onto Anaerobe Basal Agar (ABA) supplemented with 5% (v/v) laked horse blood (Oxoid) and incubated in a hydrogen-enriched microaerobic atmosphere (3% O_2, 10% CO_2, 7% H_2, 82% N_2) provided using a MACS workstation for 48 h. The strains were then sub-cultured at least once (but less than 5 times) before using for experimentation. To prepare and standardise cultures for inoculation, colonies were swept from the plate surface using a 1 µl loop and inoculated into 2 ml of sterile distilled water and adjusted to an optical density of 1.0_{492nm}. A 1 in 100 dilution of this bacterial suspension was then prepared of which 50 µl was inoculated (in triplicate) onto fresh ABA plates and spread evenly across the surface using a sterile hockey stick spreader. This produced semi-confluent growth of cultures after 48 h, which is a requirement for accurate disc diffusion readings (King 2001).

The nine antibiotics evaluated were all constituents of commonly used *Campylobacter* selective isolation media. The susceptibility breakpoints for the discs used were those recommended by the NCCLS (National Council for Clinical Laboratory Standards) (Huysmans and Turnidge 1997). The antibiotic discs (Oxoid Ltd., Basingstoke, UK) selected included amphotericin B (20 µg), cefazolin (20 µg), cefaperazone (75 µg), colistin sulphate

(50 μg), polymyxin B (300 units), rifampicin (5 μg), teicoplanin (30 μg) trimethoprim (5 μg) and vancomycin (30 μg). An additional antibiotic, cefsulodin was included half way through the study; therefore susceptibility results of certain species to this antibiotic were not included. After inoculation, sterile forceps were used to place the antibiotic disc onto the agar surface. Three discs were placed on each plate so disc diffusion zones did not overlap. The plates were incubated for 48 h in an atmosphere provided by the MACS workstation (3% O_2, 10% CO_2, 7% H_2, 82% N_2). After 48 h, zones of inhibition (diameter of circles where bacterial growth had been inhibited) were measured and recorded in millimetres (Figure 9). Susceptibility (S) was defined when a zone of inhibition with a diameter of over 20 mm was produced. Intermediate susceptibility or resistance (I) was defined as those bacteria that produced an average zone of inhibition of 10-20 mm and complete resistance (R) as any results under 10 mm diameter. Other potential competitor bacteria were also screened using the disc diffusion method. These were resuscitated from frozen stocks onto ABA (5% blood) and incubated under aerobic conditions at 37°C for 24 h. Once the plates had been inoculated and discs applied, they were incubated for a further 24 h in an atmosphere provided by the MACS workstation (3 % O_2, 10 % CO_2, 7 % H_2, 82 % N_2).

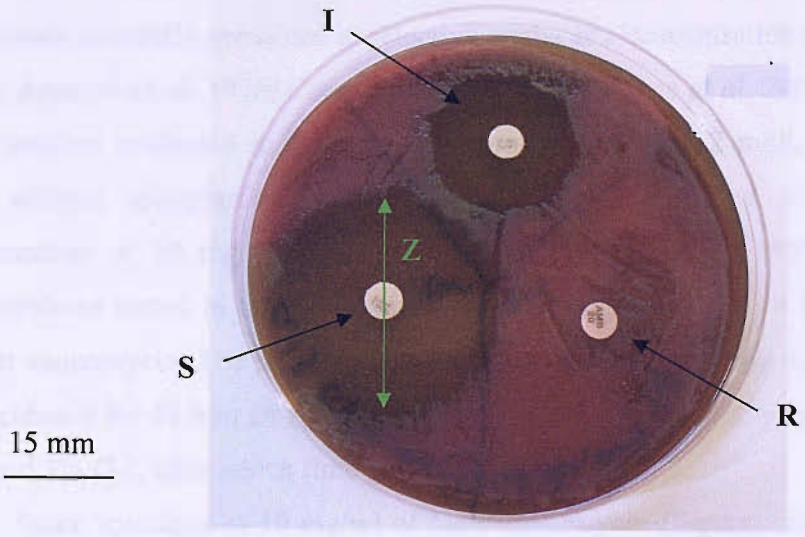


Figure 9. Antimicrobial Susceptibility Disc Diffusion Tests after incubation for 48 h. Green arrow: Diameter of zone of inhibition measured in mm (Z). R, Resistant (0 mm), I, Intermediate susceptibility (~10-20 mm), S, Susceptible (>20 mm).

2.3.8. Determination of Concentrations of Teicoplanin and Vancomycin for use in Isolation Media

The disc diffusion tests carried out previously (section 2.3.6) revealed that vancomycin and teicoplanin were active against all Gram-positive contaminants tested. Therefore it was thought that these would be useful candidates to include in a novel isolation medium for samples where Gram positive organisms would be present. However, these antibiotics also showed some activity towards certain *Campylobacter* species. These included all species of *C. helveticus* (18,19) (vancomycin only), *C. hominis* (20,21) and *C. rectus* (42,43) and one isolate each of *C. venerealis* and *C. showae* (45). It was thought that it might be possible to find a suitable concentration of these antibiotics to include in a novel isolation medium that would inhibit contaminants yet will not interfere with *Campylobacter* growth. Therefore, various agar dilutions were prepared (at concentrations usually used in selective isolation media). Representative strains of *Campylobacter* species (except *C. hominis* due to problems with reference strain recovery) were resuscitated (as described in section 2.2.3) and inoculated using the Streak Dilution method (described in section 2.3.2.4) onto ABA (5% blood) containing various concentrations of either vancomycin or teicoplanin. Concentrations were selected according to the concentrations used in currently available selective isolation media. Teicoplanin is usually contained in selective media at a concentration of 4 mg/l (Aspinall *et al.* 1993b; Aspinall *et al.* 1996; Corry and Atabay 1997; Byrne *et al.* 2001), therefore teicoplanin concentrations evaluated in these experiments were 1 mg/l, 2 mg/l, 4 mg/l and 6 mg/l and ABA without teicoplanin. Vancomycin is usually contained in selective media at a concentration of 10 mg/l (Morgan *et al.* 1990; Corry *et al.* 1995a; 2002). Vancomycin concentrations tested in this experiment were 2.5 mg/l, 5 mg/l, 10 mg/l, 15 mg/l and ABA without vancomycin. The plates were inoculated in duplicate using the streak dilution method and incubated for 48 h in an atmosphere provided using the MACS workstation (7% H_2, 10 % CO_2, and 3% O_2), after which time growth was assessed.

Stock solutions of 10 mg/ml of both vancomycin (Sigma) (100 mg into 10 ml sterile distilled water) and teicoplanin (donation from Oxoid) (100 mg into 10 ml dissolved into 60:40 sterile distilled water: ethanol) were prepared. These antibiotic stock solutions were then filter sterilised and supplemented to 1 litre of autoclaved ABA (at approx. 50°C) to achieve the appropriate concentrations.

2.3.9. Evaluation of ABA VAT Medium for the Isolation of *Campylobacteraceae* and Selective Inhibition of Other Species

The antibiotic disc susceptibility tests showed that amphotericin B and trimethoprim had no inhibitory effects at the levels tested against any of the *Campylobacteraceae* evaluated. However, vancomycin, supposedly an antibiotic inhibitory to Gram positive bacteria showed inhibitory effects against a small number of strains at 15 mg/l, growth was not affected at 10 mg/l. Therefore, these antibiotics were chosen to include in a new minimally selective medium for the isolation of all *Campylobacteraceae*, called ABA VAT medium. Although these three antibiotics had no inhibitory effects when tested individually, it is possible that when supplemented as part of an antibiotic cocktail, the action of antibiotics may have an exacerbated inhibitory effect. Therefore, this experiment was carried out to evaluate the effect of the VAT antibiotic selective supplement on the growth of *Campylobacteraceae* and a number of non-related organisms.

All 59 CAMPYCHECK strains were resuscitated onto ABA (5% blood) in an atmosphere provided by the MACS workstation (3% O_2, 10% CO_2, 7% H_2, 82% N_2). Competitor bacterial strains were resuscitated onto ABA (5% blood) in an aerobic atmosphere at 37°C for 24 h. The streak dilution method was used to inoculate plates in duplicate onto ABA (5 % blood) (without VAT supplement), ABA supplemented with VAT and ABA supplemented with CAT selective supplement.

2.3.9.1. Preparation of VAT Antibiotic Stock Solutions

VAT selective supplement contained vancomycin (10 mg/l), amphotericin B (10 mg/l) and trimethoprim (5 mg/l). Trimethoprim, amphotericin B and vancomycin were purchased in powdered form (Sigma-Aldrich, UK). Trimethoprim stock solution (5 mg/ml) was prepared by dissolving 50 mg into 10 ml of 50:50 ethanol to water; amphotericin B was prepared by adding 100 mg to 10 ml of dimethyl sulphoxide; vancomycin stock solution was prepared by adding 100 mg to 10 ml sterile distilled water. Stock solutions of these antibiotics were dissolved, filter sterilised, distributed into 2 ml aliquots and stored at -20°C. When they were required, 1 ml of these stock solutions was aseptically added to 1 litre of molten agar.

2.4. DEVELOPMENT OF INCUBATION ATMOSPHERES FOR THE GROWTH OF *CAMPYLOBACTERACEAE*

2.4.1. Initial Evaluation of Incubation Atmospheres

Eleven *Campylobacter* strains were selected for study from the CAMPYCHECK strain collection. These included two isolates each of *C. coli* (1,2), *C. jejuni* (31,32), *C. lari* (38,39), *C. upsaliensis* (49,50,51) and three isolates of *C. concisus* (4,5,6). To carry out incubation atmosphere comparisons, the streak dilution method was used to assess growth (see section 2.3.1.4). The incubation conditions tested included the commercially available CampyGen™ (CN35, Oxoid), AnaeroGen™ (AN35, Oxoid) and the Anaerobic Gas Generating Kit with catalyst (BR38, Oxoid). These atmosphere generation systems were used in conjunction with an Oxoid Anaerobic Gas Jar (HP11, Oxoid) according to manufacturers' instructions. These atmospheres were compared to an atmosphere provided using the MACS workstation (4% O_2, 10% CO_2, 7% H_2, 82% N_2). The theoretical gas concentrations provided using the commercial gas generating kits, suggested by the manufacturer are shown in Table 7. To test that anaerobic conditions (<1% oxygen) had been achieved in the anaerobic atmospheres, an Anaerobic Indicator (BR55, Oxoid) was placed inside the jars during incubation.

A further experiment was carried out to assess the requirement for hydrogen. Here the theoretical gas concentrations thought to be achieved using a CampyGen™ sachet were reproduced in the MACS workstation but supplemented with 1% hydrogen. To verify that it was hydrogen causing an effect on growth rather than the use of an Anaerobic Gas Jar and CampyGen™ sachet, experiments were carried out to compare growth in the MACS workstation containing theoretical CampyGen gaseous concentrations to that using the CampyGen™ sachet in conjunction with an Anaerobic Gas Jar. The results shown are the mean of duplicate experiments carried out independently on separate occasions.

2.4.2. Optimisation of Hydrogen and Oxygen Concentrations

Fifty CAMPYCHECK reference strains were selected for study. Organisms were resuscitated from PROTECT™ beads (stored at -80°C) directly onto ABA supplemented with 5% laked horse blood. The 'anaerobic' species were incubated in an anaerobic environment provided by the Oxoid Anaerobic Gas Generating Kit (BR38, Oxoid) in Anaerobic Gas Jars (Oxoid). Other species were incubated in an atmosphere provided using the MACS

Table 7. Gaseous Compositions of Incubation Atmospheres Evaluated. * These gaseous compositions were as stated by manufacturers' instructions (Bridson 1998).
??? = concentration of hydrogen present unknown, residual levels may be present.

Atmosphere Type	*AnaeroGen (anaerobic)	BR38* (anaerobic)	CampyGen* (microaerobic)	'CampyGen' (microaerobic)	'CampyGen' with 1% H_2 (microaerobic)	Microaerobic with 7% H_2
	Gas jar	Gas jar	Gas jar	MACS	MACS	MACS
O_2	<1%	<1%	6%	6%	7%	5%
H_2	0%	0???	0	0	6%	7%
CO_2	9- 13%	10%	14%*	14%	14%	10%

workstation at 37°C, in a microaerobic atmosphere supplemented with hydrogen (7% H_2, 4% O_2, 10% CO_2 and 79% N_2) for 48-72 h. Since the MACS workstation was required to carry out evaluations of the modifications of hydrogen and oxygen concentrations, it could not be used at the same time to grow the maintenance strains. Furthermore, the atmosphere comparisons could not be carried out in parallel. Therefore, a method had to be employed whereby the physiological state of the bacteria and inoculum concentration was fixed each time the incubation atmosphere was modified, to allow accurate and standardised growth comparisons. To achieve this, each time the cultures were used for experimentation, maintenance subcultures were also prepared. Species were sub-cultured onto fresh ABA plates and incubated in either an anaerobic atmosphere (BR38, Oxoid) for the 'anaerobic' *Campylobacter* species or in a pre-mixed gas (2.5% O_2, 10% CO_2 and 7% H_2, Air Products, UK) in self-seal polythene bags for 48 h. This involved a gas evacuation-replacement method whereby self-seal polythene bags (Sainsburys, UK) (doubled up to limit exchange and leakage of gases) were flushed three times with the pre-mixed gas in order to replace atmospheric air with the pre-mixed gas atmosphere.

The maintenance cultures were used to prepare a standardised liquid inoculum, which was inoculated onto ABA plates using the Streak Dilution method (section 2.3.1.4) in duplicate. The plates were incubated for 48 h at 37°C in the MACS workstation containing the atmosphere being assessed.

To optimise the oxygen concentration, the carbon dioxide and hydrogen concentration were kept constant at 10% CO_2 and 6% H_2 (7% hydrogen could not be combined with 5% oxygen because the MACS workstation contains pre-set safety limits which prevent explosive

atmospheres being used). Oxygen concentrations tested included 1%, 3% and 5%. Hydrogen concentrations tested included 0%, 4% and 7% with controlled oxygen and carbon dioxide concentrations (3% oxygen and 10% carbon dioxide). Each time the incubation atmosphere in the MACS workstation was modified, it was left 24 h to equilibrate before incubation of the cultures.

2.4.3. Evaluation of Conventional and Novel Incubation Atmospheres for the Growth of *Campylobacteraceae*

The optimal incubation atmosphere for growth of all *Campylobacteraceae* was 7% hydrogen, 3% oxygen and 10% carbon dioxide. There is a need to develop a universally applicable gas mix as it is likely that organisations such as routine testing laboratories and other research laboratories would not have access to a MACS workstation. Therefore, alternative approaches to achieving a comparable incubation atmosphere to the one achieved in the MACS workstation (7% H_2, 3% O_2, 10% CO_2 and 80% N_2) were evaluated. These included use of a pre-mixed gas containing 7% H_2, 2.5% O_2, 10% CO_2 and 80% N_2, used in conjunction with a gas evacuation-replacement method. Another alternative method evaluated was that provided using a combination of commercially available Oxoid gas production sachets (Peter Stephens, Oxoid, Pers. Comm.).

The growth of *Campylobacteraceae* was evaluated in conventional commercially available atmospheres (CampyGen, AnaeroGen, and the Anaerobic Gas Generating Kit, Oxoid) and novel atmospheres specifically developed for the growth of all *Campylobacteraceae*, which were provided by the MACS workstation, the gas-evacuation-replacement method with pre-mixed gas and the Oxoid gas sachet combination.

All 59 strains included in the CAMPYCHECK reference strain collection were selected for study. All strains were resuscitated onto ABA media and incubated in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2 and 7% O_2) for 48-72 h. Standardised inocula were prepared (section 2.2.1.1) and inoculated in duplicate onto ABA using the streak dilution method (section 2.2.1.4). Plates were then incubated under all six incubation atmospheres. Use of a CampyGen, AnaeroGen and the Oxoid Anaerobic Gas Generating Kit have already been described in section 2.4.1. Use of the pre-mixed gas and evacuation-replacement method was as described in the previous section (section 2.4.2). To achieve the atmosphere provided using the Oxoid gas sachet combination, after plates were

placed inside an Anaerobic Gas Jar (3.5 l), gas sachets including a CampyGen™ sachet (CN0025A, Oxoid), a CO_2Gen™ sachet (CD0025A, Oxoid) and an AnaeroGen™ Compact sachet (AN0010C, Oxoid) were added to the jar. After this 10 ml of water was added to 0.1 g of sodium borohydride (16940-66-2, Sigma) (previously weighed into a 25 ml sterile plastic container) which was placed at the bottom of the gas jar with the lid off. The gas jar was then sealed and the plates incubated as normal. This method was carried out as quickly as possible (less than 1 min) to minimise the loss of gas from the jar. The final atmosphere thought to be achieved during incubation was between 0.5% and 2.5% oxygen, 10% carbon dioxide and 7% hydrogen (Smith *et al.* 2006b). It is thought that the atmosphere is achieved due to the combination of ascorbate within the gas sachets which is thought to convert oxygen to carbon dioxide, which also leads to a reduction in oxygen concentration. Hydrogen is produced (and sodium borate as a bi-product) when the sodium borohydride is mixed with water ($NaBH_4 + 2H_20 \rightarrow NaBO_2 + 4H_2$) (Smith *et al.* 2006b).

2.5. DEVELOPMENT OF TESTS FOR THE IDENTIFICATION OF *CAMPYLOBACTERACEAE* SPECIES

2.5.1. Introduction

A number of existing and novel tests for the identification of *Campylobacteraceae* were initially evaluated against the CAMPYCHECK reference strain collection. Certain identification tests were then chosen for use in identifying presumptive *Campylobacteraceae* isolates from salad vegetable and sewage samples (Figure 10). Detailed experimental procedures for the identification tests used are described in this section.

2.5.2. Morphological Identification

2.5.2.1. Colony Morphology on Plating Medium

The first step in the identification of *Campylobacter* species is visual inspection of colonies. *Campylobacter* colonies generally have a buff coloured or dirty yellow appearance on ABA medium. Exceptions are *C. helveticus* which has a thin flat film-like growth and can initially resemble swarming *Proteus* species (Lastovica 2006). Colonies of *Arcobacter* tend to be whiter than *Campylobacter*. While this method can be used, there can be variability within species and strains. Furthermore, 'atypical' colonies often occur, especially if different types

of media and incubation atmospheres are used. Translucent colonies with a moist appearance, for example, may sometimes appear on moist plating media.

Cultures from the CAMPYCHECK strain collection were inoculated onto ABA (5% blood) and incubated for 48 h in a MACS workstation (atmosphere: 3% O_2, 10% CO_2 and 7% O_2). Descriptions of the cultures were noted and pictures of plates were taken so colony morphology of reference cultures could be compared to presumptive isolates recovered from salad vegetable and sewage samples (Chapter 6).

2.5.2.2. Differentiation by Cell Morphology using EDIC Microscopy

All 59 *Campylobacteraceae* reference strains were resuscitated from storage at -80°C. They were plated onto ABA (5% blood) and incubated for 48-72 h in a MACS workstation atmosphere (3% O_2, 10% CO_2, 7% H_2). The strains were then sub-cultured and incubated for a further 48 h. Several colonies were removed from the plate using a 10 µl loop and re-suspended in 350 ml of ultra pure filter sterilised sterile distilled water. This bacterial suspension was homogenised by vortex mixing for 5 s and 50 µl placed onto a polytetrafluoroethylene (Teflon®)-coated multispot microscope slide (C. A. H. Hendley Ltd., England) which was left at room temperature for approximately 30 min to air dry. The bacterial smears were then analysed using episcopic differential interference contrast (EDIC) microscopy. The microscope used was an ME600 Nikon microscope which had been highly modified and fitted with a combination of lenses and filters to allow the use of a combination of both transmitted visible light and episcopic UV illumination from a mercury lamp (X-Cite™ 120 Fluorescence illumination system) (Best Scientific, UK). A x100 phase objective lens (long-working-distance) in conjunction with a 2 x zoom lens was used which achieved a final magnification of x 2000. Images were captured using a Q-imaging camera and analysed using Image Pro Plus 5.0 imaging software.

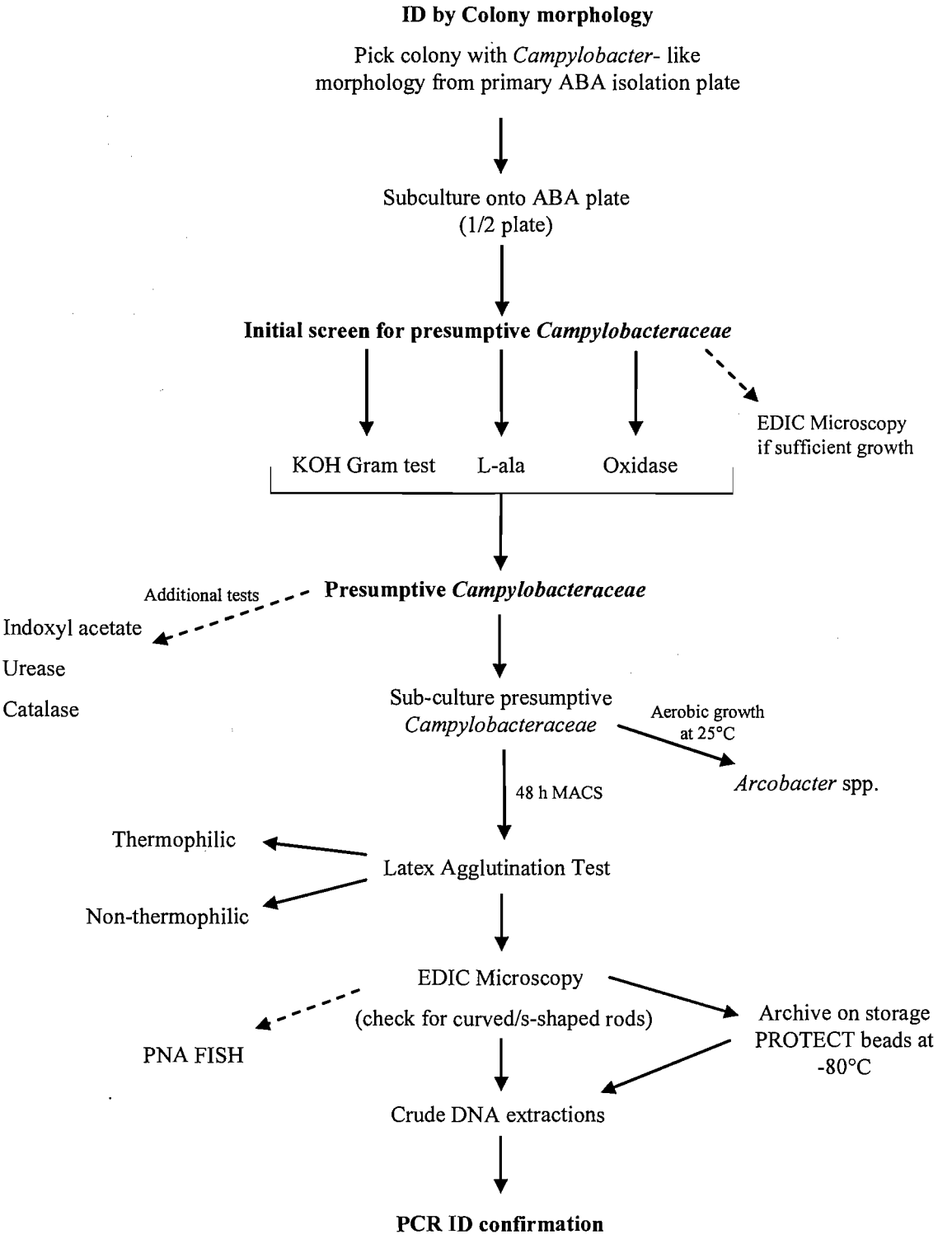


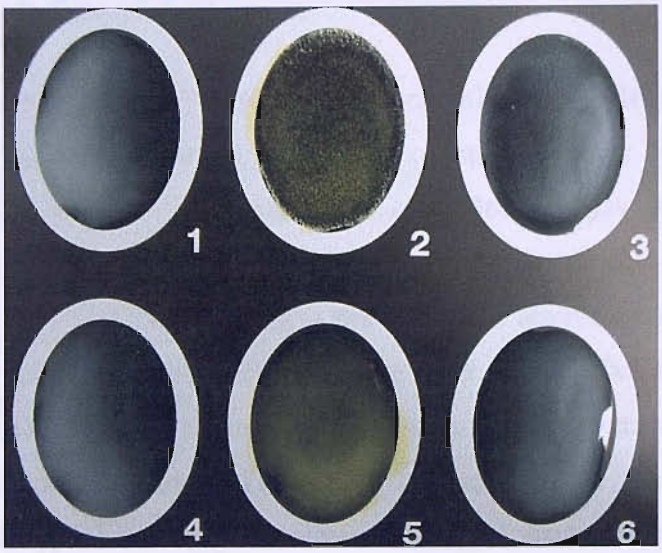
Figure 10. Overview of Procedures Used to Confirm the Identity of Presumptive *Campylobacteraceae* Isolates Recovered from Samples. Arrows with dotted lines represent ID tests that were not carried out as standard but which were tested on a number of isolates on certain occasions.

2.5.3. Immunological Identification: The Latex Agglutination Test

A commercially available latex agglutination kit, M46 Microscreen *Campylobacter*, was developed by Microgen Bioproducts (Camberley, UK) in the 1980s and has been used extensively by many laboratories for identification of thermotolerant campylobacters only (Meinersmann *et al.* 2005). Initially, during this study the specificity of this latex kit was evaluated against all 59 strains in the CAMPYCHECK strain set, demonstrating that it was unsuitable for the detection of many emerging *Campylobacteraceae* species. Subsequently, a novel latex agglutination kit was developed by Microgen. This consisted of the original latex reagent (white latex particles) (to detect the thermotolerant species) and a second latex reagent (yellow latex particles), with specificity for all *Campylobacteraceae*. This new prototype latex kit was then evaluated against all 59 strains within the CAMPYCHECK strain set. It was, therefore used to confirm the identity of isolates from samples and allowed approximate discrimination between thermotolerant and non-thermotolerant *Campylobacteraceae*.

To carry out the tests, all reagents were first allowed to reach room temperature and gently shaken to ensure a homogeneous suspension. A 50 µl aliquot of isotonic saline was dispensed onto each of three ovals of the agglutination slide. An inoculating loop was used to remove several colonies from the agar plate (after 48-72 h incubation) and if microbial growth was sparse, a broad sweep of the agar surface was taken. The bacteria were mixed into each of the three drops of isotonic saline on the slide to form even suspensions. One drop (50 µl) of control latex reagent was added to one of the bacterial suspensions on the slide. Similarly 1 drop (50 µl) of each of the two test latex reagents was dispensed onto the other two bacterial suspension wells. The bacterial suspensions were mixed with the latex reagents using a mixing stick, spreading the mixtures to the edges of the oval areas. The slide was the gently rocked from side to side to keep the fluid suspensions in constant movement for 2 min, after which the slide was observed for agglutination. An agglutination reaction was indicated by visible aggregation of the latex particles (Figure 11A). The results were interpreted as shown in Figure 11B.

A.



Control	Non-therm latex	Therm latex
Well 1	Well 2	Well 3
-	+	+
Well 4	Well 5	Well 6
-	-	-

B.

Thermophilic Latex	Species Latex	Control Latex	Interpretation
+	-	-	Thermotolerant *Campylobacter*
+	+	-	Thermotolerant *Campylobacter*
-	+	-	Non-Thermotolerant *Campylobacter*
+ or -	+ or -	+	Auto-agglutination*

Figure 11. Interpretation of the Novel Prototype *Campylobacteraceae* Latex Agglutination Test. A. Examples of positive (wells 2 and 3) and negative (wells 5 and 6) agglutination; B. Interpretation of latex reactions. *An isolate that causes the Control Latex reagent to agglutinate cannot be tested by CAMPYCHECK *Campylobacter*.

2.5.4. Biochemical Identification

2.5.4.1. Use of the Oxoid Biochemical Identification System (O.B.I.S.) to Rapidly Identify *Campylobacteraceae*

The prototype O.B.I.S. (Oxoid Biochemical Identification system) test for *Campylobacteraceae* is a novel, rapid and simple two-step procedure for identifying presumptive *Campylobacteraceae* (Smith *et al.* 2006a). It was developed by collaborators, Oxoid, as part of the CAMPYCHECK project. All members of the *Campylobacteraceae* are both Gram-negative and do not possess the L-alanine aminopeptidase enzyme, unlike other Gram-negative bacteria (Figure 12B) (Carlone *et al.* 1983). This test was initially evaluated against the CAMPYCHECK strain set and found to be very successful as a quick, simple and effective confirmation method. Thereafter, it was used as the first step in identifying presumptive *Campylobacteraceae* isolates recovered from samples. Furthermore, it was routinely used to check the purity of *Campylobacter* reference cultures.

To carry out the Gram-lysis test (first step in test), a colony from a 48-72 h plate was emulsified in a drop of 3% (w/v) KOH solution on a glass slide. If a 'string' of DNA (between the emulsifying loop and the slide) was produced within 1 min, the organism was considered Gram-negative. If the organism was Gram-positive, the solution would not become viscous and no 'string' would be seen when the loop was raised. To carry out the L-ala test, a colony was inoculated onto the card impregnated with substrate. One drop of O.B.I.S. buffer was added and after 30 s 1 drop of O.B.I.S. colour developer was applied. A strong purple colour after 30 s indicated that a positive reaction had occurred; therefore indicating the organism was not a *Campylobacteraceae* species (Figure 12A). An alternative commercially available test for the presence of L-alanine aminopeptidase available from Fluka (Cat. No. 75554) was also evaluated. To carry out this test a colony was removed with an inoculation loop and suspended in 0.2 ml distilled water in a 1.5 ml eppendorf tube. The test strip impregnated with substrate (0.5 µmole L-alanine-4-nitroanilide) was placed into the bacterial suspension and incubated for 10-30 min at 37°C. If the suspension turned yellow, L-alanine aminopeptidase was present.

A.

KOH	L-ala	Result
-	+	Not *Campylobacteraceae*
-	-	Not *Campylobacteraceae*
+	+	Not *Campylobacteraceae*
+	-	**Presumptive *Campylobacteraceae***

B.

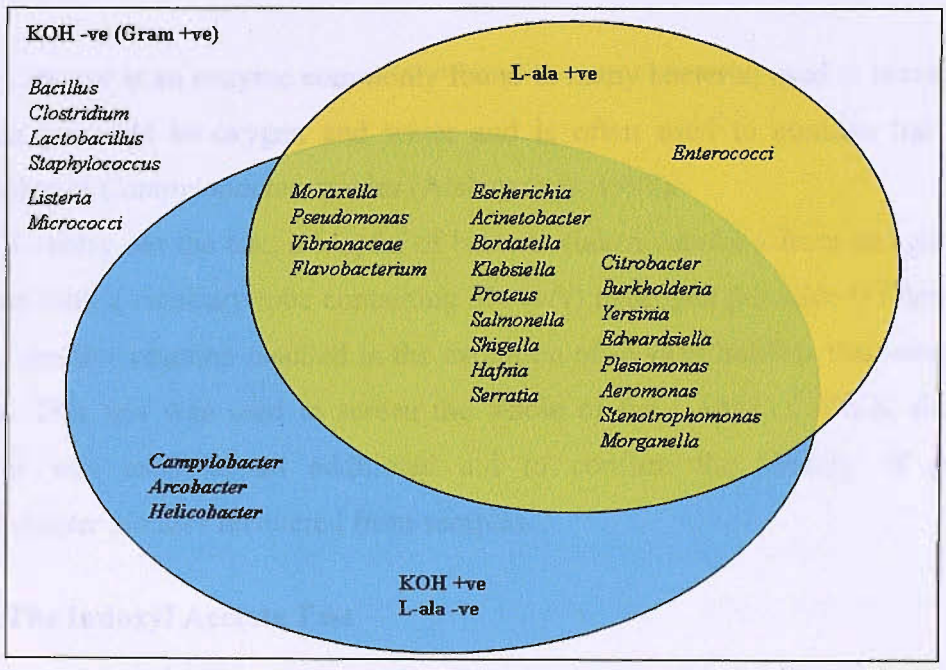


Figure 12. Interpretation of the L-Alanine Aminopeptidase and KOH Identification Test. A. Interpretation of results; B. Inclusivity and exclusivity of a range of bacteria to the KOH and L-ala test (Adapted from Smith *et al.* 2006a)

2.5.4.2. The Oxidase Test

The enzyme cytochrome oxidase is produced by many organisms, including all *Campylobacter* species. Oxidase Identification Sticks (BR064A, Oxoid) utilise a dry reagent specially stabilised to give it a long shelf life. The tip of each stick is impregnated with a solution of N,N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α-napthol, which in the presence of oxidase will be oxidized and change colour. To carry out the test a colony on the plate was touched with the impregnated end of the stick. In a positive reaction the presence of the enzyme cytochrome oxidase would combine with the substrate and turn blue after 30 s (but not longer than 3 min). No colour change indicated the organisms were oxidase negative. This test was used to screen the whole of the CAMPYCHECK strain set and thereafter the test was used to confirm the identity of presumptive *Campylobacteraceae* isolates recovered from samples.

2.5.4.3. The Catalase Test

Catalase is an enzyme commonly found in many bacteria, used to breakdown toxic hydrogen peroxide to oxygen and water and is often used to confirm the identity of thermotolerant *Campylobacter* species (Atabay *et al.* 1997).

To carry out the test, a loopful of bacteria (taken carefully from an agar plate) was picked up with a capillary tube containing 3% (v/v) hydrogen peroxide (Fluka) (about 1/3 full). A positive reaction resulted in the evolution of oxygen bubbles that were trapped in the tube. This test was used to screen the whole of the CAMPYCHECK strain set and thereafter was used as an additional aid to confirm the identity of presumptive *Campylobacter* isolates recovered from samples.

2.5.4.4.The Indoxyl Acetate Test

Some *Campylobacteraceae* are thought to produce a bacterial esterase, which in the presence of oxygen will hydrolyse indoxyl (a breakdown product of tryptophan) to an indigo colour (Lastovica and Skirrow 2000).

To prepare the test reagents a 10% (w/v) solution of indoxyl acetate (13500, Sigma) in acetone was prepared and used to saturate filter paper strips (1 cm x 4 cm) (which were stored in the dark at 4°C). To carry out the test a loopful of bacteria was rubbed onto a small area of the strip which was then covered with 200 µl of distilled water. After 10 min, a dark blue/indigo colour indicated a positive result (e.g. *C. jejuni*) and no colour change

indicated a negative result (e.g. *C. fetus*). This test was used to screen the whole of the CAMPYCHECK strain set and thereafter was used as an additional aid to confirm the identity of presumptive *Campylobacter* isolates recovered from samples.

2.5.4.5. The Urease Test

Hydrolysis of urea by the enzyme urease (present in *Helicobacter* species but not most *Campylobacter* species) releases the end product ammonia. The alkalinity causes the indicator phenol red to change from yellow to pink (Lastovica 2006). Christensen's Urea Agar (5%) was prepared by supplementing 25 ml of a 40% Urea solution (Oxoid SR20) to 475 ml Urea Agar Base (Oxoid, CM53) and pouring 25 ml plates. To carry out the test, a loopful of culture was stabbed into the agar and a rapid colour change from yellow to bright pink indicated a positive result. This test was evaluated against the CAMPYCHECK strain set and thereafter was used to deduce the identity of presumptive *Campylobacter* isolates recovered from samples, as a differential test between *Campylobacter* and *Helicobacter* species.

2.5.4.6. Hippurate Hydrolysis

Hippuricase is thought to hydrolyse hippurate to benzoic acid and glycine (Lastovica 2006). Glycine is then deaminated by the oxidising agent, ninhydrin. Hippurate hydrolysis is one of the main phenotypic tests used to differentiate *C. jejuni* from *C. coli* (On 1996). Ninhydrin in turn becomes reduced in the process and the end products of the ninhydrin oxidation form a purple-coloured dye.

To carry out the test, 1 ml hippurate solution (25 ml of 5% (w/v) hippuric acid stock solution (sodium salt (Sigma H 9380) added to 100 ml distilled water and filter sterilised) in glass tubes was heavily inoculated with a loopful of culture taken from a 48-72 h ABA plate. This was incubated overnight and subsequently 0.5 ml of ninhydrin solution (3.5 g ninhydin (Merck 6762) in 100 ml of 50:50 butanol in acetone) was added to the overnight culture and results read after 10 min. A purple colour indicated a positive result, whilst a colourless or light purple colour indicated that the test was negative. This test was carried out on all of the CAMPYCHECK strain set, however due to unexpected results (discrepancies of results observed to results from previous studies i.e. false positive and negative results (On 1996)), the test was not used in the testing of presumptive isolates.

2.5.4.7. Test for Growth in an Aerobic Atmosphere at Room Temperature

The only species within the *Campylobacteraceae* which should grow in an aerobic environment at room temperature are the *Arcobacter* species (Lastovica and Skirrow 2000).

To carry out the test, colonies were streaked onto ABA (5% blood) (5 per plate) and incubated in an aerobic environment (on the bench) at 25°C for 48 h. This test was evaluated against the CAMPYCHECK strain set and thereafter was used to differentiate between *Campylobacter* and *Arcobacter* from presumptive *Campylobacteraceae* isolates recovered from samples.

2.5.4.8. The Miniaturised Biochemical ID Test Strips

The ID system used as part of the Cape Town protocol is based on several biochemical tests which are used to determine the species within the genus *Campylobacter* (Lastovica and Le Roux 2003; Lastovica 2006). Although this system is very effective for the identification of *Campylobacter* species, it is highly labour intensive, requiring a large number of reagents and media. Microgen Bioproducts attempted to reproduce these tests in a miniaturised well format. Three tests could not be miniaturised including the oxidase test, catalase test and indoxyl acetate test, which were included as off-well pre-screen tests. Miniaturised biochemical tests included on the strip included lysine decarboxylase, ornithine, arginine dihydrolase, aryl sulphatase, hippurate hydrolysis, nalidixic acid, pyrazinamidase, ONPG (o-nitrophenyl-β-D-galactopyranoside), nitrate, urease, citrate and glucose (Table 8). In collaboration with Microgen Bioproducts (Camberley, UK) the tests were supplied at various stages of development and were evaluated against the *Campylobacter* CAMPYCHECK reference strains. Unfortunately, there were problems encountered with optimisation of the test; therefore the tests were not used for identification of isolates from samples. The *Campylobacter* ID strip tests still require further optimisation and so further testing of these kits is currently ongoing at Microgen (Stuart Clark, Pers. Comm.).

Table 8. Description of Identification Tests Included on Microgens Prototype Biochemical Identification Microwell Strip (Prototype Microgen pack insert, 2006)

	Micro-well strip	Reaction	Positive	Negative
1	Lysine	Lysine decarboxylase – Bromothymol blue changes to green / blue indicating the production of the amine cadaverine.	Green/ Blue	
2	Ornithine	Ornithine decarboxylase – Bromothymol blue changes to blue indicating the production of the amine putrescine.	Blue	Yellow / Green
3	Arginine Dihydrolase	Arginine is converted to ornithine, ammonia and CO_2 by arginine dihydrolase resulting in an increase in pH and a change in colour of the bromothymol blue from green to blue. At 48 hours green reactions are negative.	Green/ Blue Blue	Yellow Yellow / Green
4	Aryl Sulphatase	The breakdown product of phenolphthalein forms a pink colour in the presence of sodium carbonate	Bright Pink	Straw Colour / Light Pink
5	Hippurate	Hippuricase hydrolyses hippurate to benzoic acid and glycine. Glycine is deaminated by the oxidising agent Ninhydrin. Ninhydrin becomes reduced in the process and the end products of the Ninhydrin oxidation form a purple coloured dye.	Purple	Colourless / light Purple
6	Nalidixic Acid	Naladixic acid inhibits the growth of some species the incorporation of Tetrazolium blue (a vital stain) produces a colour change from clear to blue for resistant isolates	Blue	Straw colour
7	Pyrazinamidase	Pyrazinamide is hydrolysed to free pyrazinoic acid which is then revealed by the addition of Ferrous Sulphate	Red / Cherry Red	Caramel / Yellow colour
8	ONPG	Hydrolysis - ONPG hydrolysis by B-galactosidase results in the production of yellow ortho-nitrophenol.	Yellow	Colourless
9	Nitrate	Nitrate is reduced to nitrite which forms a deep red complex after the addition of α-Naphthylamine and Sulphanilic Acid	Red	Yellow
10	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink / red	Pink / Red	Yellow
11	Citrate	Utilisation of Citrate (only carbon source) leading to a pH increase giving a colour change in Bromothymol blue from green to blue	Blue	Green
12	Glucose	Glucose fermentation – Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation	Yellow	Blue / Green

2.5.5. Molecular Identification by Fluorescence *in situ* Hybridisation using 16S rRNA Targeted PNA Oligonucleotide Probes

2.5.5.1. Thermophilic *Campylobacter* Probe

A PNA oligonucleotide probe had previously been developed for detection of *C. jejuni*, *C. coli* and *C. lari* (Lehtola *et al.* 2005). The sequence (5'-CCCTACTCAAACTTGT-3') was designated CJE195 due to its 16S rRNA sequence position in the *C. jejuni* NCTC11168 strain at bases 195-209. The probe was labelled with the fluorophore, tetramethylrhodamine (TAMRA), at the 5' end (N-terminus). This probe had been developed at the University of Southampton, therefore there was access to probe stocks. The specificity of this probe sequence was re-assessed using BLAST and Ribosomal Database Project II software to verify that since the time of probe development the sequence was still only specific for *C. jejuni*, *C. coli* and *C. lari* species, as more sequences would have been entered into the database since the time of development. The database results showed that the probe was still specific for these three species. A further evaluation of the probe was undertaken to assess the probe specificity against a number of the CAMPYCHECK strain set. This thermotolerant *Campylobacter* probe was used alongside the Latex Agglutination Test to identify thermotolerant *Campylobacter* species recovered from samples.

2.5.5.2. Slide Preparation and Hybridisation Procedure

Campylobacter species (or presumptive isolates) were resuscitated from frozen stocks onto ABA (5% laked horse blood) and incubated in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2, 7% H_2) for 48-72 h. They were then sub-cultured onto ABA without blood for a further 48-72 h. Lehtola *et al.* (2005) showed that blood contained in media may contribute to bacterial autofluorescence. The following method was used to carry out hybridisations unless otherwise stated.

The hybridisation protocol was as described by Lehtola *et al.* (2005). A loopful of bacteria was suspended in 350 µl PBS, vortexed, and 25 µl of the suspension placed on polytetrafluoroethylene (Teflon®)-coated multispot microscope slides (C. A. H. Hendley Ltd., England). The smear was air dried and gently passed through a flame several times. Before hybridisation, cells were fixed by immersing in 90% (v/v) ethanol for 10 min and allowed to air dry. Smears were covered with 25 µl of the PNA probe in a hybridisation solution and covered with cover slips. The hybridisation solution contained 10% (w / v)

dextran sulfate (Sigma), 10 mM NaCl (Sigma), 30% (v / v) formamide (Sigma), 0.1% (w / v) sodium pyrophosphate (Sigma), 0.2% (w/v) polyvinylpyrrolidone (Sigma), 0.2% (w / v) Ficoll (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (v / v) Triton X-100 (Sigma), 50 mM Tris HCl (Sigma) and 200 nM PNA probe. A cover slip was placed on the top of the slide to cover the sample smear and hybridisation solution. A control (hybridisation buffer without probe) was also included. The slide was then placed in a damp incubation chamber and transferred to a hybridisation oven. Slides were incubated for 90 min at 55°C. Following incubation, slides were washed in pre-warmed (55°C) washing solution containing 5 mM Tris Base (pH 10) (Sigma), 15 mM NaCl and 1% (v /v) Triton X-100 (Sigma) and incubated at 55°C for 30 min. After the washing step, slides were rinsed with sterile distilled water and allowed to air dry. Finally, the slides were mounted with one drop of non-fluorescence immersion oil (Fluka Chemika), covered with coverslips and stored at 4°C in the dark for no longer than 48 h before analysis. The slides were viewed by epi-fluorescence (EF) microscopy (with red light filter) using a x 100 oil immersion objective (final magnification x 1000) (ME600 Nikon microscope, Best Scientific, UK). UV illumination was provided by a mercury lamp (X-Cite™ 120 Fluorescence illumination system, Best Scientific, UK) and images were captured using a Q-imaging camera and analysed using Image Pro Plus 5.0 imaging software. Hybridisation was measured by making a subjective estimate of signal intensity in 4 arbitrary classes: +, ++, +++, ++++. + = low signal, ++++ = high signal.

2.5.5.3. Development of *Campylobacteraceae* Probe

2.5.5.4. Probe Sequence Design

A literature search for previously used *Campylobacter* rRNA targeted probes revealed a sequence that specifically detected members of the genus *Campylobacter* (Schmid *et al.* 2005). The 16S rRNA targeted probe (CAMP653) sequence 5'-CTGCCTCTCTCCCTYACTCT-3' is situated at binding positions 653-670. This probe had been developed for use as a DNA probe, therefore the sequence had to be adapted and assessed for its suitability for use as a PNA probe. The sequence was shortened to 15 nucleotide bases (probe length generally required to be 12-18 bases long (Stender *et al.* 2002)). The final sequence selected for use was 5'-CTGCCTCTCTCCCTYAC-3'. The sequence specificity of this probe was assessed against other bacterial ribosomal sequences using database search software on the Ribosomal Database Project II website using a sub-program called 'Probe Match' (http://rdp.cme.msu.edu/probematch). There were 240

sequence matches (out of 226159 bacteria) all found within the *Campylobacteraceae* family. Although 230 sequence matches were found within the genus *Campylobacter*, 10 direct hits were also found within the genus *Sulphurospirillum* which is still a member of the *Campylobacteraceae* family.

2.5.5.5. Probe Synthesis and Labelling

The chosen sequence was evaluated for its suitability as a PNA probe (i.e. purine content and melting point) using the 'PNA Probe Designer' software freely available in the public domain at the Applied Biosystems website (http://www.appliedbiosystems.com/support/pnadesigner.cfm). It was found to be suitable and a green fluorophore dye was selected to attach to the probe sequence so it could be used in conjunction with the thermotolerant *Campylobacter* probe (Lehtola *et al.* 2005) which was attached to a red/orange fluorophore (TAMRA).

The green fluorophore, Alexa fluor 488 (Invitrogen), was selected for use based on its described advantages compared to other green fluorophores such as FAM (6-carboxyfluorescein). These include significantly better photostability allowing more time for observation and image capture, pH insensitive fluorescence between pH 4-10 and its solubility in water (therefore no organic co-solvents are required in labelling reactions).

Synthesis of the oligonucleotide probes was carried out by Professor Tom Brown (School of Chemistry, University of Southampton, UK). The PNA oligomer was synthesised on an Expedite™ DNA/PNA synthesiser (ABI Expedite™ 8909, AME Bioscience) using a 2.0 micromole synthesis cycle and Fmoc PNA chemistry. They were labelled in solution post-synthetically using the NHS (N-hydroxysuccinimide) esters of Alexafluor 488 and purified using reversed-phase high performance liquid chromatography (HPLC).

Preliminary tests of the synthesised probe against a panel of *Campylobacter* species (*C. jejuni* (31), *C. coli* (1), *C. concisus* (4), *C. upsaliensis* (49) and *C. lari* (38)) and other non-target bacteria (*Salmonella* spp., *Pseudomonas spp. Enterobacter cloacae)* revealed that the use of this probe was ineffective, possibly due to incompatibility linking the Alexafluor dye to the PNA sequence. Additionally, results showed that there was extremely bright green autofluorence of the bacteria. Due to these problems no further time was spent attempting to optimise the probe conditions. As an alternative, the probe was labelled with TAMRA (tetramethyl-6-carboxyrhodamine) dye (as this fluorophore had

previously been successfully used with the thermophilic *Campylobacter* PNA probe (Lehtola *et al.* 2005) and it was considered that probe yield would be much higher.

2.5.5.6. Optimisation of PNA Oligonucleotide Probe Conditions

2.5.5.6.1.1. Initial Evaluation of *Campylobacter* genus probe

Initially, the probe was tested on seven *Campylobacteraceae* strains (using the method described in section 2.5.5.2). This resulted in successful hybridisation as indicated by strong fluorescence. However, subsequent testing of the probe against a number of non-*Campylobacteraceae* strains, used as negative controls, also showed similar hybridisation results with the probe.

2.5.5.6.1.2. Media and Autofluorescence

There were problems with autofluorescence, especially in the green UV filter channel. Therefore, a number of media were tested for their effects on autofluorescence. Bacteria were sub-cultured from ABA (supplemented with blood) onto CCDA (no selective supplement), ABA (no blood) and TBA (no blood), incubated for 48 h and then hybridisations carried out. However, high autoflourescence remained on the control slides. Therefore, cultures were resuscitated from frozen stocks directly onto CCDA and with repeated sub-cultures onto CCDA (from blood plates). The green autofluorescence was reduced by repeated subcultures onto CCDA and resuscitating frozen stocks directly onto CCDA and therefore CCDA (without selective supplement) was used when preparing cultures for FISH analysis.

2.5.5.6.1.3. Temperature of Hybridisation

Preliminary experiments showed that there was strong non-specific binding with other non-target bacteria. Therefore, a number of different hybridisation temperatures were evaluated, in an attempt to try and increase the stringency. Temperatures were evaluated at 5°C increments from 55°C to 70°C. For this experiment, two *Campylobacter* species (*C. jejuni* and *C. upsaliensis*) were used as positive controls, and *Pseudomonas* and *Shigella* as negative controls.

2.5.5.6.1.4. Length of Hybridisation

To increase the specificity of binding the hybridisation time was reduced from 90 min to 60 min, 30 min and 15 min. For this experiment two *Campylobacter* species (*C.*

jejuni and *C. upsaliensis*) were used as positive controls and *Pseudomonas* and *Shigella* as negative controls.

2.5.5.6.1.5. Formamide Concentration

Formamide is an organic solvent and is included in the hybridisation buffer to reduce the thermal stability of double-stranded polynucleotides, allowing hybridisation to be performed at lower temperatures. It is thought that if the formamide concentration was decreased, the stringency of binding would increase. To evaluate the effect of formamide concentration, the hydridisation solution was supplemented with different concentrations of formamide: 0%, 15%, 20%, 25%, 30%, 35%, 40% and 50%. For this experiment two *Campylobacter* species (*C. jejuni* and *C. upsaliensis*) were used as positive controls and *Pseudomonas* and *Shigella* as negative controls.

2.5.5.6.1.6. Washing Buffer (temperature, detergent and salt concentration)

As non-specific binding remained a problem, despite alterations to hybridisation conditions, experiments were carried out to further increase stringency conditions. Steps included increasing the washing temperature and increasing the NaCl and detergent concentration. Concentrations of 1%, 2% and 3% of triton X-100 included in the washing buffer were evaluated. Sodium chloride concentration at 15 mM, 50 mM and 150 mM was also evaluated. In addition, increasing the washing temperature from 55°C to 60°C was investigated. Strains used to test these conditions included *C. jejuni* and *Pseudomonas* species.

2.5.5.6.1.7. Use of RNase to Test for Specific RNA Binding

A number of variables had been tested in an attempt to reduce non-specific binding with limited success. It was postulated that the PNA oligonucleotide probe sequence may be binding to other structures and nucleic acids within the cell other than RNA. To address these concerns, culture suspensions were pre-treated with RNase to degrade RNA prior to hybridisation. Culture suspensions were prepared as normal, except cells were then centrifuged (5000 x g for 10 min) (Biofuge Pico, Hereus, UK) to pellet the cells which were re-suspended in an RNase buffer solution (0.5 mg/ml RNase T1 (Promega), 100 mM EDTA (Sigma) and 100mM Tris-base (Sigma), pH 8). The cells were incubated for 20 min at 37°C. After incubation the suspension was centrifuged again to pellet the cells which were re-suspended in sterile distilled water. This wash step was repeated once and 25 µl of the cell suspension placed on a microscope slide to air dry. Cells were briefly heat fixed

onto the slide and hybridisations carried out as previously described (section 2.4.5.2). Parallell control experiments were also carried out alongside this experiment where the described procedure was repeated but without RNase included in the buffer. In this experiment two *Campylobacter* species (*C. jejuni* and *C. upsaliensis*) were used as positive controls and *Pseudomonas* and *Shigella* as negative controls.

2.5.5.6.1.8. BSA as a Blocking Agent to Prevent Non-Specific Binding

To reduce non-specific binding of the probe to other potential intracellular structures such as cellular proteins, amino acids or other nucleic acids, Bovine Serum Albumin (BSA) (Sigma) at a concentration of 1% (w/v) was included in the hybridisation buffer, in an attempt to reduce non-specific binding. In this experiment two *Campylobacter* species (*C. jejuni* and *C. upsaliensis*) were used as positive controls and *Pseudomonas* and *Shigella* as negative controls.

2.5.6. Molecular Identification of Presumptive *Campylobacteraceae* Using PCR

Originally, its was planned to send live cultures of isolates to collaborators so accurate typing to the species level could be achieved using a number of molecular typing methods (including protein electrophoresis and AFLP analysis), which required growth of strains and sample preparation in highly standardised conditions (Duim *et al.* 2004). Unfortunately, there were complications with obtaining authorisation to send live cultures, therefore only heat killed preparations could be sent for ID confirmation. Due to this problem, multiplex-PCR identification of isolates recovered from salad vegetables and sewage was carried out by collaborators (Dr Kurt Houf, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium), as this technique did not require high quality DNA or standardised growth conditions.

2.5.6.1. Crude DNA Extractions

Crude cell lysates were prepared for PCR analysis using a simple method to heat kill and lyse the bacteria. Presumptive *Campylobacter* isolates were cultured onto ABA for 48 h in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2 and 7% H_2). Several colonies were removed from the surface of the plate and re-suspended in 1 ml of ultra-pure sterile distilled water to an OD_{595nm} of ~1.0. This culture suspension was then incubated in a waterbath at 95°C for 15 min to lyse the cells. These heat killed lysates were

then stored at -80°C until they were sent via airmail (with frozen ice packs) to collaborators (Dr Kurt Houf, Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University) for PCR identification.

2.5.6.2. Identification of *Campylobacter* and *Arcobacter* species using PCR

Campylobacter and *Arcobacter* genus-specific multiplex-Polymerase Chain Reaction (m-PCR) analysis was performed as described by Linton *et al.* (1996) and Harmon and Wesley (1997), respectively. Arcobacters were identified to the species level using the multiplex-PCR procedure, described by Houf *et al.* (2000). *Campylobacter jejuni* and *Campylobacter coli* were differentiated from other *Campylobacter* species using the multiplex-PCR procedure described by Vandamme *et al.* (1997). If campylobacters were identified using the genus specific primers, but were not identified as *C. jejuni* or *C. coli* by species specific primers they were considered non-thermotolerant or emerging *Campylobacter* species.

2.5.7. Development of Novel Sub-Typing Method using a Proteomic Approach

A two dimensional gel electrophoresis approach (proteins separated by charge and molecular weight) was investigated for the identification of *Campylobacter* species by comparative protein profiling. The 2D gel approach also allowed for the further down stream analysis of proteins of interest which could be excised and identified, providing information regarding metabolism, structure and pathogenesis.

2.5.7.1. Sample Preparation

Selected strains from the CAMPYCHECK strain set, including *C. jejuni* NCTC 11168, *C. concisus* CCUG 13144, *C. lari* RM2100, *C. upsaliensis* RM3195, *C. hominis* CH001 and *C. coli* RM2228, were resuscitated from PROTECT™ beads stored at -80°C. Cultures were plated directly onto ABA (Oxoid) (5% laked horse blood) and incubated at 37°C for 48 h in MACS workstation (Don Whitley Scientific) containing 3% oxygen, 7% hydrogen and 10% carbon dioxide. The selected strains were subsequently sub-cultured onto fresh ABA plates in the same conditions for a further 48 h. Samples for each species were prepared in duplicate in order to a) prepare samples for 2D gel analysis and b) determine the protein concentration of samples. Loopfuls of the selected cultures were swept from the surface of four pooled plates using a 10-µl loop and re-suspended in 1 ml

of 100 mM Tris buffer (pH 7.5) at 4°C. The cells were then centrifuged at 5000 x g for 10 min to pellet the cells, which were re-suspended in 1 ml 100 mM Tris buffer. This wash step was then repeated. The cells were finally re-suspended in 1 ml of freshly made lysis buffer (400 µl Triton x-100 (Sigma), 400 µl ß-mercaptoethanol (Sigma), 400 µl ampholines (pH4-7) (Amersham Biosciences, UK), 28 mg PMSF (phenyl methyl sulfonyl fluoride) (Sigma) made up to a final volume of 16 ml with distilled deionised water) and sonicated on ice using a XL-2020 Sonicator (Misonix Inc., UK) with a mini-probe (cycle included 6 x 30 s sonication bursts with 15 s breaks in between). Solid urea was then added to these samples to a final concentration of 9 M. A 200 µl aliquot of each sample was then snap frozen in liquid nitrogen and stored at -80°C. Protein concentrations were estimated using the Bradford protein assay (Bio-Rad) on replicate culture samples suspended in 30% acetonitrile instead of lysis buffer (as components of the lysis buffer were incompatible with the protein assay).

2.5.7.2. Rehydration and Separation by Iso-electric Focusing

Protein samples were added to rehydration solution (1.2 g urea (Sigma), 50 mg CHAPS (Sigma), 50 µl 1 M DTT (dithiothreitol) (Sigma), 50 µl IPG (Immobilised pH gradient) buffer, pH 4-7 (Amersham Biosciences), 50 mg Orange G (Sigma) made up to 2.4 ml analytical grade water) so the final protein concentration loaded onto the IPGphor 18-cm strip holder (Amersham Biosciences; catalog no. 80-6417-44) was 0.5 mg in 400 µl. Once the sample had been loaded onto the strip holder, the Immobiline™ DryStrip gel (Amersham Biosciences) was placed face down onto the strip holder containing the sample, making sure the positive end of the gel was aligned toward the pointed end of the chamber. The dry strip and sample rehydration solution were then overlaid with 1.5 ml of paraffin oil and strip holder lids placed on top. The strips were then placed onto the IPGphor tank (Amersham Biosciences) and the rehydration and separation steps run for 25 h. The strips were then removed and excess oil drained before wrapping in clingfilm and freezing at -80°C for at least 10 min or overnight.

2.5.7.3. Second Dimension

The second dimension gels (separation by molecular weight) were run using a horizontal Multiphor™ II Electrophoresis Unit (Amersham Biosciences). Initially cooling towers were set at 15°C and the pre-cast gel (ExcelGel™ gradient XL 12-14, Amersham Biosciences) was positioned between columns 1-17 at the top of the tank. ExcelGel SDS

buffer strips (anode and cathode) (Amersham Biosciences) were then applied to the gel to ensure good contact between the gel and electrodes. A series of preparative wash steps were carried out on the rehydrated IEF separated sample strip before running the second dimension. An equilibrium solution (36 g urea, 1.0 g SDS (sodium dodecyl sulphate), 10 ml 500 mM Tris-HCl (pH 6.8) and 30 ml glycerol made up to 100 ml with analytical grade water) was prepared, forming the basis of the solution for the three wash steps. To carry out the first wash the IEF-separated sample strip was removed from the -80°C freezer to thaw and placed in a 12 ml glass tube containing 10 ml of equilibrium solution (with 160 µl DTT). This was sealed and placed on a rocker (Gyro rocker STR 9, Stewart UK) at 10 rpm for 15 min. The liquid was removed and for the second wash, 10 ml of equilibrium solution alone was added to the glass tube and placed on a rocker at 10 rpm for a further 15 min. The liquid was removed and replaced with 10 ml of a third wash solution (equilibrium solution with 0.45 g iodoacetamide and 50 mg bromophenol blue) and placed on a rocker at 10 rpm for 15 min. After the three wash steps the sample strip was placed gel side down on top of the pre-cast polyacrylamide gel, parallel to the centre of the clear SDS buffer strip. Two small pieces (0.5 cm^2) of filter paper (Whatman No.1) were placed at either end of the sample strip to prevent a distorted gel band. A third piece was placed just to the right of the clear buffer strip in column 18 onto which 10 µl of 10-250 kD protein marker (Rainbow™ Molecular Weight Markers, Amersham) was loaded. The gel was then run at 1000 V, 20 mA and 40 W for 45 min, after which the IEF sample strip and filter pieces were removed. The power was then adjusted to 1000 V, 40 mA and 40 W and run for a further 2 h 30 min, or until the dye front had moved into the orange SDS buffer strip at the end of the gel.

2.5.7.4. Fixing, Staining and Imaging

After separation by iso-electric focussing and electrophoresis, the gel was washed with 100 ml fixing solution (7% acetic acid, 10% methanol). The gel was then placed in a tray with 300 ml of fixing solution, covered with aluminium foil and placed on a rocker at 10 rpm for 30 min. The fixing solution was poured off and the gel rinsed with another 100 ml fixing solution. To stain, 300 ml of SYPRO ruby stain (Molecular Probes, Invitrogen, UK) was poured over the gel, covered with aluminium foil and placed on a rocker at 10 rpm overnight. After overnight staining, the gel was washed with 300 ml analytical grade water and left for at least 24 h before imaging on the VersaDoc imager (Versadoc 3000

Imaging System, Bio-Rad). The exposure time was kept constant on the gels that were to be compared.

The PDQuest® image analysis software was initially used to automatically count the number of spots on each of the gels and the manual tool was used to remove automatically detected spots that were in fact sample artefacts. It was then used to compare the 2D protein profiles and determine differential protein expression across the gels. The software was used to automatically align the gels and match the gel spots across the gels; however the manual match tool was required to match a number of protein spots manually.

2.5.7.5. Optimisation of 2D PAGE Method

2.5.7.6. Sample Preparation

Preliminary experiments included a wash step using PBS however this lead to horizontal streaking of proteins on the gels. This problem was then alleviated by using a Tris wash buffer (20 mM Tris-HCl (pH 7.5), which did not contain such high levels of ionic salts, which are thought to interfere with the isoelectric focusing step.

2.5.7.7. Evaluation of Total Protein Concentration Loaded onto Gel

C. *upsaliensis* sample preparations were used to directly compare loading 0.3 mg and 0.5 mg total protein onto the dry gel strip. Loading 0.3 mg total protein showed that some proteins on the gel were lost and loading more than 0.5 mg total protein lead to insufficient resolution of proteins due to overlapping of protein spots, therefore loading 0.5 mg protein was selected.

2.5.7.8. Comparison of Protein Gel Stains

Two dimensional gel electrophoresis of *C. jejuni* was run in duplicate and then stained with either Colloidal Coomassie brilliant blue (BioSafe™, Bio-Rad) or SYPRO Ruby stain (Molecular Probes, Invitrogen, UK). To stain with Colloidal Coomassie, 300 ml of this stain was poured over the gel to cover it and then placed on a rocker for 1 h, after which the gel was washed three times in analytical grade water and left 24 h to de-stain before image analysis. Staining with SYPRO Ruby was as described in section 2.4.7.4. SYPRO Ruby was found to be more sensitive for protein staining, therefore was selected for gel staining.

2.5.7.9. Choice of pI for Isoelectric Focussing

Running the isoelectric focussing step at a pH range of 3-10 instead of pH 4-7 was evaluated. *C. upsaliensis* was used to carry out this evaluation. Samples were prepared in duplicate, one of which was run at a pH range of 4-7 and the other at pH 3-10. Solutions (lysis buffer and rehydration solution) were prepared using IPG buffer (pH 3-10) instead of pH 4-7. In addition, Immobiline Dry-Strips (pH 3-10) were used instead of pH 4-7. Although at pH 4-7 some basic proteins were lost, a higher number of proteins could be resolved when compared to pH 3-10.

2.5.7.10. Identification of Spots using Tandem Mass Spectrometry

After visualisation with Colloidal Coomassie brilliant blue, protein spots were excised using a robotic spotcutter (BioRad Laboratories). In addition, due to problems encountered with the automated spot cutter, a scalpel was used to manually excise the spots from the gel which were placed into wells on microtitre plates. Protein samples were then subjected to *in situ* trypsin digestion using the method of Shevchenko *et al.* 1996. The resulting peptides were separated by Nano- reversed phase liquid chromatography, using a Waters C18, 3 µm, 100Å (150 mm x 75 µm, i.d.) column, and electrosprayed into a quadrupole time-of-flight tandem mass spectrometer. Separation was performed over 60 mins with a gradient of 0–85 % B (Buffer A: 5% acetonitrile in water containing 0.1% formic acid; Buffer B: 95% acetonitrile in water containing 0.1% formic acid).

All data were acquired using a Q-tof Global Ultima (Waters Ltd) fitted with a nanoLockSpray™ source to achieve better than 10 ppm mass accuracy. A survey scan was acquired from *m/z* 375 to 1800 with the switching criteria for MS to MS/MS including ion intensity and charge state. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide.

All MS/MS spectra were automatically processed and searched against a FASTA formatted listing of protein sequences from the NCBI non-redundant database (June, 2005 versions), using ProteinLynx Global Server 2.05. Proteins were only assigned if, for each peptide ion, greater or equal to three experimentally derived *y* ions could be matched to the predicted spectra.

All MS/MS proteomic analysis was carried out by Paul Skipp at the Centre for Proteomic Research, University of Southampton, UK.

2.6. DEVELOPMENT OF RECOVERY METHODS FOR SALAD VEGETABLES AND WASTE WATER

2.6.1. Rationale for Recovery Methods

As this PhD study forms part of an international collaborative project, a certain level of standardisation had to be adopted by all CAMPYCHECK partners. Although methods for recovery of *Campylobacter* will vary according to the type of sample in question, certain criteria were set (based on experiments in previous chapters and from research from other CAMPYCHECK partners) which had to be followed. Research from previous experiments (see Culture Development results section) revealed that the optimal gas environment for all *Campylobacteraceae* species was 3% oxygen, 7% hydrogen and 10% carbon dioxide and the isolation medium Anaerobe Basal Agar (ABA) supplemented with 5% blood, therefore these were chosen for use in sample recovery experiments.

The National Food Centre (Dublin, Ireland) carried out a large amount of research into the evaluation of broths and recovery times for the growth of *Campylobacteraceae*. They concluded that CEB (*Campylobacter* enrichment broth (also known as Bolton broth)) with FBP and 5% blood was optimal for resuscitation and recovery from samples for a period of 24 h and that Hunts broth was optimal for the growth and maintenance of cultures (Table 9). The use of a 0.6 μm filter (as used in the Cape Town protocol (Lastovica 2006)) was recommended as a selective step, despite the reduction in sensitivity of recovery. Maximum Recovery Diluent (MRD) was the agreed diluent for preparing serial dilutions of cultures and samples. Preliminary experiments (data not shown) compared MRD to PBS but revealed no significant difference so MRD was chosen. It was also decided that three positive control species would be used to spike samples alongside all sampling experiments carried out. These were *C. jejuni* NCTC 11168, *C. concisus* CCUG 13144 and *C. hominis* NCTC CH001. *C. jejuni* was thought to represent the well known clinically important thermotolerant *Campylobacter* species, which will grow well in any standard microaerobic conditions. *C. concisus* is a slightly more fastidious and sensitive species, requiring hydrogen for growth and *C. hominis* is representative of the slow growing *Campylobacter* species which generally prefer anaerobic conditions.

The final recovery methods for both salad vegetables and sewage sludge included both a presence/absence and direct plating method (Figure 13).

Table 9. Composition of Enrichment Broths used for Recovery of *Campylobacteraceae.* *Although it is generally recomended that CEB is supplemented with antibiotic selective supplement (Bridson 1998), it was not included during these studies.

Broth Type	Campylobacter Enrichment broth (Lab M)	Hunt and Radle Broth (Oxoid)
Basal Components (g/l)		25 g nutrient broth no. 2 (CM67, Oxoid), 6.0 g yeast extract (L21, Oxoid).
pH	7.4 ± 0.2	7.4 ± 0.2
Supplements	50 ml laked horse blood (Oxoid)	FBP supplement (Campylobacter growth supplement; SR84; Oxoid) 50 ml laked horse blood (Oxoid)

2.6.2. General Recovery Methods

2.6.2.1. Preparation of Inocula for Spiking

Positive controls which were run along all recovery experiments included *C. concisus* (CCUG 13144), *C. hominis* (NCTC CH001) and *C. jejuni* NCTC 11168) which were spiked at approximately $\log_{10}$ 4 cfu/g. Negative controls included 100 µl of MRD or 100 µl CEB (Lab M).

To prepare positive control cultures to spike samples, cultures were inoculated onto ABA and incubated for 48 h in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2 and 7% H_2). They were then sub-cultured onto fresh ABA plates for a further 48 h. A 1 µl loopful of this culture was inoculated into 30 ml of Hunts broth (Hunt and Radle enrichment broth, BAM M29, Oxoid) supplemented with 5% blood and FBP (ferrous sulphate, sodium metabisulphate and sodium pyruvate; Campylobacter growth supplement, SR84, Oxoid) and incubated in the MACS workstation (3% O_2, 10% CO_2 and 7% H_2) for 72 h. After incubation a 1 ml aliquot of this culture was diluted into 100 ml of Hunts broth (Oxoid) (supplemented with 5% blood and FBP) for 24 h (a control sample with Hunts was also included). Following incubation, the culture suspensions were inverted several times to homogenise the liquid culture which was then equally distributed into two 50 ml centrifuge tubes and centrifuged at 4°C for 15 min at 5000 x g (Legend bench top centrifuge, Sorvall). The pellet was washed by re-suspending in 10 ml of MRD, which was carried out twice. Finally the pellet was re-suspended in 45 ml of MRD (the neat spike). To enumerate this culture suspension, serial dilutions were prepared (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) by diluting 1 ml into 9 ml MRD. A 100 µl aliquot of each of these dilutions (10^{-3}, 10^{-4}, and 10^{-5}) was inoculated onto ABA (5 % blood) in triplicate and

incubated in the MACS workstation for 48 h, after which the colony forming units were counted. A 10 ml aliquot of this culture suspension was used to evenly spike a 25 g sample (to give a final inoculum of approx. $\log_{10}$ 4 cfu/g). The spiked sample was then lightly shaken for 10 s to ensure even sample coverage and left in the MACS workstation for 30 min so sample attachment could take place before the recovery method was initiated.

2.6.2.2. General Presence/Absence Enrichment Method

A 25 g (or 25 ml) sample was placed into a filtered stomacher bag (VWR International, UK) (and either spiked with the three positive control strains or left unspiked). The sample was then diluted with 225 ml of CEB (Campylobacter Enrichment Broth, Table 9) (supplemented with 5 % blood) with or without supplementation with VAT selective supplement. The sample suspension was then pulsified (Pulsifier®, Microgen Bioproducts Ltd, UK) for 15 s to detach the bacteria from the sample into suspension. The sample suspensions were then incubated in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2 and 7% H_2) for 18-24 h (an enrichment step to revive stressed cells). After the 24 h resuscitation step, the samples were gently massaged by hand for 10-15 s to homogenise the enriched sample suspension. Serial dilutions (10^{-1} to 10^{-3}) of this suspension were prepared by adding 1 ml to 9 ml MRD. Aliquots of 200 µl of these diluted sample suspensions were then either directly plated or filtered, using the Cape Town method (Lastovica 2006) onto ABA (5% blood) with and without VAT selective supplement. The samples that were inoculated using the Cape Town filtration method were placed onto a 0.6 µm pore size, 47 mm diameter, mixed cellulose ester membrane (ME26, Schleicher and Schuell) in triplicate using a spiral action (dropping suspension onto various points of the membrane to avoid flooding). After 15 min the filter was carefully removed using sterile forceps and a sterile hockey stick spreader used to spread the solution evenly over the entire plate. Samples which had been inoculated directly onto the plates were spread with sterile plastic disposable spreaders and left 15 min to dry in the laminar flow cabinet. The plates were then inverted and incubated for 48 h in an atmosphere provided using the MACS workstation (3% O_2, 10% CO_2 and 7% H_2). Plates were also analysed after 72 h and 96 h to check for growth of new colonies.

2.6.2.3. General Enumeration Method (Direct Plating)

Although a presence absence method is the generally accepted method for isolating *Campylobacter* species from food, a method for enumerating the number of

Campylobacteraceae also needed to be developed so collaborating risk assessment statisticians (RIVM, The National Institute for Public Health and the Environment, Netherlands) could use the data effectively.

A 25 g (or 25 ml) sample (spiked with *C. jejuni*, *C. concisus*, *C. hominis* or left unspiked) was diluted into 225 ml MRD and pulsified for 15 s. Serial dilutions were prepared by adding 1 ml to 9 ml (10^{-1} to 10^{-5}). Aliquots of 200 µl were then directly plated onto ABA (with or without VAT) and spread over the plate using a plastic disposable hockey stick spreader. Plates were then incubated in the MACS workstation for 48 h, after which colonies with *Campylobacter*-like morphology (CLM) were counted and colonies confirmed.

2.6.2.4. Confirmation and Identification of Presumptive *Campylobacteraceae* Isolates

Following incubation, colonies with *Campylobacter*-like morphology (CLM) were counted and five colonies randomly selected for further identification. The isolates were picked and sub-cultured onto fresh ABA plates (5 per plate and up to 30 per sample if isolated colonies across all dilutions were evaluated). The plates were then incubated for a further 48 h before subsequent tests could be performed. Primary tests that were carried out after initial isolate sub-culture included; the KOH, L-ala, and oxidase test (Figure 10). Presumptive *Campylobacteraceae* isolates were then sub-cultured (2 isolates per plate) for a further 24-48 h in the MACS workstation so further identification tests could be carried out. Presumptive isolates were also sub-cultured onto ABA (5% blood) (5 isolates per plate) to test for aerobic growth at room temperature (test for *Arcobacter* species). The prototype latex agglutination test was also carried out to further group isolates into thermotolerant and 'non-thermotolerant' *Campylobacteraceae* and EDIC microscopy to assess cellular morphology. Other secondary biochemical tests included oxidase, indoxyl acetate, catalase and the urease test. All presumptive *Campylobacteraceae* isolates were transferred to beads and stored at -80°C for further molecular identification. Detailed experimental procedures for identification methods are described in section 2.5.

2.6.3. Optimisation of Presence/Absence Method for Salad Vegetables

2.6.3.1. Evaluation of VAT Selective Supplement and Cape Town Filtration

In these experiments two types of sample were analysed including unprocessed and processed watercress, purchased from a local green grocer and a local supermarket

respectively. Preparation and enumeration of spike cultures was carried out as described in the previous section, 2.5.2.1. The recovery experiment was carried out as described in section 2.5.2.2 with the following modifications; samples were diluted in CEB with and without VAT selective supplement and then, when inoculating these samples onto plates, serial dilutions of the sample suspensions were plated onto ABA with and without VAT selective supplement in duplicate, both by direct plating and filtering through a 0.6 μm pore-size membrane.

2.6.3.2. Evaluation of a Centrifugation Step for Sample Clarification

It was thought that the sensitivity of the method might have been reduced due to particulate matter in the sample leading to clogging of the 0.6 μm pore-size mixed cellulose ester membrane filter. Therefore, it was thought that including a low speed centrifugation step might clarify the sample and allow an increased number of bacteria through the filter. The method was carried out as described in section 2.5.2.2 with the following modifications. After the 24 h enrichment step, the enriched sample was gently homogenised by hand and a 10 ml aliquot centrifuged at 800 x g for 10 s at 4°C in a 15 ml centrifuge tube (120 x 17 mm; code 62.554.502; Sarstedt, Numbrecht, Germany). Serial dilutions from this supernatant were then prepared in MRD. Aliquots of this centrifuged suspension and the pre-centrifuged sample suspension were serially diluted (1 ml into 9 ml MRD) then 200 μl either filtered through a 0.6 μm membrane or directly plated onto ABA (with and without VAT).

2.6.3.3. Sensitivity of Detection of the Presence/Absence Method

These experiments were carried out as described in section 2.5.2.2, however, instead of spiking at the standard spike concentration of 10^4 cfu/ml, extra serial dilutions were included so that 25 g watercress samples were spiked at concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 cfu/25g of *C. jejuni*, *C. concisus* and *C. hominis*. Following enrichment, plates were inoculated in duplicate onto ABA VAT at 10^{-1} and 10^{-2} for filtered plates and 10^{-3} and 10^{-4} for plates that were directly inoculated.

2.6.3.4. Development of Enumeration Method including Vacuum Filtration

It was thought that a vacuum filtration method would be more suitable than direct plating for enumerating recovery of *Campylobacter* species from the samples, since a

larger volume of sample can be analysed resulting in increased sensitivity. It was also initially thought that this method could be used in conjunction with a resuscitation step on filter pads so that an enrichment step could be included that still remained quantitative.

To evaluate this method 25 g of watercress sample was either spike inoculated with approx. 10^4 cfu/g of *C. jejuni*, *C. concisus*, *C. hominis* or left unspiked. This was then diluted into 225 ml MRD and pulsified for 15 s. Serial dilutions were prepared (10^{-2} to 10^{-5}) of which 10 ml was vacuum filtered (using a vacuum pump (Applied Vacuum Engineering, Bristol, UK)) and filtration unit (Millipore, UK)) onto a 0.1 µm polycarbonate membrane (Whatman, UK). The filters were then removed from the filter housing using sterile forceps and placed onto either ABA (with and without VAT) (and incubated for 48 h) or onto filter pads pre-impregnated with 1.7 ml Campylobacter Enrichment broth (with and without VAT), which were incubated for 24 h before being transferred onto ABA VAT and incubated for a further 48 h. This method was also directly compared to the alternative enumeration method, where 200 µl of the sample dilutions were directly plated onto ABA and ABA VAT.

2.6.4. Recovery from Waste Water and Sewage Sludge

2.6.4.1. Evaluation of VAT Selective Supplement and Cape Town Filtration as Part of a Presence/Absence Method

These experiments were carried out to investigate the effect of VAT selective supplement addition in both the agar medium and the enrichment broth and filtering the sample onto plates in addition to direct plating. The method was carried out as described in section 2.5.2.2 with the following modifications. Serial dilutions of the enriched sample were plated onto ABA (with and without VAT) by direct plating (10^{-2} to 10^{-5}) and through a 0.6 µm pore size membrane filter (10^{-1} to 10^{-3}).

2.6.4.2. Direct Plating as an Enumeration Method

These experiments were carried out as described in section 2.5.2.3 using MAD and undigested sewage sludge as sample matrices. Twenty-five g of sample was diluted in 225 ml MRD, pulsified for 15 s and serial dilutions prepared by adding 1 ml to 9 ml MRD (10^{-1} to 10^{-5}). Aliquots of 200 µl were then directly plated onto ABA (with or without VAT) and spread over the plate using a plastic disposable hockey stick spreader. Plates were then incubated in the MACS workstation for 48 h and colonies with *Campylobacter*-like morphology (CLM) counted and confirmed.

2.6.4.3. Vacuum Filtration as an Enumeration Method

Digested sludge (MAD) was used to carry out these experiments. A 25 g sewage sludge sample was either spike inoculated with approx. 10^4 cfu/g of *C. jejuni*, *C. concius*, *C. hominis* or left unspiked. This was then diluted into 225 ml MRD and pulsified for 15 s. A centrifugation step was included (section 2.3.2.1.2). Serial dilutions were prepared (10^{-2} to 10^{-5}) and 10 ml of each dilution was vacuum filtered in duplicate (Applied Vacuum Engineering, Bristol, UK) and filtration Unit (Millipore, UK) onto a 0.1 μm polycarbonate membrane (Whatman, UK). The filters were then removed from the filter housing using sterile forceps and placed onto ABA (with and without VAT) and incubated for 48 h or onto filter pads previously saturated with 1.7 ml Campylobacter Enrichment broth (with and without VAT), which were incubated for 24 h before being transferred onto ABA VAT and incubated for a further 48 h. This method was also directly compared to direct plating, where 200 μl of the sample diluted in MRD (10^{-2} to 10^{-5}) was directly plated onto ABA and ABA VAT.

2.6.5. Use of Optimised Enumeration and Presence/Absence Methods to Assess the Prevalence of *Campylobacteraceae* in Sewage Sludge and Salad Vegetables

2.6.5.1. Salad Vegetable Samples

The salad vegetable samples that were evaluated were either bought from local supermarkets and green grocers or a donation from Vitacress Salads Ltd, Hampshire, UK. The principle sample that was investigated was watercress, due to its widespread prevalence across Hampshire (UK), its increased popularity in the diet as a raw salad vegetable, and due to its increased risk of contamination due to waterborne contaminants (as it is grown underwater in irrigation channels). Unprocessed samples were obtained from a local green grocer shop. The watercress samples were transported at 4°C until processing, which was within 24 h of collection. The processed salad vegetable samples received from Vitacress had gone through various processing steps including washing in borehole water, drying in warm air (60°C) and packaging in a modified atmosphere (high N_2). Unprocessed salad vegetable samples were either purchased from local green grocery shops (samples had been freshly hand picked from the field that morning), or provided by Vitacress (samples that had come straight from Vitacress farms without processing). Samples were transported in a cool box with ice packs and then stored at 4°C until

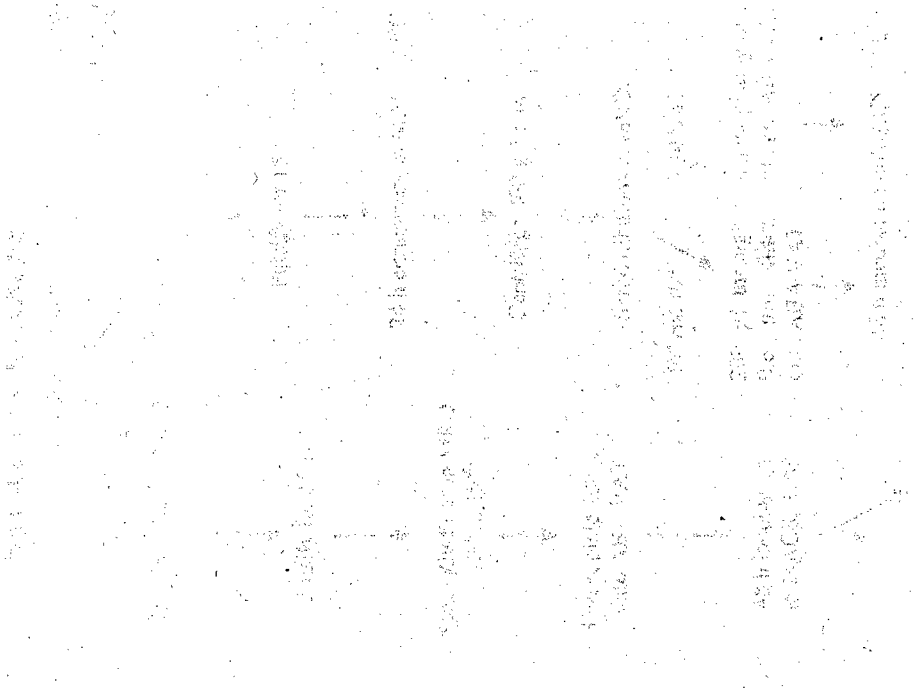
analysis, which was within 24 h of collection. Salad vegetables investigated other than watercress included spinach, wild rocket (lettuce) and red chard (lettuce). The source of the salad vegetables was principally from Vitacress farms in the UK but also Portugal and the USA. An equal number of unprocessed (harvested straight from the field) and processed (washed and ready-to-eat) samples were analysed during the study. Details of samples evaluated and dates analysed are shown in the results section of Chapter 6 (Table 40).

The optimised method used for recovery of *Campylobacteraceae* from salad vegetable samples included both an enrichment method and enumeration by direct plating (Figure 13). The optimised methods used were as described in section 2.3.2.2 and 2.3.2.2, however CEB was used without VAT selective supplement and ABA was supplemented with VAT selective supplement. The centrifugation step for sample clarification was also included after enrichment before serial dilutions were prepared. When the enumeration method was used, samples were plated directly at dilutions of 10^{-1} to 10^{-4}. When the enrichment (presence/absence) method was used samples were filtered at dilutions of 10^{-1} and 10^{-2} and directly plated at 10^{-3} and 10^{-4}.

2.6.5.2. Southern Water Milbrook Wastewater Treatment and Sludge Recycling Centre

Millbrook waste water treatment centre receives and treats wastewater from a population of approximately 138,000 and in addition takes and recycles waste from other Southern water plants, equating to nearly 250,000 people in the area. The wastewater undergoes four stages of treatment and meets stringent European requirements before it is released into the Test Valley estuary. Eventually, the solid waste (sludge) that is removed from the wastewater cleaning process is treated using thermal driers to produce granules that can be used as a fertilizer and soil conditioner. The plant produces about 10,000 tonnes of fertilizer a year. When the wastewater is first received via the sewerage system it is treated at the inlet works to remove debris, grit and grease. It is then transferred to primary settlement tanks, where removal of solid waste (about 65%) occurs. Biological treatment in aeration lanes then removes organic pollutants and it is then passed to the final settlement tanks where any remaining solid particles are removed before the treated wastewater is returned to the estuary of the River Test. For recycling of the solid waste (sludge), it is heated in anaerobic digesters at a constant temperature of 35°C for 14 days to reduce bacterial numbers. Methane gas produced during this process is stored on site

before being recycled back into the works to provide heat for the treatment processes. Water is then removed from the digested sludge in a centrifuge and it is then dried to produce fertilizer granules which are sold commercially and distributed to various farms, golf courses and land reclamation sites. The principle samples used for evaluating recovery of *Campylobacteraceae* from sewage sludge were thickened sewage sludge separated from primary settlement tanks (pre-digested) and mesophilic anaerobically digested (MAD) sludge, which was taken from a mesophilic sequential batch reactor (SBR). Details of samples evaluated and dates analysed are shown in the results section of Chapter 6 (Table 42). The optimised method used for recovery of *Campylobacteraceae* from sewage sludge included a presence/absence and enumeration method are shown in Figure 13. The method was carried out as described in section 2.3.2.2 and 2.3.2.2, however CEB was used without VAT and ABA was supplemented with VAT. When the enrichment step was carried out, the centrifugation step as described in section 2.3.2.1.2 was included. Samples were plated out at 10^{-2} to 10^{-5} dilutions when plating directly and at 10^{-1} to 10^{-3} dilutions after enrichment, where all samples were filtered through a 0.6 μm membrane.



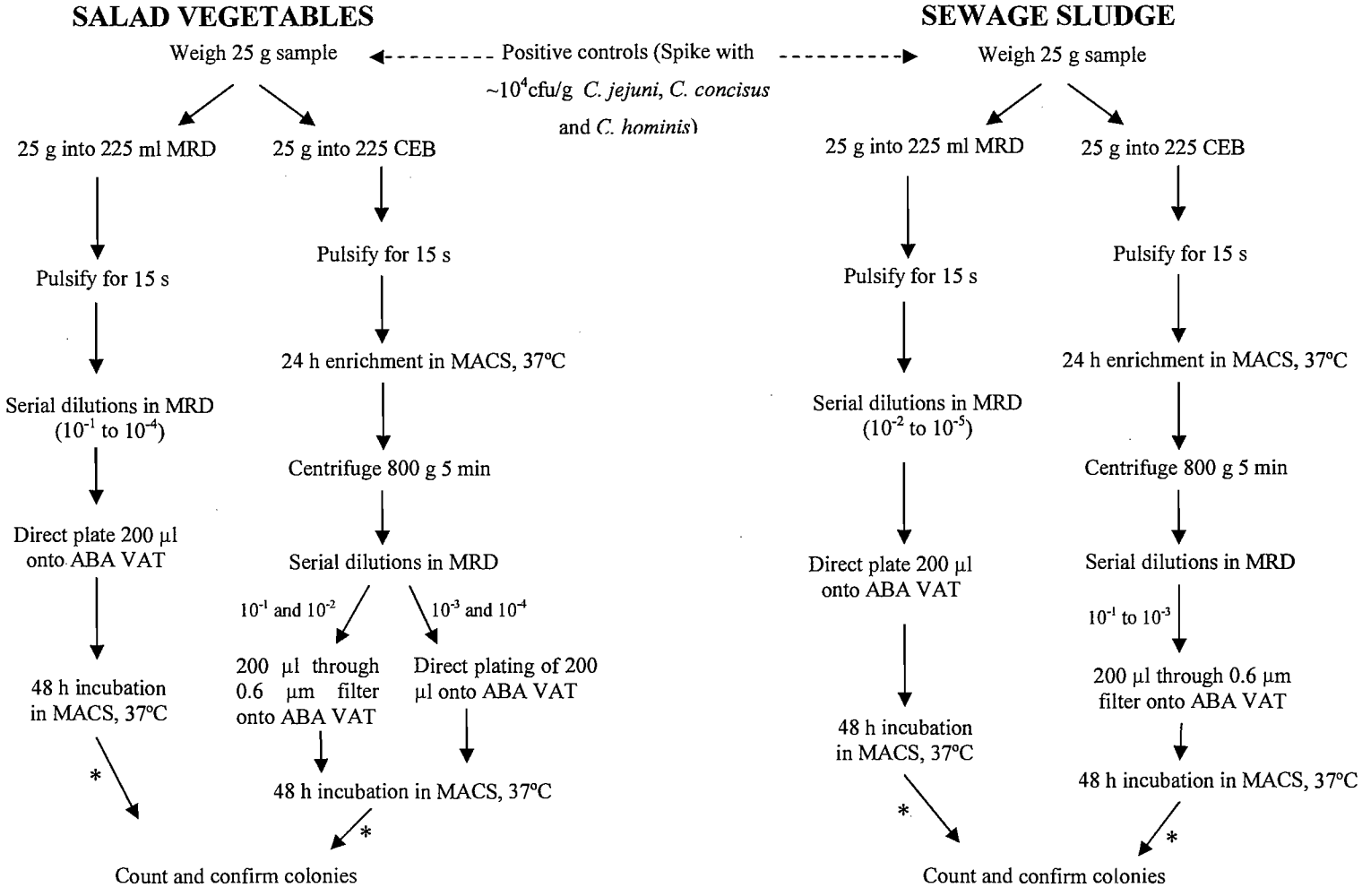


Figure 13. Overview of the Optimised Presence/Absence and Enumeration Methods Used for Recovery of *Campylobacteraceae* from Salad Vegetables and Sewage Sludge. * Plates were also examined after 72 and 96 h.

CHAPTER 3

DEVELOPMENT OF CULTURE MEDIA FOR THE RECOVERY OF *CAMPYLOBACTERACEAE*

3.1. INTRODUCTION

The methods currently available to culture and isolate *Campylobacter* species are thought to be biased towards *C. jejuni* and *C. coli* species, due to their perceived importance as clinical pathogens. Commonly used selective isolation media contain a range of antibiotics which are included to inhibit the growth of competing flora found in clinical, food and environmental samples. However, it is thought that these are also inhibitory to emerging *Campylobacteraceae* species (Engberg *et al.* 2000; Lastovica and Skirrow 2000; Lastovica 2006). Furthermore, it is considered that incubation atmospheres are not optimal for the growth of emerging species. Recent studies using the Cape Town isolation protocol, for example, have shown that other species within the *Campylobacteraceae* can be isolated in high numbers if alternative isolation methods are used (Lastovica 2006). Moreover, there is evidence that many of these emerging species (including *C. concisus*, *C. upsaliensis* and *A. butzleri*) are indeed also significant pathogens. It is likely that the true prevalence and clinical significance of these emerging pathogens will not be deduced until the methods for isolating these species are improved. Therefore, this chapter aims to address some of these issues, concentrating on the assessment and development of isolation media with the eventual goal of producing an isolation medium suitable for the growth of all *Campylobacteraceae*. Traditional method validation usually entails comparative evaluation against a 'gold standard' method. However, in this case there is no 'gold standard' *Campylobacteraceae* isolation procedure, even for the thermotolerant species, with many institutions adopting different methods. Therefore, in this study the most widely used culture methods were evaluated and used as a baseline to compare to the novel methods developed.

3.2. METHODS

Detailed accounts of the methods used in this chapter are described in section 2.3. Initially, the semi-quantitative Streak Dilution method was used to assess the growth of a range of *Campylobacteraceae* included in the CAMPYCHECK reference strain set on various media; subsequently antibiotic selective supplements were evaluated. For most experiments assessing and developing culture media, all of the strains within the CAMPYCHECK reference strain set were evaluated. However, in some circumstances not all of the strains could be included in studies due to time constraints, or if growth of certain strains was problematic due to poor growth or contamination.

3.3. RESULTS

3.3.1. Evaluation of Growth Assessment Methods

Three methods for assessing growth productivity of *C. jejuni* CCUG 11168, *C. concisus* CCUG 13144 and *C. hominis* CH001 were compared on various media. Methods included the quantitative Miles-Misra method and semi-quantitative methods including the Ecometric and Streak Dilution method.

The results showed that generally the Miles-Misra, Ecometric and Streak Dilution methods were in agreement for the assessment of growth on a range of different media, as demonstrated by their productivity ratio scores (Table 10). When the three methods were used to assess growth of *C. jejuni* on different media, results showed there were little or no differences between the growth assessments used, with optimal growth on all media tested. *C. concisus* and *C. hominis* were shown to be sensitive to a number of selective supplements, which was demonstrated by all three growth assessment methods. However, on several occasions the semi-quantitative Ecometric and streak dilution methods slightly underestimated growth on certain media compared to the Miles-Misra method. For example, according to the Miles-Misra method, *C. concisus* was able to grow on CCDA (-) and Preston media with a productivity ratio of 0.4, but was reduced to 0.1 when the Streak Dilution and Ecometric methods were used to evaluate growth. Furthermore, the growth of *C. hominis* was not detected using the Ecometric and Streak Dilution method but was detected using the Miles-Misra method (Table 10).

When carrying out experiments using the Streak Dilution method, cultures were streaked onto plates in duplicate and growth assessed. This was subsequently repeated independently on a separate occasion and a mean of the growth scores from these experiments taken. Table 12 shows raw data to demonstrate the repeatability and reproducibility of the Streak Dilution method when growth was compared on different media. An experiment comparing the growth of *Campylobacter* species on various media was carried out on two separate occasions. Table 11(A) shows the first experiment and Table 11(B) shows results from a repeat of the same experiment. Although no comparative statistics were carried out as the method was only semi-quantitative, the results showed that there was little variability between the mean of the two experiments and within duplicate inoculated plates.

Table 10. Comparison of Growth of *C. jejuni*, *C. concisus* and *C. hominis* on Various Media using Three Growth Assessment Methods (Miles-Misra, Ecometric and Streak Dilution). All results shown are a mean score of duplicate plates and duplicate experiments. The productivity ratios are shown in parentheses and were calculated as a fraction of the growth score on Tryptose Blood Agar (5% blood). [a] Results are expressed as $\log_{10}$ colony forming units per ml of MRD. [b] Growth was scored according to the absolute growth index: 100, growth on all sectors streaked; 0, no growth[c]. Growth scored according to growth on the streaked quadrants: 1, growth on first sector of streaking; 2, growth on second sector of streaking; 3, growth on third sector of streaking; 4, growth on fourth sector of streaking.

	TBA (-)		CCDA (-) /CAT (-)		CCDA (+)		CAT (+)		Preston (-)		Preston (+)		Skirrow (-)		Skirrow (+)	
***C. jejuni* NCTC11168**																
Miles-Misra[a]	9.5	(1.0)	9.56	(1.0)	9.52	(1.0)	9.60	(1.0)	9.63	(1.0)	9.43	(1.0)	9.47	(1.0)	9.50	(1.0)
Ecometric[b]	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)	100	(1.0)
Streak dilution[c]	4	(1.0)	4	(1.0)	3.5	(0.9)	4	(1.0)	4	(1.0)	3.75	(0.9)	4	(1.0)	3.75	(0.9)
***C. concisus* CCUG13144**																
Miles-Misra[a]	10.5	(1.0)	3.64	(0.4)	0	(0.0)	0	(0.0)	10.0	(1.0)	3.90	(0.4)	6.33	(0.6)	3.72	(0.4)
Ecometric[b]	97.5	(1.0)	5	(0.1)	0	(0.0)	0	(0.0)	100	(1.0)	30	(0.1)	65	(0.7)	50	(0.5)
Streak dilution[c]	3.5	(1.0)	0.5	(0.1)	0	(0.0)	0	(0.0)	3.25	(0.9)	0.38	(0.1)	2.5	(0.7)	1	(0.3)
***C. hominis* CH003**																
Miles-Misra[a]	10.3	(1.0)	4.16	(0.4)	0	(0.0)	3.9	0.38	10.3	(1.0)	0	(0.0)	9.2	(0.9)	7.32	(0.7)
Ecometric[b]	100	(1.0)	72.5	(0.8)	0	(0.0)	0	(0.0)	90	(0.9)	0	(0.0)	90	(0.9)	55	(0.6)
Streak dilution[c]	3.25	(1.0)	2	(0.6)	0	(0.0)	0	(0.0)	3.5	(1.1)	0	(0.0)	3	(0.9)	1	(0.3)

Table 11. Raw Data Demonstrating the Repeatability and Reproducibility of the Streak Dilution Method to Assess Growth on Various Media. Experiment 1 (A) and Experiment 2 (B) were carried out independently.

A.

CAMPYCHECK reference strain	Selective isolation agar medium																							
	TBA			Preston						Skirrow						CCDA/CAT			CCDA			CAT		
	Basal medium			Basal medium			Selective Supplement			Basal medium			Selective supplement			Basal medium			Selective supplement			Selective supplement		
	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**
C. coli RM 2228	4	3	**3.5**	4	4	**4**	4	3	**3.5**	4	4	**4**	3	4	**3.5**	4	3	**3.5**	4	3	**3.5**	3	3	**3**
C. coli Lab 33	4	3	**3.5**	4	4	**4**	3	4	**3.5**	4	3	**3.5**	3	3	**3**	4	4	**4**	4	4	**4**	3	4	**3.5**
C. concisus CCUG 13144	3	4	**3.5**	3	3	**3**	0	1	**0.5**	3	3	**3**	1	0	**0.5**	1	1	**1**	0	0	**0**	1	1	**1**
C. concisus CCUG 19995	4	3	**3.5**	4	3	**3.5**	1	0	**0.5**	3	4	**3.5**	0	0	**0**	2	1	**1.5**	0	1	**0.5**	1	1	**1**
C. concisus L 396/96	4	4	**4**	4	4	**4**	0	0	**0**	4	4	**4**	0	0	**0**	1	2	**1.5**	0	0	**0**	0	0	**0**
C. jejuni NCTC 11168	4	4	**4**	4	4	**4**	4	4	**4**	4	4	**4**	3	4	**3.5**	3	4	**3.5**	3	3	**3**	4	4	**4**
C. jejuni RM 1221	3	4	**3.5**	3	4	**3.5**	3	4	**3.5**	4	4	**4**	4	3	**3.5**	4	3	**3.5**	4	3	**3.5**	4	4	**4**
C. lari RM 2100	4	4	**4**	4	3	**3.5**	3	3	**3**	3	4	**3.5**	2	3	**2.5**	2	3	**2.5**	2	2	**2**	3	2	**2.5**
C. lari CCUG 22395	3	3	**3**	4	3	**3.5**	2	2	**2**	3	3	**3**	2	1	**1.5**	2	2	**2**	1	1	**1**	2	1	**1.5**
C. upsaliensis RM 3195	4	3	**3.5**	2	2	**2**	0	0	**0**	2	3	**2.5**	0	0	**0**	3	2	**2.5**	1	1	**1**	3	2	**2.5**
C. upsaliensis CCUG 19559	4	3	**3.5**	3	3	**3**	0	0	**0**	3	3	**3**	2	1	**1.5**	2	2	**2**	0	1	**0.5**	2	2	**2**

B.

CAMPYCHECK reference strain	Selective isolation agar medium																							
	TBA			Preston						Skirrow						CCDA//CAT			CCDA			CAT		
	Basal medium			Basal medium			Selective Supplement			Basal medium			Selective supplement			Basal medium			Selective supplement			Selective supplement		
	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**	1	2	**Mean**
C. coli RM 2228	3	3	**3**	4	3	**3.5**	3	3	**3**	4	4	**4**	3	3	**3**	3	3	**3**	3	3	**3**	3	3	**3**
C. coli Lab 33	3	3	**3**	3	3	**3**	3	3	**3**	4	4	**4**	3	4	**3.5**	3	3	**3**	3	3	**3**	3	3	**3**
C. concisus CCUG 13144	3	4	**3.5**	3	4	**3.5**	1	0	**0.5**	3	4	**3.5**	1	1	**1**	1	1	**1**	0	0	**0**	1	0	**0.5**
C. concisus CCUG 19995	4	4	**4**	4	4	**4**	0	0	**0**	3	3	**3**	1	1	**1**	1	1	**1**	0	0	**0**	0	0	**0**
C. concisus L 396/96	4	3	**3.5**	4	4	**4**	0	0	**0**	4	3	**3.5**	1	1	**1**	1	1	**1**	0	0	**0**	1	1	**1**
C. jejuni NCTC 11168	4	4	**4**	4	4	**4**	3	4	**3.5**	4	4	**4**	3	3	**3**	4	4	**4**	3	3	**3**	4	4	**4**
C. jejuni RM 1221	3	3	**3**	3	3	**3**	3	3	**3**	4	3	**3.5**	3	3	**3**	3	3	**3**	3	3	**3**	4	4	**4**
C. lari RM 2100	4	3	**3.5**	3	3	**3**	3	3	**3**	3	3	**3**	2	2	**2**	2	2	**2**	2	2	**2**	2	2	**2**
C. lari CCUG 22395	3	4	**3.5**	3	3	**3**	2	1	**1.5**	3	4	**3.5**	1	1	**1**	2	2	**2**	0	1	**0.5**	1	2	**1.5**
C. upsaliensis RM 3195	3	3	**3**	3	2	**2.5**	0	0	**0**	2	2	**2**	0	1	**0.5**	2	2	**2**	0	0	**0**	2	2	**2**
C. upsaliensis CCUG 19559	3	3	**3**	3	3	**3**	0	0	**0**	3	4	**3.5**	1	1	**1**	2	1	**1.5**	0	0	**0**	1	1	**1**

3.3.2. Evaluation of Isolation Media and Commercially Available Selective Supplements

3.3.2.1. Initial Media Evaluation of Basal Media and Selective Supplements

These experiments were carried to assess the growth of a number of *Campylobacteraceae* species on conventional, commercially available, selective isolation media (with and without selective supplements) using the semi-quantitative Streak Dilution method.

The isolation media (without selective supplements) which were found to be optimal for the growth of all *Campylobacteraceae* species were TBA, Campylobacter Agar Base (Preston basal agar) and Blood Agar base no. 2 (Skirrow basal agar) supplemented with 7% laked horse blood (without their respective antibiotic selective supplements) (Table 12). *Campylobacter* Blood Free Agar Base (mCCDA basal agar) supported the least growth, especially of *C. concisus* strains. *C. coli* and *C. jejuni* species grew equally well on all of the media tested, irrespective of the presence of antibiotic selective supplements. All of the strains of *C. concisus* and *C. upsaliensis* were inhibited by Preston, Skirrow and mCCDA antibiotic selective supplements. *C. lari* strains were also slightly inhibited by these selective supplements. When CAT selective supplement was included in Campylobacter Blood Free Agar Base it permitted the growth of both of the *C. upsaliensis* strains (not recovered when supplemented with mCCDA selective supplement), however the growth of *C. concisus* strains was still suppressed. *Arcobacter butzleri* and *A. cryaerophilus* were both inhibited when Preston and Skirrow selective supplements were included in the Campylobacter Blood free Agar base, however their growth was not affected by CAT and CCDA selective supplements.

3.3.2.2. Evaluation of Tryptose Blood Agar (TBA) and Anaerobe Basal Agar (ABA)

Anaerobe Basal Agar (ABA) is a complex medium, often used for the growth of fastidious bacteria (Bridson 1998). It was proposed by Peter Stephens (Oxoid Ltd. Pers. Comm., 2004) as a medium that may be comparable or even better than Tryptose Blood Agar (TBA) (the medium used as part of the Cape Town protocol) for the growth of *Campylobacter* species (Lastovica 2006). In the following experiments, growth of all *Campylobacteraceae* in the CAMPYCHECK reference strain set was compared on TBA and ABA using the Streak Dilution method.

Table 12. Growth of *Campylobacteraceae* Species on Various Isolation Agar Media With and Without Antibiotic Selective Supplements. Growth was assessed according to the Streak Dilution method (a semi-quantitative growth scale). Strains were incubated in a microaerophilic environment supplemented with hydrogen (7% H_2, 4% O_2, 10% CO_2) at 37°C for 48 h. Results are a mean of duplicate experiments carried out independently. Growth was scored according to growth on the streaked quadrants: +, growth on first sector of streaking; ++, growth on second sector of streaking; +++, growth on third sector of streaking; ++++, growth on fourth sector of streaking.

CAMPYCHECK reference strain	Selective isolation agar medium							
	TBA	Preston		Skirrow		CCDA/CAT	CCDA	CAT
	Basal medium	Basal medium	Selective supplement	Basal medium	Selective supplement	Basal medium	Selective supplement	Selective supplement
C. coli RM 2228	+++	++++	+++	++++	+++	+++	+++	+++
C. coli Lab 33	+++	+++	+++	+++	+++	+++	+++	+++
C. concisus CCUG 13144	++++	+++	-/+	+++	+	+	-	+
C. concisus CCUG 19995	++++	++++	-/+	+++	+	+	-	-
C. concisus Lastovica 396/96	++++	++++	-	++++	+	+	-	-
C. jejuni NCTC 11168	++++	++++	+++	++++	+++	++++	+++	++++
C. jejuni RM 1221	+++	+++	+++	++++	+++	+++	+++	++++
C. lari RM 2100	++++	+++	++	+++	++	++	++	++
C. lari CCUG 22395	+++	+++	++	+++	+	++	+	++
C. upsaliensis RM 3195	+++	++	-	++	-	++	+	++
C. upsaliensis CCUG 19559	+++	+++	-	+++	+	++	-	++
A. butzleri SSI 71032	++++	+++	-	++++	+	+++	+++	+++
A. cryaerophilus CCUG 71032	+++	+++	+	+++	-	+++	+++	+++

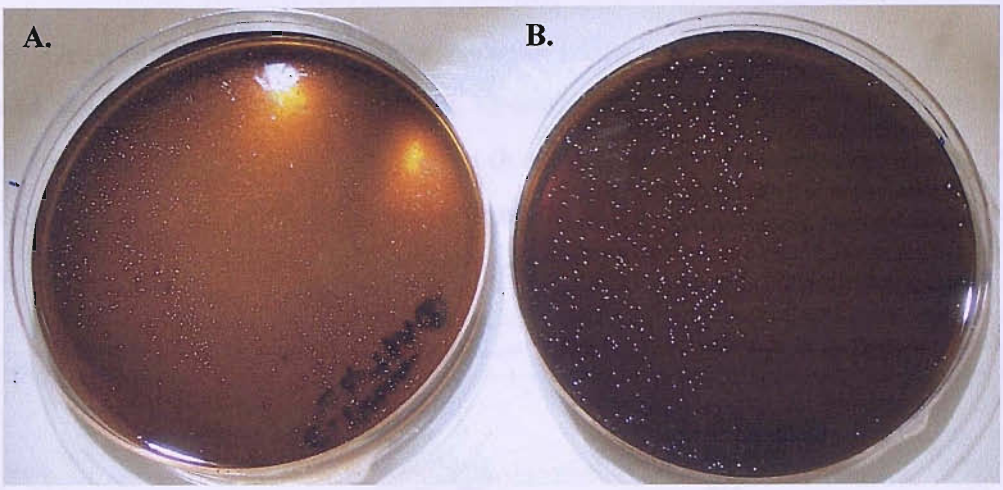


Figure 14. Growth of *C. showae* on ABA showing bigger colonies when grown on ABA medium. A) TBA; B) ABA

The results showed that there were no significant differences in growth of the tested species when plated onto ABA and TBA, with all species being able to grow well on both media (Table 13). Certain strains, including *C. hyointestinalis* (22), *C. sputorum* (48), *C. gracilis*, *C. rectus* and *C. showae*, showed slightly higher recoveries on ABA medium. Although, for the majority of species tested there was no significant difference in growth, according to the streak dilution method it was observed that the colony sizes of certain species, especially those that are known to prefer anaerobic growth conditions (*C. showae*, *C. hominis*, *C. rectus* and *C. curvus*), appeared to be bigger and more defined when plated onto ABA compared to TBA (Figure 14).

3.3.2.3. Blood Supplementation

Defibrinated or laked horse blood is commonly included in media at concentrations of 5% to 10% to counteract the effects of toxic oxygen derivatives (Corry *et al.* 1995a; Bridson 1998). This experiment compared the growth of various *Campylobacteraceae* species from the CAMPYCHECK reference strain set, on media supplemented with laked and defibrinated horse blood at 5% and 10% using the Streak Dilution method.

The results showed that, generally, there was very little difference in the growth of the tested species on all of the media combinations tested (Table 14). The majority of *Campylobacteraceae* (12 out of 18 species) showed slightly higher growth with

Table 13. Growth Evaluation of *Campylobacteraceae* Species on Tryptose Blood Agar (TBA) and Anaerobe Basal Agar (ABA). Growth was assessed using the streak dilution method. Growth was scored according to growth on the streaked quadrants: +, growth on first sector of streaking; ++, growth on second sector of streaking; +++, growth on third sector of streaking; ++++, growth on fourth sector of streaking.

Species		TBA	ABA
***Campylobacter* spp.**			
C. coli (1)	RM 2228	+++	+++
C. coli (2)	Lab 33	++++	++++
C. coli (3)	300.97	++++	++++
C. concisus (4)	CCUG 13144	++++	+++
C. concisus (5)	CCUG 19995	+++	+++
C. concisus (6)	L 396/96	+++	+++
C. fetus subsp. fetus (10)	CCUG 32114	+++	++++
C. fetus subsp. fetus (11)	Abdn 1076	+++	+++
C. fetus subsp. fetus (12)	241.99	++++	+++
C. fetus subsp. venerealis (13)	CCUG 11287	+++	+++
C. fetus subsp. venerealis (14)	Armi 4402	+++	+++
C. fetus subsp. venerealis (15)	Abdn SM5	+++	+++
C. helveticus (18)	CCUG 30566	+++	+++
C. hyointestinalis subsp. hyo. (22)	LMG 7538	++	++++
C. hyointestinalis subsp. hyo. (23)	LMG 9260	++++	+++
C. hyointestinalis subsp. hyo. (24)	176.96	+++	+++
C. hyointestinalis subsp. lawsonii (26)	CHY 5	+++	+++
C. jejuni subsp. doylei (28)	SSI 5384	+++	++++
C. jejuni subsp. jejuni (31)	NCTC 11168	++++	++++
C. jejuni subsp. jejuni (32)	RM 1221	++++	++++
C. lanienae (36)	NCTC 13004	++++	+++
C. lanienae (37)	DARDNI G718D	++++	+++
C. lari (38)	RM 2100	+++	+++
C. lari (39)	CCUG 22395	+++	++++
C. mucosalis (40)	CCUG 21559	++++	+++
C. mucosalis (41)	CCUG 23201	+++	+++
C. sputorum bv. sputorum (46)	Lastovica 86.92	+++	+++
C. sputorum bv. paraureolyticus (47)	LMG 11764	++	++++
C. sputorum bv. fecalis (48)	CCUG 20703	+++	++++
C. upsaliensis (49)	RM 3195	++	+++
C. upsaliensis (50)	CCUG 19559	+++	+++
C. upsaliensis (51)	CCUG 19607	+++	+++
Anaerobic-like *Campylobacter* spp.			
C. curvus (7)	SSI 19296	+++	++++
C. curvus (8)	L 525.92	+++	++++
C. gracilis (16)	CCUG 27720	++	+++
C. hominis (20)	NCTC CH001	+++	+++
C. hominis (21)	NCTC CH003	+++	+++
C. rectus (42)	CCUG 20446	++	++++
C. rectus (43)	CCUG 11645	++	+++
C. showae (45)	CCUG 11641	++	+++
Related non-*Campylobacter* spp.			
B. ureolyticus (53)	Rig 9880	++++	+++
A. butzleri (54)	SSI 71032	++	+++
A. butzleri (55)	29.97	+++	++++
A. butzleri (56)	167.97	++++	++++
A. cryaerophilus (57)	CCUG 178011	++++	++++
A. cryaerophilus (58)	SSI 70952	+++	+++
A. skirrowii (59)	BU30CC 8B1	+++	+++

defibrinated horse blood, irrespective of the concentration added. The difference was particularly evident with *C. fetus*, *C. venerealis*, *C. hyointestinalis, C. doylei*, *C. mucosalis* and *C. sputorum*. However, *C. lawsonii* grew better with laked horse blood. There was very little effect on growth when the blood concentration was increased. Defibrinated horse blood performed better, however laked horse blood was also suitable for the growth of all of the species tested.

3.3.2.4. Confirmation of Inhibition by Selective Supplements

Experiments carried out previously showed that a number of selective supplements contained in various basal agar media inhibited the growth of some *Campylobacteraceae* species (section 3.3.2.1). In the following experiments, various commercially available antibiotic selective supplements (including CCDA, CAT, Preston and Skirrow) were evaluated for growth of a wide range of *Campylobacteraceae* using only TBA as a basal medium. It is possible that the growth inhibition effect, as previously observed, could have been partly due to the use of a less than optimal basal medium, which when supplemented with the antibiotic selective supplements may have caused a synergistic inhibitory effect. Furthermore, it was anticipated that one of these selective supplements tested would be suitable for use in a novel selective medium to use in future isolation studies.

The results were in agreement with the previous study (section 3.3.2.1) in that the inhibitory effects of the selective supplements were similar (Table 15). However, the inhibitory effects were somewhat less pronounced when the species were grown on TBA, compared to some other basal media. This was particularly evident for Campylobacter Blood-free agar base which, when supplemented with both CCDA and CAT selective supplements, completely inhibited growth of *C. concisus*. However when TBA was supplemented with the same supplements, the inhibitory effects were reduced, allowing growth of *C. concisus*.

The results showed that none of the selective supplements tested was completely suitable for the growth of all *Campylobacteraceae* species, although CAT selective supplement was the most suitable candidate. CAT selective supplement supported good growth of the majority of species tested, inhibiting the growth of only 24% (14/59) of the *Campylobacteraceae* strains tested. The only species that were inhibited by CAT selective

Table 14. Evaluation of Blood Type and Concentration When Supplemented to ABA Basal Medium for the growth of *Campylobacteraceae* Species. Growth was assessed using the streak dilution method. Growth was scored according to growth on the streaked quadrants: +, growth on first sector of streaking; ++, growth on second sector of streaking; +++, growth on third sector of streaking; ++++, growth on fourth sector of streaking.

Species and strain		Defibrinated Horse Blood		Laked Horse Blood	
Campylobacter spp.		10%	5%	10%	5%
C. coli (1)	RM 2228	+++	+++	+++	+++
C. coli (2)	Lab 33	+++	+++	+++	+++
C. concisus (4)	CCUG 13144	++	++	++	++
C. concisus (5)	CCUG 19995	+++	+++	+++	+++
C. concisus (6)	L396/96	++	++	++	++
C. fetus subsp. fetus (10)	CCUG 32114	++++	++++	+++	+++
C. fetus subsp. fetus (11)	Abdn 1076	++++	++++	++++	+++
C. fetus subsp. venerealis (13)	CCUG 11287	++++	++++	+++	+++
C. fetus subsp. venerealis (14)	Armi 4402	++++	++++	+++	+++
C. helveticus (18)	CCUG 30566	+++	+++	+++	+++
C. helveticus (19)	CCUG 34016	+++	+++	+++	+++
C. hyointestinalis subsp. hyo. (22)	LMG 7538	+++	+++	+++	+++
C. hyointestinalis subsp. hyo. (23)	LMG 9260	+++	+++	+++	+++
C. hyointestinalis subsp. lawsonii (26)	CHY 5	+++	+++	+++	+++
C. hyointestinalis subsp. lawsonii (27)	CCUG 27631	++++	+++	+++	++++
C. jejuni subsp. doylei (28)	SSI 5384	++++	++++	+++	+++
C. jejuni subsp. doylei (29)	CCUG 18266	++++	++++	+++	+++
C. jejuni subsp. jejuni (31)	NCTC 11168	+++	+++	+++	+++
C. jejuni subsp. jejuni (32)	RM 1221	+++	+++	+++	+++
C. lanienae (36)	NCTC 13004	+++	+++	+++	+++
C. lanienae (37)	DARDNI G718D	++++	++++	++++	++++
C. lari (38)	RM 2100	+++	+++	+++	+++
C. lari (39)	CCUG 22395	+++	+++	+++	+++

Table 14 Continued.

C. mucosalis (40)	CCUG 21559	+++	+++	++	++
C. mucosalis (41)	CCUG 23201	++++	++++	+++	++
C. sputorum bv. sputorum (46)	Lastovica 86.92	+++	+++	+++	++
C. sputorum bv. paraureolyticus (47)	LMG 11764	+++	+++	+++	+++
C. upsaliensis (49)	RM 3195	++++	+++	+++	+++
C. upsaliensis(51)	CCUG 19607	+++	+++	+++	+++
Anaerobic-like *Campylobacter* spp.					
C. curvus (7)	SSI 19296	++++	++++	++++	++++
C. curvus (8)	Lastovica 525.92	++++	++++	+++	+++
C. gracilis (16)	CCUG 27720	+++	+++	+++	+++
C. gracilis (17)	CCUG 13143	++++	++++	+++	+++
C. hominis (20)	NCTC CH001	+++	+++	+++	+++
C. hominis (21)	NCTC CH003	+++	+++	+++	++
C. rectus (42)	CCUG 20446	+++	++++	+++	++++
C. rectus (43)	CCUG 11645	+++	+++	++++	+++
C. showae (44)	CCUG 30254	++++	++++	+++	+++
C. showae (45)	CCUG 11641	+++	+++	+++	+++
Related non-*Campylobacter* spp.					
B. ureolyticus (52)	CCUG 18470	++++	++++	+++	++
B. ureolyticus (53)	Rig 9880	+++	++++	++++	++++
A. butzleri (54)	SSI 71032	++++	++++	++++	++++
A. butzleri (55)	29.97	++++	++++	+++	+++
A. cryaerophilus (57)	CCUG 178011	+++	+++	+++	+++
A. cryaerophilus (58)	SSI 70952	+++	+++	+++	+++
A. skirrowii (59)	BU30CC 8B1	++++	++++	+++	+++

supplement were all three strains of *C. concisus*, all strains two strains of *C. sputorum*, *C. gracilis* and *C. rectus* and one strain each of *C. doylei*, *C. showae*, *A. skirrowii* and *B. ureolyticus*.

TBA supplemented with Preston selective supplement inhibited the growth of the majority of the *Campylobacteraceae* strains tested. It was the most inhibitory of all of the supplements tested, inhibiting 78% (46/59) of the strains tested. The only strains not to be affected by the Preston selective supplement included all strains of *C. venerealis*, *C. hyointestinalis*, *C. jejuni*, *C. curvus* and one strain each of *C. fetus* and *C. lawsonii*.

When TBA was supplemented with CCDA and Skirrow selective supplements, both inhibited the growth of a range of strains tested, with growth inhibition of 60% (35/59) and 65% (38/59) of the species tested, respectively. Species inhibited by CCDA selective supplement included all strains of *C. concisus*, *C. venerealis*, *C. doylei*, *C. sputorum*, *C. upsaliensis* and one strain each of *C. fetus*, *C. lanienae* and *C. lari*. 'Anaerobic' species including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus* and *C. showae* as well as *B. ureolyticus* were also susceptible to CCDA selective supplement. Furthermore, growth of *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* was inhibited by CCDA selecteive supplement to varying degrees depending on strain. The strains inhibited by Skirrow selective supplement included strains of *C. concisus*, *C. helveticus*, *C. lanienae*, *C. sputorum*, *C. upsaliensis* and one strain each of *C. fetus*, *C. lari*, *C. mucosalis*. All strains of the 'anaerobic' species, including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus C. showae* and *B. ureolyticus* and all strains of *Arcobacter* species, were also inhibited by Skirrow selective supplement. Growth of the control species, *S. aureus*, *E. coli* and *Salmonella* species, were all inhibited by all of the selective supplements tested, although low level growth was possible when TBA was supplemented with Skirrow selective supplement.

3.3.2.5. Antibiotic Susceptibility Testing using Disc Diffusion Tests

The previous experiments (section 3.3.2.4) demonstrated that a number of antibiotic selective supplements, commonly included in selective isolation media, were inhibitory to the growth of many *Campylobacteraceae* species. Therefore, experiments were carried out using antibiotic susceptibility disc tests, to assess a range of individual antibiotics commonly included in selective isolation media. It was thought that results would be useful in determining which antibiotics would be suitable for use in a novel selective medium. The antibiotics evaluated included trimethoprim, teicoplanin, vancomycin, amphotericin B, cefoperazone, cefsulodin, cefazolin, rifampicin, polymyxin

Table 15. Evaluation of Commercially Available Antibiotic Selective Supplements Including CCDA, CAT, Skirrow and Preston for the Growth of Campylobacters and Related Species. Bacteria were inoculated onto TBA with and without selective supplements, incubated for 48 h in a CAMPYCHECK atmosphere in the MACS workstation and growth assessed using the streak dilution method.

Species and strain		TBA	CCDA	CAT	Skirrow	Preston
***Campylobacter* spp.**						
C. coli (1)	RM 2228	++++	++++	+++	+++	++
C. coli (2)	Lab 33	++++	++++	+++	+++	++
C. coli (3)	300.97	++++	+++	+++	+++	++
C. concisus (4)	CCUG 13144	++++	++	++	+	-
C. concisus (5)	CCUG 19995	++++	-	++	++	-/+
C. concisus (6)	L 396/96	++++	+	++	++	+
C. fetus subsp. fetus (10)	CCUG 32114	+++	+++	+++	++	+++
C. fetus subsp. fetus (11)	Abdn 1076	++++	++	+++	++	++
C. fetus subsp. fetus (12)	241.99	++++	+++	+++	+++	++
C. fetus subsp. venerealis (13)	CCUG 11287	+++	+	++++	+++	+++
C. fetus subsp. venerealis (14)	Armi 4402	++++	+	++++	++++	++++
C. fetus subsp. venerealis (15)	Abdn SM5	++++	+	++++	++++	++++
C. helveticus (18)	CCUG 30566	++++	+++	+++	++	++
C. helveticus (19)	CCUG 34016	++++	++++	++++	-	-
C. hyointestinalis subsp. hyo. (22)	LMG 7538	++++	++++	++++	++++	+++
C. hyointestinalis subsp. hyo. (23)	LMG 9260	++++	++++	++++	+++	++++
C. hyointestinalis subsp. hyo. (24)	176.96	++++	++++	++++	++++	++++
C. hyointestinalis subsp. hyo. (25)	234.95	++++	++++	++++	++++	++++
C. hyointestinalis subsp. lawsonii (26)	CHY 5	++++	+++	++++	+++	++
C. hyointestinalis subsp. lawsonii (27)	CCUG 27631	++++	++++	++++	+++	+++
C. jejuni subsp. doylei (28)	SSI 5384	++++	+	+++	+++	+
C. jejuni subsp. doylei (29)	CCUG 18266	++++	-/+	++	+++	-/+
C. jejuni subsp. doylei (30)	269.97	++++	++	+++	++++	+
C. jejuni subsp. jejuni (31)	NCTC 11168	++++	++++	++++	++++	+++
C. jejuni subsp. jejuni (32)	RM 1221	++++	++++	++++	++++	++++
C. jejuni subsp. jejuni (33)	SVS 4039	++++	++++	++++	++++	++++
C. jejuni subsp. jejuni (34)	RM 1864	++++	++++	++++	++++	++++
C. jejuni subsp. jejuni (35)	47.97	++++	++++	+++	++++	++++
C. lanienae (36)	NCTC 13004	++++	+++	+++	+	++

Table 15 Continued.

C. lanienae (37)	DARDNI G718D	++++	++	+++	++	++
C. lari (38)	RM 2100	++++	++	+++	+	++
C. lari (39)	CCUG 22395	++++	+++	++++	+++	++
C. mucosalis (40)	CCUG 21559	++++	+++	+++	+++	++
C. mucosalis (41)	CCUG 23201	++++	+++	++++	-	-
C. sputorum bv. sputorum (46)	Lastovica 86.92	++++	-	+++	+	-/+
C. sputorum bv. paraureolyticus (47)	LMG 11764	++++	-/+	+	-/+	-
C. sputorum bv. fecalis (48)	CCUG 20703	++++	-	++	+	-
C. upsaliensis (49)	RM 3195	+++	++	++	-	-
C. upsaliensis(50)	CCUG 19559	+++	-	++	-	-
C. upsaliensis(51)	CCUG 19607	++++	-/+	+++	+	-/+
Anaerobic-like *Campylobacter* spp.						
C. curvus (7)	SSI 19296	++++	+	+++	++	+++
C. curvus (8)	Lastovica 525.92	++++	++	++++	+	+++
C. curvus (9)	Lastovica 13A	++++	+	+++	++	+++
C. gracilis (16)	CCUG 27720	++++	-	-	-/+	-
C. gracilis (17)	CCUG 13143	++++	-	-	-	-
C. hominis (20)	NCTC CH001	++++	-	++++	++	-
C. hominis (21)	NCTC CH003	+++	-/+	++	-	+
C. rectus (42)	CCUG 20446	++++	-	-	-	-/+
C. rectus (43)	CCUG 11645	++++	-	-/+	-	-
C. showae (44)	CCUG 30254	++++	++	++++	++	-
C. showae (45)	CCUG 11641	++++	-	++	++	-/+
Related non-*Campylobacter* spp.						-
B. ureolyticus (52)	CCUG 18470	+++	-	-	-	-
B. ureolyticus (53)	Rig 9880	++++	-	+	+	+
A. butzleri (54)	SSI 71032	++++	++	++++	-	-
A. butzleri (55)	29.97	++++	-	++++	-	-
A. butzleri (56)	167.97	++++	-/+	++++	-	-
A. cryaerophilus (57)	CCUG 178011	++++	++	++++	-	-
A. cryaerophilus (58)	SSI 70952	+++	+	++	+	-
A. skirrowii (59)	BU30CC 8B1	++++	-	++	+	-
% strains inhibited		**0% (0/59)**	**60% (35/59)**	**24% (14/59)**	**65% (38/59)**	**78% (46/59)**
Control non-related bacteria						
E. coli		++++	-/+	-/+	+	-
MRSA		++++	-	-	+	-
Salmonella		++++	-/+	+	+	-

B and colistin sulphate. Antibiotics were tested on all of the *Campylobacteraceae* strains in the CAMPYCHECK strain set and were also tested on a range of potential competitor bacterial strains.

3.3.2.6. Evaluation of Antibiotic Susceptibility of *Campylobacteraceae* Species

The only antibiotics that all *Campylobacteraceae* strains were completely resistant to were amphotericin B and trimethoprim (51/51) (Table 16). The majority of *Campylobacter* species tested were resistant to teicoplanin (46/51) and vancomycin (45/51). Strains that were susceptible to teicoplanin included one strain each of *C. curvus* (9), *C. showae* (45), *A. butzleri* (57) and *C. rectus* (42,43). Strains susceptible to vancomycin included *C. rectus* (42,43), *C. showae* (44,45), *C. helveticus* (18,19) and one strain of *C. curvus* (9). It is worth noting that these two glycopeptide antibiotics had similar modes of action in that the strains susceptible to these antibiotics were comparable.

Approximately half of the *Campylobacteraceae* strains tested were resistant to rifampicin (22/46), and less than half were resistant to cefoperazone (17/51) and cefazolin (18/45). The only species that were completely resistant to cefoperazone were all strains of *C. coli* (1,2,3), *C. fetus* (7,8,9), *C. jejuni* (31-35), *C. lanienae* (36) and *C. lari* (38), two strains of *Arcobacter butzleri,* one strain of *C. hyointestinalis* (22) and *C. mucosalis* (41). Strains resistant to cefazolin included *C. coli* (2,3), *C. fetus* (10-12), *C. fetus* subsp. *venerealis* (15), *C. jejuni* subsp. *jejuni, C. lari, A. butzleri* (54-56) and one strain of *A. cryaerophilus*. Species resistant to rifampicin included all strains of *C. fetus* (10-12), *C. fetus* subsp. *venerealis* (14,15), *C. hyointestinalis* (22-25), *C. jejuni* (31-35) and *C. lari* (38,39). Species which varied in their susceptibility, depending on strain, included *C. concisus,* where two of the three strains were resistant (4,6), one out of two strains of *C. upsaliensis* (51) and *B. ureolyticus* (53).

Antibiotics which were particularly inhibitory towards the tested *Campylobacteraceae* species included cefsulodin and colistin B, of which only 8/43 and 3/51 strains were resistant, respectively. The only species resistant to cefsulodin were all *A. butzleri* (54-56) and *A. cryaerophilus* (57,58) strains and three strains of *C. jejuni* (32-34). The only strains resistant to colistin sulphate included *C. lanienae* (36) and both strains of C. *rectus* (42,43).

Polymyxin B was inhibitory to the growth of all of the *Campylobacteraceae* strains tested, with 0/50 strains showing resistance.

In most cases, all of the strains within the same species showed similar antibiotic susceptibility profiles, however there were a few discrepancies. Overall, species which showed the highest degree of resistance to eight out of the 10 antibiotics tested were *C. jejuni* subsp. *jejuni* and one strain of *A. butzleri* (56). However, they were not resistant to rifampicin and polymyxin B. All of the *C. fetus* subsp. *fetus* strains also displayed high resistance to seven out of ten antibiotics excluding cefsuludin, colistin sulphate and polymixin B. One particular strain of *C. showae* (45) was most susceptible, being resistant to only amphotericin B and trimethoprim. The other strain tested, *C. showae* (44), was resistant to teicoplanin and vancomycin.

3.3.2.6.1. Evaluation of Antibiotic Susceptibility of Potential Competitor Species

The results showed that when the antibiotics were tested on a range of potential competitor bacterial species, cefoperazone was the most inhibitory and wide ranging antibiotic in terms of its action against the non-*Campylobacteraceae* species tested (Table 17). All 10 competitor species tested (*Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Listeria monocytogenes, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens, Staphylococcus aureus* and *Streptococcus* species were susceptible to its action; however cefoperazone was also shown to be inhibitory to many *Campylobacter* species.

The most effective antibiotic in terms of its action towards both competitor species and *Campylobacter* species was trimethoprim. Nine out of the ten species tested were susceptible to its action; exceptions included *A. bangeri* and *P. aeruginosa*. Trimethoprim displayed a widespread action against a number of the competitor species, yet it did not inhibit growth of any of the *Campylobacter* species tested. Teicoplanin and vancomycin displayed similar activities against both competitor species and *Campylobacter* species. The activity of these antibiotics appeared to be restricted to the Gram positive organisms including *L. monocytogenes*, *S. aureus*, and *Streptococcus* species and the majority of *Campylobacter* species were resistant to their action. Amphotericin B showed no effect on any of the species tested due to it being active against yeasts and fungi, none of which were tested in this study. Polymyxin B was active against a number of competitor strains but also inhibited a number of *Campylobacter* species. Colistin sulphate inhibited the majority of competitor strains (except *L. monocytogenes* and *S. aureus*). However it also significantly inhibited the growth of all

Table 16. Antibiotic Disc Diffusion Test Results for *Campylobacteraceae* Strains from the CAMPYCHECK (CC) Reference collection. D/mm = Diameter/mm. R=Resistant, S = Susceptible. For disc susceptibility break points see Method. 1-3, *C. coli*, 4-6, *C. concisus*, 7-9, *C. curvus*, 10-12, *C. fetus ss fetus*, 13-15, *C. fetus ss venerealis*, 16-17, *C. gracilis*, 18-19, *C. helveticus*, 20-21, *C hominis*, 22-25, *C. hyointestinalis*, 26-27, *C. lawsonii*, 28-30, *C. doylei*, 31-35, *C. jejuni*, 36-37 *C. lanienae*, 38-39, *C. lari*, 40-41, *C. mucosalis*, 42-43 *C. rectus*, *B. ureolyticus*, 54-56 *A. butzleri*, 57-58, *A. cryaerophilus*, 59 A. *skirrowii*. ABA (5% laked horse blood) was used as the basal medium.

CC.	Amphotercin B		Cefoperazone		Cefazolin		Cefsulodin		Colistin		Polymyxin B		Rifampicin		Teicoplanin		Trimethoprim		Vancomycin	
No.	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R
1	0	R	0	R	NT	N/A	N.T	N/A	30	S	30	S	12	I	0	R	0	R	0	R
2	0	R	0	R	0	R	N.T.	N/A	25	S	20	S	15	I	0	R	0	R	0	R
3	0	R	0	R	0	R	N.T	N/A	20	S	20	S	12	I	0	R	0	R	0	R
4	0	R	8.5	I	0	R	35	S	21.5	S	19.5	I	3.8	R	0	R	0	R	0	R
5	0	R	23.5	S	24.5	S	32	S	30	S	25.5	S	11	I	0	R	0	R	0	R
6	0	R	27.5	S	40	S	30	S	35	S	35	S	0	R	0	R	0	R	0	R
7	0	R	15	I	NT	N/A	N.T.	N.A.	15.3	I	15	I	9.3	R	0	R	0	R	0	R
8	0	R	14	I	24	S	31	S	23.5	S	22	S	N.T.	0	0	R	0	R	0	R
9	0	R	30	S	30	S	30	S	12	I	12	I	37.5	S	20	S	0	R	24	S
10	0	R	0	R	0	R	33.5	S	14	I	13.8	I	0	R	0	R	0	R	0	R
11	0	R	0	R	1.75	R	40	S	31.8	S	27	S	0	R	0	R	0	R	0	R
12	0	R	0	R	8.5	R	40	S	15	I	13	I	0	R	0	R	0	R	0	R
14	0	R	36	S	57.5	S	54	S	21.3	S	20	S	0	R	0	R	0	R	0	R
15	0	R	10	I	5	R	40.8	S	16	I	15	I	0	R	0	R	0	R	0	R
16	0	R	43.3	S	N.T		N.T	N.A.	35	S	30	S	20.7	S	0	R	0	R	0	R
18	0	R	18.3	I	N.T		N.T.	N.A.	35	S	40	S	18.3	I	0	R	0	R	21.5	S
19	0	R	19	I	30	S	17	I	41	S	37.5	S	17.5	I	0	R	0	R	23.5	S
22	0	R	0	R	17	I	31.5	S	22.3	S	19.8	S	0	R	0	R	0	R	0	R
24	0	R	20	S	27	S	30	S	15.5	I	14	I	0	R	0	R	0	R	0	R
25	0	R	12	I	21	S	41	S	22.5	S	20	S	0	R	0	R	0	R	0	R
26	0	R	23.3	S	23.5	S	40	S	26	S	24	S	15.3	I	0	R	0	R	0	R

Table 16 Continued.

27	0	R	23.5	S	40	S	48	S	21	S	21	S	0	R	0	R	0	R	0	R
28	0	R	11	I	10	I	29.5	S	28	S	24.8	S	11	I	0	R	0	R	0	R
29	0	R	34	S	10	I	30	S	25.5	S	25	S	14	I	0	R	0	R	0	R
30	0	R	11.5	I	10	I	28	S	30	S	28	S	12.5	I	0	R	0	R	0	R
31	0	R	0	R	0	R	13	I	22.8	S	20.8	S	0	R	0	R	0	R	0	R
32	0	R	0	R	0	R	0	R	22.8	S	22.5	S	0	R	0	R	0	R	0	R
33	0	R	0	R	0	R	0	R	23.5	S	22.5	S	0	R	0	R	0	R	0	R
34	0	R	0	R	0	R	0	R	21.5	S	23	S	0	R	0	R	0	R	0	R
35	0	R	0	R	0	R	15	I	24	S	22.5	S	15	R	0	R	0	R	0	R
36	N.T		0	R	N.T		N.T.		0	R	30	S	35	S	0	R	0	R	0	R
38	0	R	3.3	R	0	R	10.5	I	31	S	28	S	0	R	0	R	0	R	0	R
40	0	R	50	S	48	S	40	S	32.5	S	25	S	N.T.	0	0	R	0	R	6.5	R
41	0	R	0	R	0	R	0	S	30	S	18.3	I	0	R	0	R	0	R	0	R
42	0	R	37.5	S	30	S	25	S	0	R	10	I	41.7	S	21	S	0	R	30	S
43	0	R	31	S	31	S	25.5	S	6	R	12	I	N.T.	0	20.5	S	0	R	29.3	S
44	0	R	15.5	I	26	S	25	S	23.5	S	21	S	12.5	I	0	R	0	R	9.5	I
45	0	R	30	S	33.5	S	23.5	S	25	S	21	S	27	S	20	S	0	R	23.5	S
46	0	R	30.3	S	N.T.		30	S	33.7	S	N.T	0	13	I	0	R	0	R	0	R
47	0	R	61.7	S	23.3	S	50	S	42.5	S	40.7	S	20.5	S	0	R	0	R	0	R
48	0	R	32.5	S	25	S	50	S	41.8	S	43.5	S	13.5	I	0	R	0	R	0	R
49	0	R	30	S	51	S	30	S	43.3	S	37.7	S	17.7	I	0	R	0	R	0	R
51	0	R	19	I	30	S	10.5	I	38.5	S	35	S	0	R	0	R	0	R	0	R
52	0	R	57.5	S	46.5	S	54	S	40	S	28	S	25	S	0	R	0	R	0	R
53	0	R	44	S	46	S	50	S	32.5	S	28	S	0	R	0	R	0	R	0	R
54	0	R	0	R	0	R	0	R	30	S	25	S	11	I	0	R	0	R	0	R
55	0	R	20	S	0	R	0	R	26	S	22.5	S	12	I	0	R	0	R	0	R
56	0	R	0	R	0	R	0	R	25	S	21.5	S	0	R	0	R	0	R	0	R
57	0	R	18.5	I	23	S	0	R	27	S	22	S	22.5	S	19.5	S	0	R	0	R
58	0	R	19	I	0	R	0	R	30	S	25	S	19	S	0	R	0	R	0	R
59	0	R	16.5	I	19	I	11.5	I	30	S	26.5	S	21	S	0	R	0	R	0	R

Table 17. Antibiotic Disc Diffusion Test Results for Non-*Campylobacteraceae* Strains. D/mm = Diameter/mm. R = resistant, S = Susceptible. For disc susceptibility break points see Method.

CC. No.	Amphotercin B		Cefoperazone		Cefazolin		Cefsulodin		Colistin		Polymixin B		Rifampicin		Teicoplanin		Trimethoprim		Vancomycin	
	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R	D/mm	S/R
Enterobacter cloacae	0	R	30	S	0	R	N.T.	N.T.	30	S	16	I	10	R	0	R	21	S	0	R
Escherichia coli	0	R	13	I	N.T.	N/A	N.T.	N.T.	15	I	14	I	8	R	0	R	30	S	0	R
Klebsiella oxytoca	0	R	10	I	0	R	N.T.	N.T.	10	I	15.5	I	0	S	0	R	29	S	0	R
Listeria monocytogenes	0	R	25	S	N.T.	N/A	N.T.	N.T.	0	R	0	R	31	S	17	I	40	S	20	S
Proteus vulgaris	0	R	37	S	13.5	I	N.T.	N.T.	37	S	15	I	15	I	0	R	17	I	0	R
Pseudomonas aeruginosa	0	R	30	S	0	R	N.T.	N.T.	30	S	17.5	I	0	R	0	R	0	R	0	R
Salmonella typhimurium	0	R	15	I	N.T.	N/A	N.T.	N.T.	16	I	15	I	8	R	0	R	30	S	0	R
Serratia marcescens	0	R	15.5	I	0	R	N.T.	N.T.	15.5	I	15	I	0	R	0	R	28	S	0	R
Staphylococcus aureus	0	R	17	I	N.T.	N/A	N.T.	N.T.	0	R	0	R	19	I	12	I	22	S	14.5	I
Streptococcus spp.	0	R	30	S	30	S	N.T.	N.T.	30	S	1	R	30	S	19	I	23.5	S	22.5	S

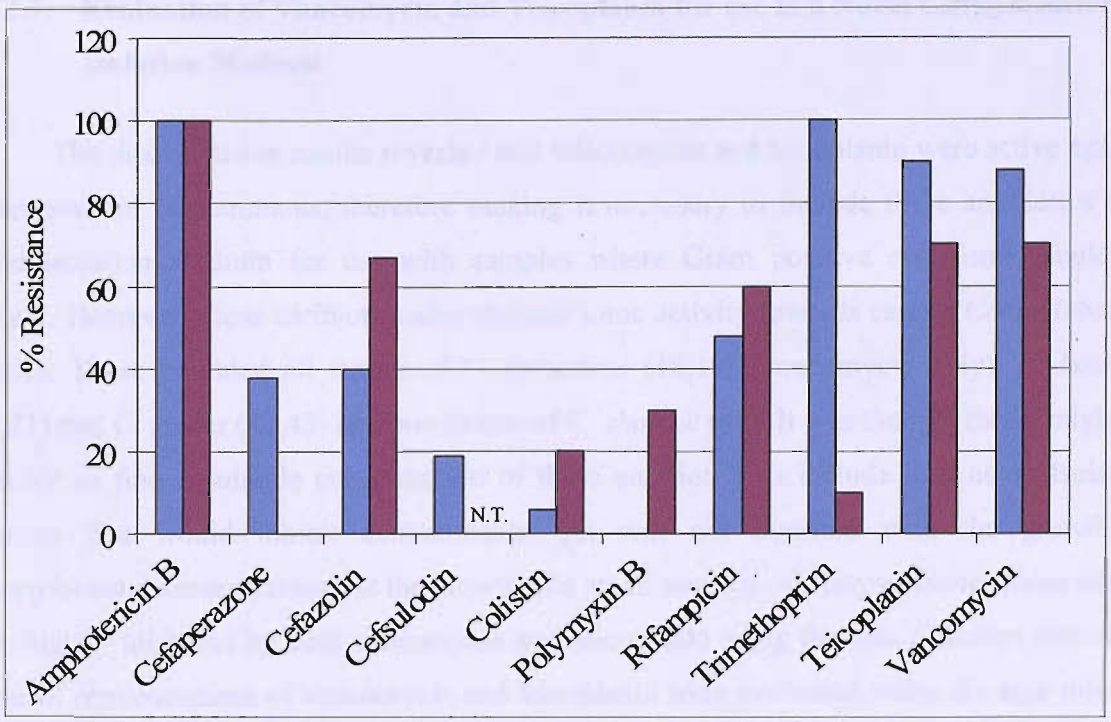


Figure 15. Summary of Overall Percentage Resistance to Antibiotics used in Selective Media to *Campylobacter* and non-*Campylobacter* species.

■ = *Campylobacter* species (n=51); ■ = potential competitor species (n=10).

Percentages were calculated from results in Table 16 and 17.

tested *Campylobacter* species. Polymyxin B also inhibited a high proportion of competitor species but unfortunately it also targeted all of the tested *Campylobacter* species. Cefazolin and rifampicin were considered ineffective because they were inhibitory to only a narrow range of competitor species, and inhibitory to a number of *Campylobacter* species.

To summarise, antibiotics that had little or no effect on *Campylobacteraceae* species included trimethoprim, teicoplanin and vancomycin (Figure 15). Furthermore, these antibiotics did inhibit the growth of a range of competitor bacteria. Although, they had no effect on *P. aeruginosa* which was only inhibited by antibiotics which also affected *Campylobacter* growth. Unfortunately, cefsulodin was not tested against competitor species, however this antibiotic is unlikely to be useful as it was shown to inhibit growth of the majority of *Campylobacter* species.

3.3.2.7. Evaluation of Vancomycin and Teicoplanin for use in a Novel *Campylobacter* Isolation Medium

The disc diffusion results revealed that vancomycin and teicoplanin were active against Gram-positive contaminants, therefore making it necessary to include these antibiotics in a novel isolation medium for use with samples where Gram positive organisms would be present. However, these antibiotics also showed some activity towards certain *Campylobacter* species. These included all strains of *C. helveticus* (18,19) (vancomycin only), *C. hominis* (20,21) and *C. rectus* (42,43) and one isolate of *C. showae* (45). It was thought that it might be possible to find a suitable concentration of these antibiotics to include in a novel isolation medium that would inhibit contaminants, yet will not interfere with the growth of *Campylobacteraceae* species. As the growth of a small number of *Campylobacteraceae* strains was slightly inhibited by both vancomycin and teicoplanin using the disc diffusion method, a range of concentrations of vancomycin and teicoplanin were evaluated, using the agar dilution method, for the growth of a number of *Campylobacteraceae* species.

When ABA agar medium was supplemented with teicoplanin, a number of *Campylobacteraceae* strains were inhibited (5/17 strains) (Table 18). The most sensitive species was *C. curvus* whose growth was completely inhibited by just 1 mg/l teicoplanin. Species which were inhibited at 6 mg/l included *C. concisus* (6), *C. lanienae* (36), *C. showae* (45) and *C. sputorum* (46). Therefore the only strain to be inhibited at 4 mg/l or below was *C. curvus* (8). *S. aureus* (Gram positive control strain) was inhibited by teicoplanin at just 1 mg/l and growth was completely suppressed at 2 mg/l. *Salmonella* (negative control) was not affected by teicoplanin at any concentration tested.

Vancomycin did not inhibit the growth of any *Campylobacter* strains at a concentration of 10 mg/l and below. However, at a concentration of 15 mg/l, a number of strains were inhibited (7/17 strains). These included *C. coli* (1), *C. curvus* 8 (although poor growth to start with), *C. fetus* (10), *C. helveticus* (18), *C. mucosalis* (40), *C. showae* (45) and *C. upsaliensis* (49). *S. aureus* was completely inhibited at a concentration of 2.5 mg/l vancomycin but growth of *Salmonella* was not affected at any concentration tested.

3.3.2.8. Evaluation of ABA VAT Medium for Isolation of all *Campylobacteraceae* Species

Accordingly, a novel selective isolation medium, ABA VAT, including vancomycin, trimethoprim and amphotericin B (VAT) was developed for the isolation of all

Table 18. Growth of *Campylobacteraceae* Species on ABA Supplemented with Various Concentrations of Teicoplanin and Vancomycin. Growth relates to the areas of growth on the four point growth scale. Growth is inhibited if growth with a higher concentration of antibiotics is less than growth on ABA without antibiotics. The last column ('inhibited?') describes whether the antibiotics at any of the tested concentrations had an effect on growth, the concentration stated shows at what concentration the *Campylobacter* were inhibited, No = no inhibitory effect.

Species	CC	Teicoplanin (mg/L)						Vancomycin (mg/L)					
	No.	0	1	2	4	6	Inhibited?	0	2.5	5	10	15	Inhibited?
C. coli	1	++++	+++	+++	+++	+++	No	++++	++++	++++	++++	++	15 mg/l
C. concisus	4	++++	++++	+++	+++	++	6 mg/l	+++	+ +++	+++	+++	++	No
C. curvus	8	+++	++	+	+	+	1 mg/l	+++	+++	+++	+++	+	15 mg/l
C. fetus	10	+++	++++	+++	+++	+++	No	+++	+++	+++	+++	+	15 mg/l
C. venerealis	13	+++	+++	+++	+++	+++	No	+++	+++	+++	++	++	No
C. gracilis	16	+++	+++	+++	+++	+++	No	+++	+++	+++	+++	+++	No
C. helveticus	18	+++	+++	+++	+++	+++	No	+++	+++	+++	++	+	15 mg/l
C. hyointestinalis	23	+++	+++	+++	+++	+++	No	+++	+++	+++	+++	+++	No
C. lawsonii	26	+++	+++	+++	++	+++	No	+++	+++	+++	+++	+++	No
C. doylei	28	+++	+++	+++	+++	+++	No	+++	+++	+++	+++	+++	No
C. jejuni	31	++++	+++	+++	+++	+++	No	+++	++++	++++	++++	++++	No
C. lanienae	36	+++	+++	+++	+++	+	6 mg/l	+++	+++	+++	++	++	No
C. lari	38	+++	+++	+++	+++	+++	No	+++	++++	+++	+++	+++	No
C. mucosalis	40	+++	+++	+++	+++	++ +	No	+++	+++	++	+++	+	15 mg/l
C. showae	45	+++	+++	+++	+++	+	6 mg/l	++++	+++	+++	+++	+	15 mg/l
C. sputorum	46	+++	+++	+++	+++	+	6 mg/l	+++	+++	++	+++	+++	No
C. upsaliensis	49	+++	+++	+++	+++	+++	No	+++	+++	+++	++	+	15 mg/l
S. typhimurium		++++	++++	++++	++++	++++	No	++++	++++	++++	++++	++++	No
S. aureus		+++	+	-	-	-	1 mg/l	+++	-	-	-	-	2.5mg/l

Table 19. Evaluation of the Novel VAT Selective Supplement Compared to CAT Selective Supplement and Without Selective Supplement for the Growth of *Campylobacteraceae* Species. Growth was assessed according to the Streak Dilution method.

		ABA	VAT	CAT
Campylobacter spp.				
C. coli	RM 2228	++++	+++	+++
C. coli	Lab 33	+++	+++	+++
C. coli	300.97	+++	+++	+++
C. concisus	CCUG 13144	++++	+++	++
C. concisus	CCUG 19995	++++	++++	+
C. concisus	Lastovica 396/96	+++	+++	++
C. fetus subsp. fetus	CCUG 32114	++++	+++	++
C. fetus subsp. fetus	Abdn 1076	++	++	++
C. fetus subsp. fetus	241.99	++	++	+++
C. fetus subsp. venerealis	CCUG 11287	++++	++++	++++
C. fetus subsp. venerealis	Armi 4402	+++	++++	++++
C. fetus subsp. venerealis	Abdn SM5	+++	+++	++++
C. helveticus	CCUG 30566	+++	+++	+++
C. helveticus	CCUG 34016	+++	+++	++++
C. hyointestinalis subsp. hyointestinalis	LMG 7538	++++	+++	+++
C. hyointestinalis subsp. hyointestinalis	LMG 9260	+++	+++	+++
C. hyointestinalis subsp. hyointestinalis	176.96	+++	+++	+++
C. hyointestinalis subsp. hyointestinalis	234.95	+++	++++	+++
C. hyointestinalis subsp. lawsonii	CHY 5	+++	+++	+++
C. hyointestinalis subsp. lawsonii	CCUG 27631	++++	++++	++++
C. jejuni subsp. doylei	SSI 5384	+++	+++	+++
C. jejuni subsp. doylei	CCUG 18266	++++	+++	++
C. jejuni subsp. doylei	269.97	++++	+++	+++
C. jejuni subsp. jejuni	NCTC 11168	++++	++++	++++
C. jejuni subsp. jejuni	RM 1221	++++	++++	++++
C. jejuni subsp. jejuni	SVS 4039	+++	+++	+++
C. jejuni subsp. jejuni	RM 1864 (=81-	+++	++++	+++
C. jejuni subsp. jejuni	47.97	+++	+++	+++
C. lanienae	NCTC 13004	+++	+++	+++
C. lanienae	DARDNI G718D	+++	+++	+++
C. lari	RM 2100	+++	+++	+++
C. lari	CCUG 22395	++++	+++	+++
C. mucosalis	CCUG 21559	+++	+++	+++
C. mucosalis	CCUG 23201	+++	+++	++++
C. sputorum bv. sputorum	Lastovica 86.92	+++	+++	+++
C. sputorum bv. paraureolyticus	LMG 11764	++++	++++	++
C. sputorum bv. fecalis	CCUG 20703	+++	+++	++
C. upsaliensis(49)	RM 3195	++++	+++	++
C. upsaliensis(50)	CCUG 19559	+++	+++	++
C. upsaliensis(51)	CCUG 19607	+++	+++	+++

Table 19 Continued.

Anaerobic-like *Campylobacter* spp.				
C. curvus (7)	SSI 19296	+++	+++	+++
C. curvus (8)	Lastovica 525.92	++++	++++	++++
C. curvus (9)	Lastovica 13A	+++	+++	+++
C. gracilis (16)	CCUG 27720	+++	+++	-
C. gracilis (17)	CCUG 13143	+++	+++	+
C. hominis (20)	NCTC CH001	+++	++++	++++
C. hominis(21)	NCTC CH003	+++	+++	++
C. rectus(42)	CCUG 20446	+++	+++	-
C. rectus (43)??	CCUG 11645	+++	++++	-/+
C. showae (44)	CCUG 30254	+++	+++	++++
C. showae (45)	CCUG 11641	+++	+++	++
Related non-*Campylobacter* spp.				
B. ureolyticus (52)	CCUG 18470	+++	+++	-
B. ureolyticus (53)	Rigshospitalet	++++	++++	+
A. butzleri (54)	SSI 71032	+++	+++	+++
A. butzleri (55)	29.97	++++	++++	++++
A. butzleri (56)	167.97	+++	+++	++++
A. cryaerophilus (57)	CCUG 17801 1)	+++	+++	+++
A. cryaerophilus (58)	SSI 70952	++++	++++	++
A. skirrowii (59)	BU 30CC 8B1	++++	++++	++
Control non-related bacteria				
Escherichia coli		++++	-	-/+
Staphylococcus aureus		+++	-	-
Salmonella typhimurium		++++	-	+
Bacillus cereus		++++	-	N/T
Bacillus lichenoformis		++++	-	N/T
Shigella sonnei		++++	-	N/T
Serratia marcescens		++++	++	N/T
Enterobacter cloacae		++++	-	N/T
Proteus vulgaris		++++	++	N/T
Proteus mirabilis		++++	+++	N/T
Acinetobacter baumannii		+++	+++	N/T
Klebsiella oxytoca		+++	-	N/T
Klebsiella pneumoniae		+++	-	N/T
Pseudomonas aeruginosa		++++	+++	N/T
Citrobacter freundii		++++	-	N/T
Citrobacter youngii		+++	-	N/T
Enterococcus faecalis		++++	-	N/T

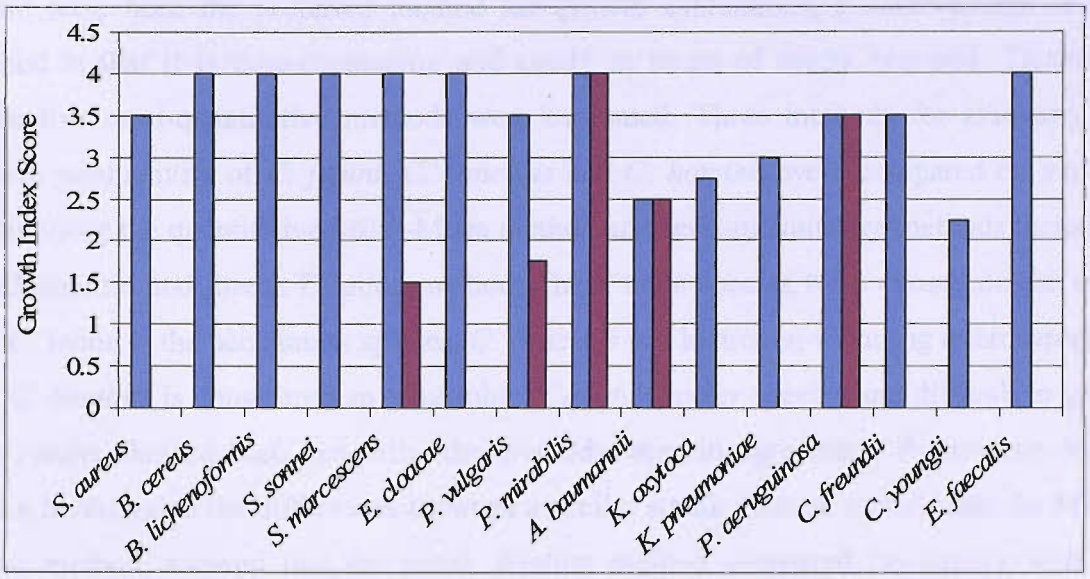


Figure 16. Growth of Potential Competitor species on ABA and ABA Supplemented with VAT. ■ = ABA (without VAT); ■ = ABA VAT. VAT included 10 mg/l vancomycin, 5 mg/l trimethoprim and 10 mg/l amphotericin B. Growth was scored according to the Streak Dilution method. For full names of species refer to Table 19.

growth of any *Campylobacteraceae* species tested but did suppress the growth of a number of potential background non-*Campylobacteraceae* bacteria (Table 19). Growth of 13 out of the 17 non-*Campylobacteraceae* strains tested was completely inhibited by the VAT supplement. These strains included *Staphylococcus aureus*, *Bacillus* species (*B. cereus* and *B. licheniformis*), *Shigella sonnei*, *Enterobacter cloacae*, *Klebsiella (K. pneumonia* and *K. oxytoca*), *Citrobacter freundii*, *Citrobacter youngii* and *Enterococcus faecalis*. Strains that were only slightly inhibited included *Serratia marcescens* and *Proteus vulgaris*. The VAT supplement had no effect on the growth of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* or *Proteus mirabilis* (Figure 16).

3.4. DISCUSSION

3.4.1. Choice of Growth Assessment Method

During this PhD study, a large number of culture comparisons with a wide variety of strains needed to be carried out. In view of this, along with considerations of strict project deadlines and time constraints, a growth assessment method needed to be used which would allow accurate yet quick comparisons, without having to use an excessive number of plates. Although, ideally, a quantitative method such as the Miles-Misra method

would have been the preferred method for growth evaluations, a disadvantage of this method is that it is time-consuming and costly in terms of media required. Therefore, alternative semi-quantitative methods were evaluated. Three methods for assessing the growth productivity of *C. jejuni*, *C. concisus* and *C. hominis* were compared on various media using the quantitative Miles-Misra method and semi-quantitative methods including the Ecometric and Streak Dilution method. These three species were chosen on the basis that *C. jejuni* is the benchmark species, *C. concisus* is a hydrogen-requiring microaerophile and *C. hominis* is considered an 'anaerobic' *Campylobacter* species and difficult to grow. The results showed that, generally, the methods were in agreement. A previous study, which investigated the differences between a similar streak dilution method and the Miles-Misra method, showed that the streak dilution method compared favourably with the quantitative Miles-Misra method and that the degree of accuracy was sufficient to enable detection of 10-fold differences in viable counts (Urquhart and Gould 1965). It was concluded that although there was a slightly lower degree of accuracy compared to the Miles-Misra method, this was more than outweighed by the increased speed and simplicity of the streak dilution technique with reduced costs due to the number of plates required and technical labour costs. Although the ecometric method was selected by previous authors to assess growth on various media (Corry and Atabay 1997), as there was little difference in the accuracy of growth measurement between this method and the streak dilution method, the latter was chosen due to its simplicity of use and ease of reading results. It is thought that a five-point growth scale (streaking in 5 areas of the plates rather than four) may have increased the accuracy of the method.

3.4.2. Media Comparisons

During this study a number of selective isolation media was investigated for the growth of a wide range of *Campylobacter* species and related organisms. A number of basal media and blood supplements were compared and, in addition, detailed investigations into antibiotic selective supplements were carried out, which ultimately resulted in the development of a new selective medium for the isolation of all *Campylobacteraceae*.

3.4.2.1. Evaluation of Tryptose Blood Agar (TBA) and Anaerobe Basal Agar (ABA)

In these experiments, the growth of *Campylobacter* species on Anaerobe Basal Agar (ABA) and Tryptose Blood Agar (TBA) was evaluated. The results showed that there

were no marked differences in the growth of *Campylobacter* species on the two basal media. However on certain occasions growth was slightly reduced on TBA medium. Moreover, the colony sizes of certain anaerobic *Campylobacter* species were much bigger on ABA compared to TBA. Previous to this study, TBA medium was considered as the 'gold standard', since it is routinely used to successfully recover all types of *Campylobacter* species from clinical samples as part of the Cape Town protocol (Lastovica 2006). Anaerobe Basal Agar was proposed by Oxoid (Peter Stevens, personal communication) as a successful basal medium for the isolation of *Campylobacter* species, due to its complex nature and its extensive use for the culture of fastidious organisms. In addition, preliminary studies carried out by Oxoid demonstrated comparable results to TBA medium (Smith *et al.* 2006c). The National Food Centre, Dublin also carried out comparisons of these two media (Claire Cagney, Pers. Comm.) when isolating *Campylobacter* from beef samples and also found that, although there was no significant difference in *Campylobacter* growth, the growth of contaminants in the sample were greatly reduced when ABA was used. Therefore, ABA medium was selected as the basal medium for the recovery of *Campylobacteraceae* species. It was thought that ABA was successful for the isolation of *Campylobacteraceae* species due to the complex mixture of its components containing many substrates used in *Campylobacter* respiration. ABA has previously been described as a 'nutritious agar for the growth of anaerobic micro-organisms, particularly *Bacteroides* species and other fastidious anaerobes' (Bridson 1998). The medium contains peptones, carefully selected to support good growth of anaerobic bacteria, and yeast extract as a vitamin source (composition is shown in Table 6 in Materials and Methods section). Starch is present to absorb certain toxic metabolites, whilst haemin and vitamin K are growth factors thought to be required by many *Bacteroides* species. Sodium succinate is an organic acid thought to improve the growth of anaerobic bacteria, including *Prevotella melaninogenica* and *Bacteroides* species and has also been shown to increase the growth of a number of *Campylobacter* species (Hinton 2006). Sodium pyruvate is thought to be an energy source and also acts similarly to catalase to degrade traces of hydrogen peroxide, which may be produced by the action of molecular oxygen on media components. L-cysteine hydrochloride and dithiothreitol are reducing agents, which have also been shown to stimulate the growth of some anaerobes (Bridson 1998). In comparison, TBA (Tryptose Blood agar), is also reported to be a highly nutritious medium specially developed to support the growth of fastidious organisms, however it is much simpler in its composition (Bridson 1998).

ABA was selected as the basal medium of choice, since it is composed of a large number of substrates previously reported to be able to be efficiently utilised by the metabolism of *Campylobacter* species. Campylobacters do not metabolise carbohydrates, but they may obtain energy by oxidising amino acids and intermediates of the tricarboxylic acid (TCA) cycle. Many of these, including sodium succinate, pyruvate and cysteine are included in ABA medium.

3.4.2.2. Blood Supplementation

Many *Campylobacter* isolation media contain either laked or defribrinated blood (at 5-10% (v/v)) to quench toxic reactive oxygen species (e.g. hydrogen peroxide and superoxide anions) (Bolton *et al.* 1984a), which can form when media are exposed to light and air. In addition, blood is thought to neutralise trimethoprim antagonists (Corry *et al.* 1995b). In this experiment growth was compared on ABA when supplemented with laked and defibrinated horse blood at concentrations of 5% and 10%.

Growth was generally higher when supplemented with defibrinated horse blood, although these differences were not significant. Defibrinated horse blood is produced by agitation to denature fibrinogen. It is thought that this process may also cause some lysis of red blood cells; however it is thought that there is also a release of NAD, thought to stimulate the growth of certain bacteria such as *Haemophilus influenzae,* and this may also be the case for certain *Campylobacter* species. A previous study has shown that lysed horse blood was the best supplement, out of 22 supplements screened by Bolton and Coates (Bolton and Coates 1983), for compounds that facilitate aerotolerance, and that saponin-lysed horse blood has generally been found to be the most suitable. Lysed blood is stimulatory for many bacteria, including *Clostridium* and *Haemophilus* species. The lytic agent used for lysed horse blood is white saponin, which appears not to affect the growth of bacteria at the level required for lysis (Bridson 1998). Studies have shown that campylobacters are capable of haemolytic activity (the lysis of haemoglobin to release iron), which can usually be readily seen as loss of red colour on blood agar plates (Tay *et al.* 1995). The same authors found that campylobacters may possess a cell-associated haemolysin, and haemolytic activity was found in 94% of *Campylobacter* species tested, therefore suggesting a potential role as a virulence factor in campylobacter gastroenteritis (Tay *et al.* 1995). It is worth commenting that the two different types of blood might show different reactions with regard to reactive oxygen species, known to be harmful to *Campylobacter* species. For example, one might show more catalase and superoxide dismutase activity, intrinsic to red blood cells (Moran and Upton 1987). Although blood

has been widely documented to have favourable effects on growth of many bacteria, including *Campylobacter,* it is worth noting that the increase of iron in the media is also likely to trigger the Fenton reaction, which in itself is likely to produce free radicals, which are harmful to *Campylobacter* growth (Ishikawa 2003).

Although the results indicated that defibrinated blood at 10% produced slightly higher recovery of certain species, it was decided that laked horse blood should be used, due to its longer shelf life compared to that of defibrinated horse blood, which is approximately one week.

3.4.2.3. Evaluation of Selective Media and Selective Supplements

Enhancing media selectivity while not adversely affecting recovery of the target organism is a common microbiological dilemma (Line and Berrang 2005). The non-*Campylobacter* colonies growing on less selective agars can mask *Campylobacter* colonies, making enumeration a challenging and tedious task. However, antibiotics added to increase selectivity of a medium may harm recovery of the target organism, especially if the target consists of cells that have undergone some form of stress. This is especially a problem for isolation of emerging *Campylobacteraceae,* since the majority of selective isolation media available have been developed to isolate *C. jejuni* and *C. coli* species.

Experiments were carried out to evaluate a range of basal agar media with and without selective supplements, including Preston, Skirrow, CCDA and CAT for the growth of a range of *Campylobacteraceae* species.

The least inhibitory selective supplement was CAT selective supplement, inhibiting growth of 24% of the *Campylobacteraceae* species tested when supplemented to TBA medium. Preston selective supplement inhibited growth of the highest number (78%) of species. Both Skirrow and CCDA selective supplements also inhibited growth, inhibiting 65% and 60% of the *Campylobacteraceae* species tested, respectively.

A large number of antibiotic supplements and basal media have been developed for the isolation of *Campylobacter* species from a wide variety of matrices over the past 30 years (Corry *et al.* 1995a; 2002). Although many previous studies in the literature have evaluated the recovery of *Campylobacter* species on different media, these comparisons have almost always been concerned with only *C. jejuni* and *C. coli* species, due to their perceived importance as human pathogens. As there are so many different media currently available for the isolation of *Campylobacter* species, it was impossible to carry out a comprehensive evaluation of all available media, since the scope and scale of the project would not allow this. Therefore, the selective supplements chosen for evaluation were

those that had been most extensively used, according to the literature. In addition, the media most likely to be able to recover non-*jejuni/coli* species were selected for evaluation. To date, few studies have shown the development of a selective medium suitable for the recovery of all *Campylobacteraceae* species, where generally filtration onto an antibiotic-free blood-containing medium is used (Lastovica 2006).

Despite selective media being developed for the isolation of *C. jejuni* and *C. coli* species, findings from several studies have shown that nearly all available selective media also inhibit the growth of a number of these strains (Corry *et al.* 1995b; Lastovica and Skirrow 2000). Indeed, *C. coli* strains have been shown to be inhibited by most of the antibiotic combinations used in selective media, but especially by those containing cephalosporins and rifampicin (Ng *et al.* 1985b). During this study, although most of the antibiotic selective supplements evaluated (including mCCDA, CAT, Skirrow and Preston), did not inhibit the growth of any of the *C. jejuni* or *C. coli* strains, Preston selective supplement slightly inhibited the growth of all three *C. coli* strains. Preston selective supplement was found to be the most inhibitory selective supplement tested in this experiment. It is likely that this is due to a combination of antibiotics included in the medium, particularly rifampicin, which was likely to be the cause of growth inhibition of Preston selective supplement.

TBA basal medium (5% blood) (without selective supplement) was previously considered the 'gold standard' medium since, used as part of the Cape Town protocol, it has been shown to recover all species of *Campylobacter* from clinical samples (Lastovica and Le Roux 2000). This study showed that all of the *Campylobacteraceae* species tested grew well on TBA medium. However, Preston and Skirrow basal media (no antibiotic supplements) performed equally well for the isolates tested (and in later studies the superiority of ABA basal medium was demonstrated).

When Preston supplement was included in the Preston basal medium, growth of certain species was inhibited, including *C. concisus*, *C. lari*, and *C. upsaliensis*, whereas growth of *C. coli* and *C. jejuni* species was unaffected. The constituents of Preston supplement include polymyxin B, rifampicin, trimethoprim, and cycloheximide. Therefore, one of these antibiotics in particular, or more likely the combined effect of these antibiotics, most likely of polymyxin B and rifampicin, was responsible for inhibiting growth.

When Skirrow supplement was included in Skirrow basal medium, growth of *C. upsaliensis*, *C. lari* and *C. concisus* was inhibited. However *C. jejuni* and *C. coli* species

were unaffected (as for Preston supplement). Antibiotics included in Skirrow supplement were vancomycin (10 mg/l), trimethoprim (5 mg/l) and polymyxin B.

Despite most media being developed for the isolation of *C. jejuni* and *C. coli* species, CAT selective medium, which contains reduced levels of cefoperazone (8 µg/l), was developed especially to include recovery of *C. upsaliensis* species (Aspinall *et al.* 1993b). The results from the present study are in agreement with results from previous experiments, which have shown that CAT agar supported the growth of a wider variety of *Campylobacter* and *Arcobacter* species than mCCDA (Corry and Atabay 1997). It was concluded that there was likely to be an inhibitory synergistic effect between deoxycholate in the basal medium and the higher level of cefoperazone (32 µg/l) in the antibiotic supplement used in mCCDA medium. This was supported by results from this study, which showed that mCCDA and CAT supplements were less inhibitory when supplemented to TBA basal medium rather than the charcoal based agar containing deoxycholate. Furthermore, antibiotic susceptibility disc diffusion tests also showed that cefoperazone was inhibitory to a number of *Campylobacter* and *Arcobacter* species. Byrne *et al.* (2001) showed that the differences between mCCDA and CAT were minimal and that the majority of *C. upsaliensis* strains were able to grow well on mCCDA. Corry and Atabay (1997) assessed growth of various *Campylobacteraceae* on CAT, mCCDA and a non-selective blood agar using the semi-quantitative ecometric method. Results showed there were no differences in growth between all isolates of *C. jejuni*, *C. coli*, *C. fetus* and *C. lari* on the three media. However, growth of emerging species including *C. hyointestinalis*, *C. sputorum* biovar. *faecalis*, *A. butzleri* and some strains of *A. cryaerophilus* were severely inhibited by mCCDA. Although CAT agar was less inhibitory towards these species it still inhibited the growth of certain strains of *C. hyointestinalis*, *C. sputorum* biovar. *faecalis*, *A. butzleri* and *A. cryaerophilus*, but generally to a lesser degree than CCDA. This can be attributed to the lower level of cefoperazone (8 mg/l) contained in this medium. Results from the present study support this finding in that when TBA was supplemented with mCCDA selective supplement, growth of *C. sputorum*, *A. butzleri* and *A. cryaerophilus* was inhibited. However, results from this study also showed that when mCCDA supplement was added to TBA basal medium instead, growth of *C. hyointestinalis* strains was not affected. Some studies have shown that it is possible to recover some *C. upsaliensis* strains on CCDA medium (at a concentration of 32 mg/l cefoperazone) from both human (Taylor *et al.* 1989) and dog and cat faeces (Burnens and Nicolet 1992). However, Aspinall *et al.* (1996) has shown that, although it may be possible to isolate some *C. upsaliensis* strains on CCDA medium, the majority will be inhibited by

the high concentration of cefoperazone contained in this medium. The study showed that when using CAT medium, the isolation rate was improved from 29% to 84%, whilst membrane filtration (no antibiotic selection) recovered 90% of isolates from faecal samples. Species which showed reduced growth in the current study when CCDA selective supplement was included in the medium were *C. concisus*, *C. lari* and *C. upsaliensis*. Growth of *C. coli* and *C. jejuni* strains was largely unaffected. The antibiotics included in the CCDA supplement were 32 mg/l cefoperazone and 10 mg/l amphotericin B. Amphotericin B is an antifungal agent and it is unlikely that this would interfere with the growth of *Campylobacter* species. However, many previous studies have documented the inhibitory effects of cefoperazone on *Campylobacter* species (Corry and Atabay 1997; Lastovica and Skirrow 2000).

The fact that CCDA medium is sub-optimal for the growth of a number of emerging *Campylobacteraceae* species is disconcerting, since CCDA is a widely approved medium used for isolation of *Campylobacter* species in many clinical and epidemiological surveys (Korhonen and Martikainen 1990; Bhadra *et al.* 1991; Piersimoni *et al.* 1995; Corry and Atabay 1997; Arzate Barbosa *et al.* 1999; Engberg *et al.* 2000; Byrne *et al.* 2001; Diergaardt *et al.* 2004; Oyarzabal *et al.* 2005). It is likely that this could be contributing to the underdetection of these organisms.

The media evaluated in this study were based on those that have been most widely used for the recovery of *Campylobacter* species from a range of samples and on those thought to be the least inhibitory to *Campylobacteraceae* species. A number of other media have been developed for the isolation of *Campylobacter* species, but were not evaluated during this study. Campy-Line agar and Campy-Cefex agar have been used to recover *C. jejuni* and *C. coli* species from poultry samples. One study showed that Campy-Line agar was the method of choice due to its high selectivity (Line and Berrang 2005). However, this medium contains a range of antibiotics, including a high concentration of cefoperazone (33 mg/l). Therefore it was thought unlikely that this would be a suitable candidate for the isolation of emerging *Campylobacteraceae*. The selective supplements included in Campy-Cefex agar are similar to CCDA except the basal medium is different. Because CCDA has been used more extensively, compared to Campy-Cefex, this was the chosen medium to be evaluated in this study. Campy-BAP is similar to Skirrow medium but contains amphotericin B; however, Skirrow medium was selected over this for evaluation in the present study as it has been used more extensively.

Since initiation of this project and after having undertaking these experiments, reports of a number of novel *Campylobacter* isolation media with the potential to isolate

emergent *Campylobacteraceae* species have been reported. A selective medium called AAV medium was developed by Thomas (2005), which was evaluated for the isolation of six types of *Campylobacter* species of pathogenic importance. However, the growth of *C. upsaliensis* was unsuccessful on this medium due to its antibiotic susceptibility towards even low levels of the antibiotic aztreonam, which was included in the medium. The composition of the AAV selective supplement is similar to VAT selective supplement (developed during the present study) in that it included vancomycin and amphotericin B. However, it is possible that the presence of aztreonam (in place of trimethoprim in VAT selective supplement), included to inhibit other Gram negative organisms, would also be inhibitory to a number of *Campylobacteraceae* species as studies assessing susceptibility of *Campylobacteraceae* to aztreonam have been limited to *C. jejuni* and *C. coli* strains (Fernandez 2000).

A novel method has recently been developed which is reported to successfully isolate antibiotic-sensitive *Campylobacter* species on a blood- and antibiotic-free differential, Kapadnis-Baseri medium (KB medium) (Baserisalehi *et al.* 2005). The method used to select against competing bacteria consisted of an initial centrifugation step to sediment all bacteria which after a short space of time permitted actively motile *Campylobacter* species only, to migrate away from the pellet (with dart-like movements) before other motile bacteria which are much slower. In addition, malachite green was included in the medium as selective agent for inhibiting growth of Gram-positive bacteria and lactose was used for differentiation of lactose- and nonlactose-fermenting bacteria and phenol red as a pH indicator for their discrimination. The medium was compared to CAT medium and showed that although enumeration data were comparable, a number of strains subsequently found to be susceptible to certain antibiotics commonly included in selective media were isolated using the KB but not the CAT medium (Baserisalehi *et al.* 2004).

3.4.3.6 Antibiotic Susceptibility Testing

Generally, when assessing antimicrobial susceptibility and determining minimum inhibitory concentration (MIC), the most commonly used and standardised methods have been to use an agar dilution protocol (McDermott *et al.* 2004; 2005). However, these methods are extremely labour intensive. Generally, standard methods (formulated by the NCCLS (National Committee for Clinical Laboratory Standards) have only been formulated for antimicrobial agents such as fluoroquinolones used in a clinical environment. As very few antibiotics used in selective media are used in a clinical environment, there remains a lack of standardised methods for testing these antibiotics, and

certainly no standard methods exist for some of the more fastidious emerging *Campylobacteraceae* species. The antibiotic disc diffusion tests were applied to assess the antimicrobial susceptibility of these species.

The antibiotic disc diffusion tests were carried out according to the NCCLS (Gaudreau and Gilbert 1997; Huysmans and Turnidge 1997) with a small number of modifications. ABA medium (supplemented with 5% laked horse blood) was used as opposed to Muller-Hinton agar (supplemented with 5% sheep defibrinated sheep blood) and also the incubation atmosphere contained hydrogen as opposed to standard microaerobic incubation. However, preliminary results with *C. jejuni*, *C. coli*, *C. concisus* and *C. upsaliensis* showed that there was no significant difference in the disc diffusion results achieved using Muller-Hinton agar and ABA medium.

A number of antibiotics commonly incorporated into selective media, including trimethoprim, polymyxin B, colistin sulphate, cefoperazone, vancomycin, teicoplanin, cefazolin and cefsulodin, were assessed for their antibiotic susceptibilities against the CAMPYCHECK reference strain set and a number of other bacteria. It was thought that this would then provide information regarding which antibiotics would be suitable for use in a novel selective medium for isolation of all *Campylobacteraceae*.

All of the *Campylobacteraceae* strains tested were resistant to the action of amphotericin B. This is usually included in selective media to suppress growth of background fungi and yeasts, found in many sample matrices. It acts on ergosterol in the fungal membrane and therefore its action is unlikely to affect bacteria (Williams *et al* 1996). There is no reported evidence that amphotericin is inhibitory to bacteria and it has been widely used in *Campylobacter* selective isolation media as a successful agent to suppress the growth of fungi (Corry and Atabay 1997; Murinda *et al.* 2006)

Trimethoprim showed no activity against *Campylobacteraceae* species, however, it was effective against most of the competitor strains tested. It is not surprising then that trimethoprim has been included in such a wide range of selective media including Preston and Skirrow media (Bolton and Robertson 1982). Trimethoprim is a potent inhibitor of the bacterial enzyme dihydrofolate reductase, which is responsible for the conversion of dihydrofolate to tetrahydrofolate, and its interference is thought to have multiple effects on metabolism (Gibreel and Skold 1998; Quinlivan *et al.* 2000). The reaction most severely affected is thymine synthesis, ultimately affecting DNA synthesis. It is possible that the ABA medium may contain extracellular thymidine, thought to inhibit trimethoprim activity (Bolton and Robertson 1982). However, it has also been shown that when the medium is supplemented with blood, the effects are reversed due to the presence of

thymidine phosphorylase in red blood cells (Bolton and Robertson 1982). It was evident that trimethoprim activity was not being inhibited in this medium, as the growth of a number of non-*Campylobacteraceae* strains was reduced. Although *Campylobacter* has been regarded as endogenously resistant to trimethoprim, there is evidence that *Campylobacter* are resistant to trimethoprim due to the possession of trimethoprim-resistant dihydrofolate reductase enzymes, determined by R-plasmids rather than the chromosome (Franklin and Snow 1989; Gibreel and Skold 1998). Furthermore, it has been suggested that the frequent occurrence of high-level trimethoprim resistance in clinical isolates of *C. jejuni* could be related to the heavy exposure of food producing animals to antibacterial drugs, which could lead to the acquisition of foreign resistance genes in naturally transformable strains of *C. jejuni* (Gibreel and Skold 1998). However, a recent study has shown that using a disc diffusion method 6.3% of *C. lari* species and 5.5% of unidentified catalase negative *Campylobacter* species were susceptible to trimethoprim using a 5 μg disc, suggesting that not all species are completely resistant.

Teicoplanin and vancomycin showed similar activities against *Campylobacter* and competitor strains. The majority of *Campylobacteraceae* strains were resistant to these two glycopeptide antibiotics. However, surprisingly, a limited number of species (*C. helveticus, C. mucosalis* and *C. showae*) showed susceptibility. The mode of action of both teicoplanin and vancomycin is to inhibit the second stage of cell wall (peptoglycan) synthesis in Gram positive bacteria only (Williams and Kalman 1977; Parenti *et al.* 2000). However, there is also evidence that vancomycin can alter the permeability of the cell membrane and selectively inhibit ribonucleic acid synthesis, affecting both Gram positive and Gram negative bacteria (Watanakunakorn 1984). Therefore, it is possible that the susceptible *Campylobacter* species in this study were being affected through this mode of action. Teicoplanin or vancomycin is commonly included as a selective supplement in many *Campylobacter* selective isolation media and antibiotic susceptibility tests have previously shown no effects on *Campylobacter* species. However these studies were mainly restricted to *C. jejuni* and *C. coli* isolates and in some cases *C. upsaliensis* (Karmali *et al.* 1986; Preston *et al.* 1990; Aspinall *et al.* 1996; Byrne *et al.* 2001).

Approximately half of the *Campylobacteraceae* species tested were susceptible to rifampicin. Rifampicin, thought to be an inhibitor of transcription, is also included in a number of selective media such as Preston medium (Bolton and Robertson 1982) and many studies have also reported its inhibitory effects on *Campylobacter* species (Humphrey and Cruickshank 1985; Corry *et al.* 1995b). Other antibiotic susceptibility tests have shown that *Campylobacter* species vary in their susceptibility to rifampicin,

depending on species and strain type, although again, only a limited number of thermotolerant species have been tested in previous studies (Freland *et al.* 1987; Richter *et al.* 1989; Ballabene and Terzolo 1992). Rifampicin is thought to inhibit DNA-dependent RNA polymerase in susceptable bacterial cells by binding to its beta subunit, thus preventing transcription of messenger RNA (mRNA), and subsequent translation to proteins (Williams *et al* 1996). Studies have shown that this effect is more pronounced in injured cells (e.g. by the freezing process), as damage to their outer membrane would allow the the ingress of antibiotics such as rifampicin, which would generally be excluded by undamaged cells (Humphrey and Cruickshank 1985).

Many of the *Campylobacteraceae* species tested were inhibited by cefoperazone, which is a constituent of many routinely used selective media including CCDA and CAT medium (Corry *et al.* 1995b; Piersimoni *et al.* 1995; Corry and Atabay 1997; Lastovica and Skirrow 2000; Byrne *et al.* 2001). Cefoperazone is thought to act on peptidoglycan biosynthesis and it has been widely reported to inhibit growth of a number of *Campylobacter* species (Lastovica and Skirrow 2000; Williams *et al.* 1996). Some previous studies have shown that cefoperazone does not affect the growth of *Campylobacter*, however, these studies have been largely limited to *C. jejuni* and *C. coli* species. Furthermore, there is also evidence to show that a number of *Campylobacter* species are inhibited by cefoperazone (Bolton and Robertson 1982; Czinn *et al.* 1989; Lachance *et al.* 1993).

The majority of *Campylobacter* species were susceptible to cefazolin and cefsulodin, which, like cefoperazone are β-lactam antibiotics that affect peptidoglycan biosynthesis (Williams *et al.* 1996). These antibiotics have been included in isolation media to a lesser extent than cefoperazone, and their inhibitory effects on *Campylobacter* species have been widely reported (Glupczynski *et al.* 1988; Corry *et al.* 1995a).

The antimicrobial action of both colistin sulphate and polymyxin B are thought to be similar, which is reflected by their similar antibiotic susceptibility profiles in this study. Both of these antibiotics, thought to act on the LPS and outer membrane, affect the cell membrane permeability causing cytoplasmic leakage in Gram negative bacteria, inhibited the majority of *Campylobacter* species tested (Vaara 1992). Colistin sulphate is a common constituent of many selective isolation media (Goossens *et al.* 1986), however many studies have also shown that it is inhibitory at the concentrations used in isolation media to certain *C. jejuni/coli* strains as well as emerging *Campylobacter* species (Kiehlbauch *et al.* 1992).

Although, a number of selective isolation media have been developed for the recovery of *Arcobacter* species (Houf *et al.* 2001a), the majority of studies do not test for *Arcobacter* species *per se*. Instead, studies generally use *Campylobacter* isolation media to detect the prevalence of *Arcobacter* species as well as *Campylobacter* species. However, a number of studies have documented the susceptibility of *Arcobacter* species to a number of antibiotics contained in selective *Campylobacter* isolation media as well as *Arcobacter* isolation media (Houf *et al.* 2001b).

In this study, results showed that all strains of *Arcobacter* species were susceptible to both colistin sulphate and polymyxin B. All strains of *A. cryaerophilus, A. skirrowii* and one strain of *A. butzleri* were also susceptible to cefaperazone and certain strains were also susceptible to cefazolin, cefsuludin and rifampicin. The only antibiotics which did not inhibit growth of any *Arcobacter* species were amphotericin B, trimethoprim, vancomycin and teicoplanin. Previous studies evaluating the susceptibility of *Arcobacter* species to various selective agents included in isolation media are generally limited, probably due to their lack of perceived importance as clinical pathogens compared to *Campylobacter* species. A study using the agar dilution method to determine the antibiotic susceptibilities of 12 antibiotics commonly included in selective media to over 100 strains within the genus *Arcobacter* showed similar results to this study (Houf *et al.* 2001b). All three of the *Arcobacter* species tested, including *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*, were susceptible to colistin and rifampin at concentrations used in selective media. The study also showed that the MIC levels for cefoperzone to *A. cryaerophilus* and *A. butzleri* were only marginally above the concentration included in CCDA medium (32 μg/ml) and therefore might be detrimental to stressed or injured cells of these species. Generally, all of the *Arcobacter* species evaluated were resistant to trimethoprim (MIC of >256 μg/ml) and vancomycin (MIC of >256 μg/ml), as was found during this study. *A. skirrowii* was the most susceptible, with a number of strains with MICs of >128 μg/ml and 64 μg/ml for trimethoprim and vancomycin respectively, but these levels are well in excess of the concentrations included in selective media (Houf *et al.* 2001b).

Although previous studies in the literature have reported on antibiotic susceptibilities to a number of antibiotics included in selective media, these have been generally limited to *C. jejuni* and *C. coli* species. Furthermore, these strains would have initially been isolated using selective media and so would show biased resistance towards certain antibiotics, as only those strains which resisted antibiotics during primary isolation would be represented.

Although the results showed that a number of *Campylobacteraceae* reference strains were resistant to the activity of antibiotics such as trimethoprim and vancomycin, the true susceptibilities to these antibiotics to 'wild-type' *Campylobacteraceae* species is not known. It is possible that isolates recovered from environmental or processed samples would be in a stressed state and sub-lethally injured, and the susceptibility of these strains to the antibiotics evaluated is likely to be different.

It is unlikely that these susceptibility profiles could be used as an identification tool to differentiate species within the *Campylobacteraceae*, since the differences between species were not consistent, with considerable overlap and variation between resistance patterns within a species.

Results from both studies on the evaluation of selective supplements and the antibiotic disc diffusion tests have shown that the use of commonly used selective supplements are not suitable for the recovery of emerging *Campylobacteraceae* species including *Arcobacter* species, and may be contributing to the underdetection of these organisms. However, a limited number of antibiotics showed potential for use as selective agents, which could be incorporated into an improved minimally selective medium, suitable for isolation of all *Campylobacteraceae* species, avoiding commonly used selective antibiotics which are now known to inhibit a number of *Campylobacteraceae* species.

3.4.3.7. Formulation of a Novel Plating Medium; ABA VAT

Many different combinations of antibiotics have been included in selective media for isolation of thermophilic campylobacters. During this study a number of these commonly used selective supplements including CCDA, Preston, Skirrow and CAT, were evaluated to reveal that none was suitable for the isolation of emerging *Campylobacters*. The rationale for choosing antibiotics contained in VAT medium was firstly based on the fact that none of these antibiotic agents was shown to be inhibitory towards the *Campylobacter* strains tested using antibiotic disc diffusion tests. Furthermore, the selective combination contains three agents, each active against a certain group of organisms: vancomycin, active only against the Gram positive organisms; trimethoprim, predominantly active against Gram negative organisms and amphotericin B, active against yeasts and fungi.

The antibiotic disc diffusion tests showed that *Campylobacteraceae* species were not inhibited by these antibiotics individually. However it was not known if the antibiotics would inhibit species at their working concentrations and when included in combination

with other antibiotics. Therefore the antibiotics were supplemented to the ABA basal medium at the concentrations usually used in selective media. These concentrations were 5 mg/l trimethoprim, 10 mg/l amphotericin B, 10 mg/l vancomycin (teicoplanin was not chosen due to its greater level of inhibition, expense and lack of availability for use). ABA VAT medium was evaluated for growth with all three antibiotics in combination at their selected concentrations against *Campylobacteraceae* species and potential competitor species. The results showed there were no inhibitory effects on *Campylobacteraceae* species, but the growth of the majority of competitor species was inhibited. However, growth of *Pseudomonas aeruginosa* and *Proteus* species was not inhibited, which might pose a problem in future recovery studies (Chapter 6).

Although VAT selective supplement has been shown to work on reference strains in pure culture, when working with actual sample matrices there could be a considerable difference between these adapted lab cultures and 'wild-type' *Campylobacteraceae* isolates, complicated by the presence of high levels of contaminating flora and differential chemical constituents of the sample matrix. The background contaminants present in samples may be diverse and high in number compared to *Campylobacteraceae* isolates, which may be present. It is possible that the antibiotic concentrations of the VAT selective supplement, developed and used in this experiment (testing sample contaminants which are pure with relatively low inoculum levels of) may not be effective when applied to sample matrices. There is also a possibility that the selected antibiotics and concentrations will inhibit certain *Campylobacter* isolates in real samples as they may be physiologically stressed and/or damaged, unlike the strains tested in this experiment. Furthermore, only a limited number of strains was tested (n =59) in this study, and it is possible that if a larger number of isolates were tested, they might well be susceptible to the antibiotics evaluated.

Based on results from the experiments carried out, all of the *Campylobacteraceae* tested were resistant to VAT selective supplement, which also compared well to other CAT selective supplement. Due to the slow-growing and fastidious nature of *Campylobacteraceae* species, they would soon be overgrown by competitor bacteria present in the sample if antibiotics were not included in the media. Further evaluations of the VAT selective supplement are discussed in Chapter 6.

CHAPTER 4

DEVELOPMENT OF INCUBATION ATMOSPHERES FOR THE RECOVERY OF *CAMPYLOBACTERACEAE*

4.1. INTRODUCTION

It has generally been assumed that *Campylobacter* species are microaerophilic, requiring low levels of oxygen for growth, typically 4-10%, but are inhibited by atmospheric oxygen levels. It has also been suggested that they require increased carbon dioxide levels and that in addition, growth of certain species can be enhanced by supplementing with hydrogen (On 1996; Lastovica and Skirrow 2000; Lastovica 2006). However, most studies have only reported growth conditions for *C. jejuni* and *C. coli* species and few reports have systematically investigated these assumptions, especially for some of the emerging *Campylobacteraceae* species (Fraser *et al.* 1992; Annable *et al.* 1998).

Recent genetic and physiological findings suggest that C*ampylobacter* species are in fact not as fastidious as at first thought, containing branched and complex metabolic pathways with enzymes for both aerobic and anaerobic respiration (Mohammed *et al.* 2004; Fouts *et al.* 2005). Generally, most routine isolation studies tend to utilise commercially available gas generating systems such as the CampyGen™ and AnaeroGen™ systems, with anaerobic jars to achieve a microaerophilic incubation environment. Although there have been several studies assessing these gas generation systems, they have generally been restricted to the study of *C. jejuni* and *C. coli* species (Popovic-Uroic and Sterk-Kuzmanovic 1992; Bolton *et al.* 1997; Van Horn and Toth 1999). Many emerging *Campylobacter* species are extremely fastidious, requiring hydrogen for growth. In addition, other *Campylobacter* species, generally those isolated from the gingival crevices of the human mouth, have been shown to require strictly anaerobic conditions for growth (Lastovica and Skirrow 2000) and so will not be isolated in standard microaerophic environments.

Elaborate gas incubation systems such as the MACS VA500 Microaerophilic Workstation (Don Whitley Scientific, UK) allow tight control and manipulation of various gaseous concentrations such as oxygen, hydrogen and carbon dioxide. Some attempts have been made to optimise gaseous atmospheres for the growth of a small number of thermophilic *Campylobacter* species (Annable *et al.* 1998). However these experiments tested only a small subset of *Campylobacter* species and only a limited range of atmospheric concentrations.

It is clear that optimal incubation conditions for the recovery of all *Campylobacteraceae* species need to be developed and used in routine isolation studies so

the clinical importance and prevalence of emerging *Campylobacteraceae* species in the food chain can be fully established.

4.2. METHODS

During this study, a number of routinely used, commercially available incubation atmospheres were assessed for the growth of *Campylobacteraceae* species using the semi-quantitative Streak Dilution method. This revealed that these atmospheres were sub-optimal for the growth of a number of emerging *Campylobacteraceae* species. Following this, the MACS workstation was used to evaluate a number of incubation atmospheres at different hydrogen and oxygen concentrations and an optimal atmosphere for growth of all *Campyloabcter* species was developed. Three versions of this atmosphere, achieved using different techniques were then evaluated for the growth of *Campylobacteraceae* species.

4.3. RESULTS

4.3.1. Initial Evaluation of Incubation Atmospheres for the Growth of *Campylobacteracece* Species

Initial experiments were conducted to assess the growth of a selection of *Campylobacter* strains in conventional commercially available atmospheres, and in modifications of these incubation atmospheres using the semi-quantitative Streak Dilution method. Results showed that all of the species tested grew optimally in a microaerobic atmosphere containing 7% hydrogen, 4% oxygen and 10% carbon dioxide (Table 20) provided by the MACS workstation. In addition all species tested, including all of the *C. concisus* strains, were able to grow in a severely restricted oxygen environment provided by the Oxoid Anaerobic Gas Generating Kit (BR38, Oxoid). The atmospheric conditions provided by the CampyGen™ and AnaeroGen™ gas generating systems, supported growth of all strains *C. coli*, *C. jejuni* and *C. lari*; however, growth of *C. upsaliensis* was poor and *C. concisus* did not grow at all in these conditions. To ascertain whether there was a lack of growth of *C. concisus* in the CampyGen™ atmosphere due to lack of hydrogen, the estimated gas concentrations (thought to be achieved using the CampyGen sachet), were reproduced in the MACS workstation and growth compared. The results showed that when the CampyGen atmosphere was supplemented with 1% hydrogen, all of the *C. concisus* strains were able to grow, along with all other species. To verify that it was hydrogen influencing growth rather than the use of the Anaerobic Gas Jar and CampyGen™ sachet, a subsequent control experiment was undertaken. This experiment

Table 20. Growth of *Campylobacter* Species on TBA (7% blood) Incubated in Various Atmospheric Conditions. Growth was assessed according to a semi-quantitative growth scale (Streak Dilution method).

CAMPYCHECK reference strain	AnaeroGen (gas jar) <1% O_2, 9-13% CO_2 *	BR38 (gas jar) <1% O_2, 10% CO_2, H_2??*	CampyGen (gas jar) 6% O_2, 14% CO_2*	'CampyGen' (MACS) 6% O_2, 14% CO_2	'CampyGen' with 1% H_2 (MACS) 1% H_2, 6% O_2, 14% CO_2	Microaerophilic with 7% H_2 (MACS) 7% H_2, 4% O_2, 10% CO_2
C. coli RM 2228	++	++	+++	+++	+++	+++
C. coli Lab 33	+++	++	+++	+++	++++	++++
C. concisus CCUG 13144	-	++	-	-	++	+++
C. concisus CCUG 19995	-	++	-	-/+	++	+++
C. concisus Lastovica 396/96	-	+++	-	-/+	+++	+++
C. jejuni NCTC 11168	++	++	+++	++++	++++	++++
C. jejuni RM 1221	++	++	+++	+++	+++	++++
C. lari RM 2100	++	++	++	+++	++++	+++
C. lari CCUG 22395	++	++	++	++	+++	+++
C. upsaliensis RM 3195	+	+	++	++	+++	++++
C. upsaliensis CCUG 19559	+	+	++	++	++	+++

Results are the mean of duplicate experiments carried out independently.

Growth was scored according to growth on the streaked quadrants: +, growth on first sector of streaking; ++, growth on second sector of streaking; +++, growth on third sector of streaking; ++++, growth on fourth sector of streaking.

*These gas compositions are estimations based on manufacturers instructions and have not been strictly characterised so may not reflect actual concentrations.

directly compared growth in the MACS workstation containing the theoretical CampyGen gas concentrations to that provided by a CampyGen™ sachet in an anaerobic gas jar. The results showed that *C. concisus* was still unable to grow when the CampyGen atmosphere (standard microaerobic conditions) was provided using the MACS workstation, thus confirming that it was the addition of hydrogen that was exerting the stimulatory growth effect.

4.3.2. Optimisation of Hydrogen and Oxygen Gaseous Concentrations in the Incubation Atmosphere for the Growth of all *Campylobacteraceae*

The growth of all *Campylobacteraceae* included in the CAMPYCHECK reference strain collection was evaluated using the Streak Dilution method. The MACS workstation was used to modify hydrogen and oxygen concentrations and evaluate various incubation atmsospheres (Table 21).

4.3.2.1. Optimisation of Oxygen Concentration

All of the microaerophilic *Campylobacter* species, *Bacteriodes ureolyticus* and *Arcobacter* species grew optimally in an atmosphere containing 5% oxygen (supplemented with 6% hydrogen and 10% carbon dioxide) (Table 21). Furthermore, certain 'anaerobic' *Campylobacter* species including *C. curvus*, and selected isolates of *C. gracilis* and *C. showae* grew in an incubation atmosphere containing 5% oxygen. However, the 'anaerobic' species including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus* and *C. showae* generally grew poorly, or not at all, in an atmosphere containing 5% oxygen.

The 'anaerobic' *Campylobacter* species grew optimally when the atmosphere was supplemented with 1% oxygen. However, although growth of most other species was supported in this atmosphere, there was a marked reduction in growth of many of the microaerophilic *Campylobacter* species including *C. coli*, *C. helveticus*, *C. jejuni subsp. doylei* and selected strains of *C. hyointestinalis subsp. hyointestinalis*, *C. hyointestinalis subsp. lawsonii*, *C. jejuni*, *C. lanienae*, *C. lari*, *C. sputorum bv. fecalis* and *C. upsaliensis*. Species (other than the 'anaerobes') which were able to grow well when the atmosphere was supplemented with 1% oxygen included *C. concisus*, *C. mucosalis, Arcobacter* species and certain strains of other species.

An incubation atmosphere containing 3% oxygen supported growth of all of the *Campylobacteraceae*. However, the thermotolerant *Campylobacter* species grew optimally when the atmosphere was supplemented with 5% oxygen, and the 'anaerobic' *Campylobacter* species grew optimally in an atmosphere supplemented with 1% oxygen.

Arcobacter species including *A. butzleri*, *A. skirrowii* and *A. cryaerophilus* grew equally well under all atmospheric concentrations tested, irrespective of the hydrogen and oxygen concentrations.

4.3.2.2. Optimisation of Hydrogen Concentration

When the incubation atmosphere was not supplemented with hydrogen, the growth of many species was reduced and some species did not grow at all (Table 21). Species which did not grow without hydrogen included all of the 'anaerobic' *Campylobacter* species, including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus* and *C. showae*. Species with reduced growth without hydrogen included *C. concisus*, *C. mucosalis*, *C. fetus subsp. venerealis*, *C. upsaliensis* and *B. ureolyticus*. There were strain-specific differences in growth within a species with certain strains of *C. hyointestinalis*, *C. lawsonii*, and *C. lanienae* not requiring hydrogen for growth. Species which grew well in the absence of hydrogen included *C. coli*, *C. fetus*, *C. helveticus*, *C. doylei*, *C. jejuni*, *C. lari* and all of the *Arcobacter* species, although growth of a number of strains within these species slightly increased when supplemented with 4% and 7% hydrogen, and the addition of hydrogen certainly did not inhibit growth of any of these species. When the atmosphere was supplemented with 4% hydrogen, the majority of species tested grew well, including those which showed low or no growth without hydrogen. Furthermore, increasing the hydrogen concentration from 4% to 7% further increased the growth of many species including *C. concisus*, *C. upsaliensis*, *C. lari*, *B. ureolyticus* and many of the 'anaerobic' *Campylobacter* species.

4.3.3. Evaluation of Conventional and Novel Atmospheres for the Growth of *Campylobacteraceae*

From results in previous experiments, optimising concentrations of oxygen and hydrogen in the incubation atmospheres, it was demonstrated that all tested *Campylobacteraceae* could grow under one atmosphere type (7% hydrogen, 3% oxygen, 10% carbon dioxide). A number of laboratories do not have access to use of the MACS workstation; therefore a number of similar incubation atmospheres, achieved using alternative technologies were evaluated. These included the use of a gas cylinder containing pre-mixed gas mixture containing the novel gaseous concentrations (Note: 2.5% oxygen was used instead of 3% for safety reasons with pressurised gas mixtures). The gas evacuation-replacement method was used to fill self seal polythene bags containing inoculated culture plates with the contents of the cylinder. In addition, in

Table 21. Growth of *Campylobacteraceae* on Anaerobe Basal Agar in Various Oxygen and Hydrogen Incubation Atmospheres Using a Semi-quantitative Method. Numbers in brackets represent the CAMPYCHECK number. When varying the oxygen concentration other gases were set at 7% oxygen and 10% carbon dioxide and when varying the hydrogen concentration, 3% oxygen and 10% caron dioxide.

CAMPYCHECK reference strain		Optimisation of oxygen concentration			Optimisation of hydrogen concentration		
		1 % O_2	3 % O_2	5 % O_2	0 % H_2	4 % H_2	7 % H_2
C. coli (1)	RM 2228	++	+++	++++	+++	+++	+++
C. coli (2)	Lab 33	+	+++	++++	+++	+++	+++
C. concisus (4)	CCUG 13144	++++	++++	++++	+	+++	+++
C. concisus (5)	CCUG 19995	+++	+++	++++	-/+	++	++++
C. concisus (6)	Lastovica 396/96	+++	++++	++++	-	++	++++
C. fetus subsp. fetus (10)	CCUG 32114	++	+++	+++	+++	+++	+++
C. fetus subsp. fetus (11)	Abdn 1076	++	+++	+++	+++	+++	+++
C. fetus subsp. fetus (12)	241.99	++	+++	++++	+++	+++	+++
C. fetus subsp. venerealis 13)	CCUG 11287	++++	+++	+++	+	+++	+++
C. fetus subsp. venerealis (14)	Armi 4402	++	+++	+++	++	+++	+++
C. helveticus (18)	CCUG 30566	+	+++	+++	+++	+++	++++
C. helveticus (19)	CCUG 34016	+	+++	++++	+++	+++	+++
C. hyointestinalis subsp. hyointestinalis (22)	LMG 7538	+++	+++	+++	+	+++	+++
C. hyointestinalis subsp. hyointestinalis (23)	LMG 9260	+	+++	+++	++	+++	+++
C. hyointestinalis subsp. lawsonii (26)	CHY 5	+	++++	+++	-/+	++	++++
C. hyointestinalis subsp. lawsonii (27)	CCUG 27631	+	+++	++++	+	+++	+++
C. jejuni subsp. doylei (28)	SSI 5384	+	+++	++++	+++	+++	++++
C. jejuni subsp. doylei (29)	CCUG 18266	+	+++	++++	++	+++	+++
C. jejuni subsp. jejuni (31)	NCTC 11168	++	++++	+++	+++	++++	++++
C. jejuni subsp. jejuni (32)	RM 1221	++	+++	+++	+++	+++	+++
C. jejuni subsp. jejuni (33)	SVS 4039	+	++++	+++	+++	++++	++++
C. lanienae (36)	NCTC 13004	+++	+++	+++	+	+++	+++
C. lanienae (37)	DARDNI G718D	+	+++	++	++	++	+++

Table 21 Continued.

C. lari (38)	RM 2100 (NARTC),	++	++++	++++	++	+++	++++
C. lari (39)	CCUG 22395 (UPTC)	+	++++	+++	+++	+++	++++
C. mucosalis (40)	CCUG 21559	+++	+++	++++	+	+++	+++
C. mucosalis (41)	CCUG 23201	+++	+++	+++	-/+	++	+++
C. sputorum bv. sputorum (46))	Lastovica 86.92	++	+++	++++	++	+++	+++
C. sputorum bv. paraureolyticus (47)	LMG 11764	++	+++	++++	++	+++	+++
C. sputorum bv. fecalis (48)	CCUG 20703	+	+++	+++	++	+++	+++
C. upsaliensis (49)	RM 3195	++	+++	++	+	++	+++
C. upsaliensis (50)	CCUG 19559	+	+++	+++	++	++	+++
B. ureolyticus (52)	CCUG 18470	++	+++	++	+	+++	++++
B. ureolyticus (53)	Rigshospitalet 9880	++	++++	+++	+	+++	++++
A. butzleri (54)	SSI 71032	+++	+++	+++	++++	+++	+++
A. butzleri (55)	29.97	+++	+++	+++	+++	+++	+++
A. cryaerophilus (57)	CCUG 17801	+++	+++	++++	++++	+++	+++
A. cryaerophilus (58)	SSI 70952	+++	+++	+++	++++	+++	+++
A. skirrowii (59)	BU 30CC 8B1	+++	+++	++++	+++	+++	+++
Anaerobic-like *Campylobacter* spp.							
C. curvus (7)	SSI 19296	++++	+++	+++	+	++	+++
C. curvus (8)	Lastovica 525.92	++++	+++	++	-/+	+++	+++
C. curvus (9)	Lastovica 13A	+++	+++	++	+	++	+++
C. gracilis (16)	CCUG 27720	+++	++++	+	+	+++	++++
C. gracilis (17)	CCUG 13143	+++	+++	++	-/+	++	+++
C. hominis (20)	NCTC CH001	++++	+++	+	-/+	++	+++
C. hominis (21)	NCTC CH003	+++	+++	+	-/+	++	+++
C. rectus (42)	CCUG 20446	+++	+++	+	+	++	+++
C. rectus (43)	CCUG 11645	+++	+++	+	+	++	+++
C. showae (44)	CCUG 30254	++++	++++	++	-/+	+++	++++
C. showae (45)	CCUG 11641	++++	++++	+	-/+	+++	++++

collaboration with Oxoid Ltd, an atmosphere containing comparable gas mixtures to the novel atmosphere was developed by use of a combination of Oxoid's existing commercially available gas sachets. In this experiment growth of campylobacters and related species in conventional commercially available atmospheres (CampyGen, AnaeroGen and the Anaerobic Gas Generating Kit (BR38)) and novel atmospheres (provided by the MACS workstation, the gas evacuation-replacement method and the Oxoid gas sachet combination) was evaluated.

The results showed that the growth of *Campylobacteraceae* species could be enhanced when incubated in all three types of novel incubation atmospheres irrespective of the method used to produce the atmosphere (Table 22). However, an atmosphere provided by the Oxoid Anaerobic Gas Generating Kit also performed well. When incubated in an atmosphere provided using Oxoid's AnaeroGen and CampyGen gas sachets, growth of a number of species was inhibited. Species that did not grow in an AnaeroGen atmosphere were able to grow in a similar anaerobic environment provided by the Oxoid Anaerobic Gas Generating Kit. These included *C. concisus* strains, *C. hyointestinalis*, *C. mucosalis*, *C. curvus*, *C. gracilis*, *C. hominis* and *C. rectus*. Species that did not grow, or grew poorly in the Oxoid Anaerobic atmosphere included two strains of *C. fetus* subsp. *venerealis*, one strain of *C. hyointestinalis*, *C. helveticus*, *C. jejuni* subsp. *doylei*, *C. jejuni*, *C. lanienae*, *C. lari*, one strain each of *C. sputorum*, *C. upsaliensis* and *A. butzleri*.

The CampyGen atmosphere thought to provide standard microaerophilic conditions permitted the growth of only a limited number of species tested including *C. coli*, *C. fetus*, *C. fetus* subsp. *venerealis*, *C. jejuni* subsp. *doylei*, *C. jejuni*, *C. lari*, many of which are deemed to be the thermotolerant species, and all of the *Arcobacter* species. Species which did not grow in a CampyGen atmosphere included *C. concisus*, *C. helveticus*, *C. hyointestinalis*, *C. mucosalis*, *C. sputorum*, *C. upsaliensis* and *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus* and *B. ureolyticus*, many of which are deemed to be the 'emerging' species.

Of the three novel atmospheres evaluated, an atmosphere provided by the MACS workstation supported optimal growth of all species tested. However, when incubated in a novel incubation atmosphere provided by the pre-mixed gas evacuation-replacement method and the Oxoid gas sachet combination, although growth was comparable to the MACS workstation there were differences in growth of certain species. Growth of species including *C. helveticus* and certain strains of *C. jejuni* subsp. *doylei,* was reduced in an atmosphere provided using Oxoid gas sachets when compared to the novel atmsophere

Table 22. Growth of *Campylobacteraceae* on Anaerobe Basal Agar in Commercially Available and Novel Incubation Atmospheres.

The semi-quantitative Streak Dilution method was used to assess growth.

Species and strain		Existing commercial atmospheres			Novel 'CAMPYCHECK' atmospheres		
		AnaeroGen	CampyGen	BR38	MACS	Pre-mixed	Oxoid
C. coli (1)	RM 2228	++	+++	++	+++	+++	++
C. coli (2)	Lab 33	++	++	++	+++	+++	+++
C. coli (3)	300.97	++	++	++	+++	++++	+++
C. concisus (4)	CCUG 13144	-	-	++	+++	+	+++
C. concisus (5)	CCUG 19995	-	-	++	++	++	+++
C. concisus (6)	L 396/96	-	-	+++	+++	++	+++
C. fetus subsp. fetus (10)	CCUG 32114	++	+	++	+++	+++	+++
C. fetus subsp. fetus (11)	Abdn 1076	++	++	++	+++	+++	++
C. fetus subsp. fetus (12)	241.99	++	++	++	+++	+++	++
C. fetus subsp. venerealis (13)	CCUG 11287	+++	++	+++	+++	+++	+++
C. fetus subsp. venerealis (14)	Armi 4402	++	+	+	+++	+++	++
C. fetus subsp. venerealis (15)	Abdn SM5	+	+	+	++	+++	++
C. helveticus (18)	CCUG 30566	-/+	-	-/+	+++	+++	+
C. helveticus (19)	CCUG 34016	+	-	-/+	+++	+++	+
C. hyointestinalis subsp. hyo. (22)	LMG 7538	-/+	-/+	++	++++	++	++
C. hyointestinalis subsp. hyo. (23)	LMG 9260	-	-	+	+++	+	+++
C. hyointestinalis subsp. hyo. (24)	176.96	-	-	++	+++	+	+++
C. hyointestinalis subsp. hyo. (25)	234.95	-	-	++	+++	++	++
C. hyointestinalis subsp. lawsonii (26)	CHY 5	-	-	++	+++	+++	+++
C. hyointestinalis subsp. lawsonii (27)	CCUG 27631	+	+	+	+++	+++	++
C. jejuni subsp. doylei (28)	SSI 5384	+	+++	+	+++	++	+
C. jejuni subsp. doylei (29)	CCUG 18266	++	++	+	+++	+++	++
C. jejuni subsp. doylei (30)	269.97	++	+	+	+++	++	+
C. jejuni subsp. jejuni (31)	NCTC 11168	++	+++	++	++++	+++	++
C. jejuni subsp. jejuni (32)	RM 1221	+	++	++	+++	+++	++
C. jejuni subsp. jejuni (33)	SVS 4039	+	++	+	+++	+++	++
C. jejuni subsp. jejuni (34)	RM 1864	++	+++	+	+++	++	++
C. jejuni subsp. jejuni (35)	47.97	+	++	+	++	+++	+++

Table 22 Continued.

C. lanienae (36)	NCTC 13004	+	-	+	+++	+++	+++
C. lanienae (37)	DARDNI G718D	+	+	+	+++	+++	+++
C. lari (38)	RM 2100	+	+	+	+++	++	++
C. lari (39)	CCUG 22395	+	++	+	+++	+++	++
C. mucosalis (40)	CCUG 21559	-	-	++	++	++	+++
C. mucosalis (41)	CCUG 23201	-	-	++	+++	++	++
C. sputorum bv. sputorum (46)	Lastovica 86.92	-	-	+	+++	+	++
C. sputorum bv. paraureolyticus (47)	LMG 11764	++	-	++	+++	++	+++
C. sputorum bv. fecalis (48)	CCUG 20703	++	-	++	+++	+	+++
C. upsaliensis (49)	RM 3195	-	-	+	+++	+++	++
C. upsaliensis(50)	CCUG 19559	-	-	+	+++	++	++
C. upsaliensis(51)	CCUG 19607	+	-	+	+++	+++	+++
Anaerobic-like *Campylobacter* spp.							
C. curvus (7)	SSI 19296	-	-	++	+++	+	+++
C. curvus (8)	Lastovica 525.92	-	-	++	+++	++	++++
C. curvus (9)	Lastovica 13A	-	-	++	++	+	+++
C. gracilis (16)	CCUG 27720	-	-	+++	++	+	+++
C. gracilis (17)	CCUG 13143	-	-	+++	+++	++	+++
C. hominis (20)	NCTC CH001	-	-	++	++	+	+++
C. hominis (21)	NCTC CH003	-	-	++	++	-/+	+++
C. rectus (42)	CCUG 20446	-	-	++	+++	++	+++
C. rectus (43)	CCUG 11645	-	-	++	+++	+	+++
C. showae (44)	CCUG 30254	+	-	++	+++	+	+++
C. showae (45)	CCUG 11641	+	-	+++	+++	-/+	+++
Related non-*Campylobacter* spp.							
B. ureolyticus (52)	CCUG 18470	-/+	-	++	+++	++	++
B. ureolyticus (53)	Rig 9880	++	-	+++	+++	+	++
A. butzleri (54)	SSI 71032	++	+++	+	+++	++++	++
A. butzleri (55)	29.97	++	++	+	+++	+++	++
A. butzleri (56)	167.97	+++	++	+	+++	+++	+++
A. cryaerophilus (57)	CCUG 178011	++	++	++	+++	+++	+++
A. cryaerophilus (58)	SSI 70952	++	+++	++	+++	++	++
A. skirrowii (59)	BU30CC 8B1	++	+++	++	++++	+++	+++

using the pre-mixed gas-evacuation method and MACS workstation. Furthermore, growth of certain strains of *C. concisus*, *C. hyointestinalis*, *C. sputorum* and 'anaerobic' species including *C. curvus*, *C. gracilis*, *C. hominis*, *C. rectus*, *C. showae* and *B. ureolyticus* was slightly lower in an atmosphere using the pre-mixed gas evacuation-replacement method as opposed to an atmosphere provided by the MACS workstation and Oxoid Anaerobic Gas Generating Kit.

4.3.4. Evaluation of Novel Gaseous Environments using a Servomex Gas Analyser

A study was conducted to evaluate the actual gaseous concentrations of two novel atmospheres. Sample bags from the MACS workstation and the Air Products cylinder (pre-mixed gas) were analysed using a Servomex gas analyser at Oxoid Ltd, Basingstoke, UK. The samples were analysed 8 h after sampling and were much higher than expected for oxygen and much lower than expected for carbon dioxide and hydrogen. It was suspected that the gas bag material may have been permeable, and a re-test of the bags 9 days later was carried out. The expected values were 2.5-3% O_2, 10% CO_2, 7% H_2. The results showed that after 9 days, the oxygen levels had doubled (from 8.4% to 16.6%), the carbon dioxide concentrations had decreased from 5% to 0.3% and hydrogen from 2% to 0.1%, thus confirming that the bags were permeable and that concentrations measured would not have reflected the actual gaseous concentrations of the incubation atmospheres.

4.3.5. Evaluation of the pH Buffering Capacity of Isolation Media During Incubation in a Hydrogen Enriched Microaerophilic Atmosphere

An experiment was carried out to investigate the pH buffering capacity of a number of plating and broth media during incubation in a hydrogen enriched microaerobic atmosphere, as growth of *Campylobacter* species is thought to be optimal at approximately pH 7. The results showed that the pH remained constant (between pH 7.0-7.5) during incubation in an aerobic atmosphere. However, when incubated in a hydrogen enriched microaerobic atmosphere, the pH of Preston, CAT and CCDA media decreased from pH 7.0-7.5 to pH 6.0-6.5 after incubation for 24 h and was further reduced after 48 h. Media including ABA, ABAVAT, Skirrow and TBA remained more constant with only a minimal reduction in pH after 48 h. The pH of CEB broth and Hunts broth was also evaluated during incubation. Results showed that the pH was reduced from 7.0 to 6.3 and 7.1 to 6.5 during incubation in a hydrogen enriched microaerobic atmosphere for CEB and Hunts broth respectively.

4.4. DISCUSSION

4.4.1. Development of a Universal Incubation Atmosphere for *Campylobacteraceae*

When the MACS workstation was used to allow modification of oxygen concentrations in the incubation atmosphere, the optimal concentration for the growth of all *Campylobacteraceae* was 3%. When the incubation atmosphere was supplemented with 1% oxygen growth of certain species was inhibited. Similarly a concentration of 5% oxygen inhibited the growth of those species which prefer anaerobic conditions.

Historically, incubation atmospheres used for the isolation of *Campylobacter* species have been microaerobic (with carbon dioxide) (Wang *et al.* 1983; Annable *et al.* 1998), principally provided by commercially available gas production systems such as the CampyGen sachet (6% O_2, 14% CO_2). It is clear from results in this study that this atmosphere was not sufficient for the recovery of emerging *Campylobacteraceae*. Results showed that this was most likely to be due to the requirement of hydrogen for growth of certain species and also some species preferring lower oxygen levels than that provided when using the CampyGen gas generating system. Many studies have also shown that the presence of an increased concentration of carbon dioxide is required for growth of *Campylobacter* species. However, this requirement has not been comprehensively investigated and was not studied here.

During this study a number of novel incubation atmospheres were evaluated and compared to existing commercially available atmospheres, including the AnaeroGen, CampyGen and the Anaerobic Gas Generating Kit. The novel incubation atmospheres were based on findings from experiments optimising hydrogen and oxygen concentrations using the MACS workstation. The optimised gas atmosphere for isolation of all *Campylobacteraceae* contained 3% oxygen, 10% carbon dioxide, and 7% hydrogen. Three variations of this atmosphere were evaluated during this study which included an atmosphere provided by the MACS workstation, the Oxoid gas sachet combination and the gas evacuation-replacement method. All three of these novel methods were shown to be adequate for the growth of all *Campylobacteraceae*. However growth was slightly enhanced using the MACS workstation, probably due to the tight control achieved by constant automated rectification of gases throughout incubation. Furthermore, when using gas sachets there would be a time lapse of approximately 30-60 min before the desired incubation atmosphere inside the jar would be achieved. Furthermore, the gas evacuation-replacement method may not reflect the exact atmosphere required, due to residual atmospheric gases remaining in the bags and the escape of other gases.

Surprisingly, results from this study showed that most of the species tested were able to grow in an environment provided by the commercially available Oxoid Anaerobic Gas Generating Kit. According to manufacturers' instructions this atmosphere is reported to contain less than 1% oxygen and 10% carbon dioxide, without hydrogen (it is thought that any hydrogen produced as a by-product is absorbed by a palladium catalyst inside the jar). However, a similar anaerobic atmosphere achieved using the commercially available AnaeroGen (thought to contain less than 1% oxygen and 9-13% carbon dioxide) was shown to be inadequate for growth of the majority of species tested. It can only be assumed that the differences in growth are due to gaseous concentrations reported by manufacturers' instructions not being a true description of the actual gaseous concentration achieved i.e. it is possible that low levels of oxygen could persist inside the jar. Furthermore, an atmosphere generated using the Oxoid Anaerobic Gas Generating Kit could contain low levels of hydrogen, which may not have been completely removed by the palladium catalyst. However, these assumptions are purely based on speculation since the gas concentrations were not actually measured as part of this study. Furthermore, these gas production systems are used extensively around the world for the successful growth of strictly anaerobic bacterial species (Bridson 1998). It is even possible that anaerobic growth in such an environment is possible for a number of *Campylobacter* species as a result of the complex basal medium used in combination (ABA). Furthermore, when incubated in the MACS workstation containing 1% oxygen, 7% hydrogen and 10% carbon dioxide, a number of species were able to grow and it is possible that this is due to the presence of hydrogen, which becomes fundamentally important when oxygen is in short supply (i.e. 1% and below). It is possible that some species don't have the capacity to grow well in a severely oxygen limited environment (<1%). For example, a number of species including *C. coli*, C. *jejuni subsp doylei*, *C. helveticus* and certain strains of other microaerophilic species grew poorly in this environment, thought to be because they are unable to utilise alternative electron acceptors/donors for respiration effectively, or may be dependent on oxygen for other important processes.

Recent evidence suggests that the respiratory chain of *Campylobacter* species is clearly branched, and significantly more complex than might be expected for an organism with such a small genome. The presence of enzymes characteristic of both aerobic and anaerobic respiration has been demonstrated, which are thought to allow the use of alternative electron acceptors to oxygen (Smith *et al.* 1999). Many studies have documented the ability of a range of *Campylobacter* species to utilise formate and

fumarate (amongst many others) as alternative electron donors and acceptors in the electron transport chain, as do many anaerobic bacteria. These mechanisms may exist to allow *Campylobacter* to continue respiration at extremely low oxygen concentrations, as might be found in the niches which it occupies in the gut of animals or humans (Parkhill *et al.* 2000; Kelly 2005). Studies have shown that many species and strains of *Campylobacter* contain a certain type of oxidoreductase, usually restricted to obligate anaerobes (Daucher and Krieg 1995). Furthermore, the genome sequence of *C. jejuni* 11168 clearly has revealed the presence of the gene for this oxidoreductase type of enzyme (Parkhill *et al.* 2000). Studies have also revealed that *Campylobacter* species have two types of terminal oxidases, which may allow the bacterium to continue respiration at extremely low oxygen concentrations, as might be found in the niches which it occupies in the gut of animals or humans (Parkhill *et al.* 2000; Ketley 2005) or in the Oxoid Anaerobic Gas Generating system, or at oxygen levels of 1-3% as tested in this study. However, there is extensive metabolic diversity between and within *Campylobacter* species with regard to their enzymes and ability to utilise alternative electron donors and acceptors (Sellars *et al.* 2002; Mohammed *et al.* 2004; Fouts *et al.* 2005). Early research on campylobacters revealed anaerobic growth was possible in *C. fetus*, using fumarate as an electron acceptor, but this capacity was absent in *C. jejuni* (Véron 1981). A further study has also shown that *C. jejuni* was unable to grow in the absence of oxygen, but *C. coli* and *C. lari* could grow, provided carbon dioxide levels were above 8% (Annable *et al.* 1998). Despite findings from the present study, and other evidence pointing towards the ability of many species to grow anaerobically, Sellars *et al.* (2001) demonstrated that anaerobic growth of *C. jejuni* with fumarate and other electron acceptors was not possible under strictly anaerobic conditions. It was postulated that this was because *C. jejuni* only has the oxygen dependent class 1 type ribonucleotide reductase (RNR), required to synthesise DNA (Parkhill *et al.* 2000; Sellars *et al.* 2002). However, only low levels of oxygen are thought to be required for the enzyme to function, and growth was able to continue in severely oxygen-limited conditions. During the present study *C. jejuni* and many other species were able to grow in an atmosphere containing 1% oxygen, and it is possible that this concentration may be sufficient for type 1 RNR enzymes to function and allow DNA synthesis to continue. It is also likely that alternative mechanisms exist in other *Campylobacter* species which may utilise different enzymes. In addition, effective growth in this incubation atmosphere may also be permitted due to the use of Anaerobe Basal Agar supplemented with 5% blood; a complex medium containing a range of potential alternative metabolic substrates

(including alternative electron acceptors/donors) that could be utilised effectively in such oxygen-limited conditions.

The results showed that, although species which were deemed to be anaerobic including *C. rectus*, *C. hominis*, *C. curvus*, *C. gracilis* and *B. ureolyticus* grew optimally in an atmosphere supplemented with 1% oxygen and in the Oxoid Anaerobic Gas Generating Kit, they also grew well in an atmosphere supplemented with 3% oxygen, and could even tolerate a concentration of 5% oxygen. Moreover, these strains demonstrated a definitive requirement for hydrogen. (Han *et al.* 1991) have also demonstrated that *C. rectus*, *C. showae*, *C. gracilis* and *B. ureolyticus* are in fact microaerophiles, rather than strict anaerobes, capable of oxygen dependent growth in 2 to 8% oxygen, with the ability to respire with oxygen as the terminal electron acceptor if hydrogen was provided as the electron donor. The growth of *C. rectus* has been shown to occur in the absence of oxygen when 10% hydrogen and 10% CO_2 were present (Gillispie and Barton 1996). Another study (Wexler *et al.* 1996) also showed that *C. gracilis* could grow in the presence of up to 6% oxygen. Therefore, these species can no longer be thought of as anaerobes; rather they are hydrogen requiring microaerophiles, requiring low levels of oxygen (<5%) for growth. It is likely that oxygen levels higher than 5% would inhibit the growth of these species as these strains are adapted to growth in anaerobic environments and may lack protective oxidative stress enzymes, causing them to be more susceptible to reactive oxygen species that would damage proteins, lipids and DNA. It is also possible that levels as high as 5% oxygen could be tolerated here due to the protective effects of the medium used (Anaerobe Basal Agar supplemented with 5% laked horse blood).

As mentioned previously, results from this study showed that the presence of hydrogen in the incubation atmosphere was crucial for the growth of a number of species. Many species did not grow when the atmosphere was not supplemented with hydrogen, but were able to grow when supplemented with 4% hydrogen. Furthermore, a concentration of 7% further enhanced growth of many species. Many reports have documented the requirement of hydrogen for growth of certain *Campylobacter* species including *C. concisus*, although comprehensive assessments of this hydrogen requirement have not been carried out (Sellars *et al.* 2002; Lastovica 2006). The Cape Town protocol utilises the Oxoid Anaerobic Gas Generating Kit without the use of a palladium catalyst. It is thought that this atmosphere contains extremely high levels of hydrogen (>40%), thought to support and enhance the growth of all species contained within the *Campylobacteraceae* family (Lastovica 2006). However, use without a catalyst is not recommended by

manufacturers and is deemed to be unsafe due to high pressures, combined with the flammable nature of the gas composition.

Although the exact mechanism for the requirement of hydrogen is unclear, several discreet pathways, which almost certainly require molecular hydrogen, are either likely to or do exist in *Camplyobacter* species. In *C. jejuni*, metabolic pathways have been reported to exist which utilise hydrogen as an alternative terminal electron donor to the oxygen acceptor for respiration and growth pathways (Smith *et al.* 1999; Kelly 2001; Maier 2005).

There is a definite relationship between the cultural atmospheric requirements of *Campylobacter* species and the ecological niche they occupy in nature. It has been proposed that hydrogen could be very important for growth of campylobacters in gingival pockets of the human mouth or in periodontal pockets of diseased gums. In these areas there are many obligate anaerobes (including *Eubacterium*, *Peptococcus* and *Peptostrepococcus* species) contained in biofilms, that produce hydrogen from redox reactions associated with fermentation. The high prevalence of species such as *C. curvus*, *C. concisus*, *C. rectus*, *C. showae*, *C. hominis*, *C. gracilis* and *B. ureolyticus* in these areas is reflected in findings from this study, which showed that these species had an essential requirement for hydrogen and which preferred anaerobic atmospheres for growth.

It is thought that uptake hydrogenases mediate the transfer of electrons from hydrogen to the quinone pool and that hydrogen and hydrogenase may be important for *C. jejuni* (and possibly other species) to colonise the avian gut (Kelly 2005; Maier 2005). Many studies have reported the ability of *Campylobacter* species to utilise hydrogen as an electron donor in the electron transport chain. It has been suggested that in *C. jejuni,* hydrogen could feed into the methoquinone pool where other alternative electron donors and acceptors, such as the fumarate and formate would also be important (Sellars *et al* 2002). It is likely that hydrogen may also be a factor in other important processes. For example, it may act independently as a growth stimulus or concomitantly as a generator for the proton motive force, required for respiration. One possible requirement is for molecular-supplied hydrogen to form part of the proton flow in molecular motors such as the ATPase-requiring bacterial flagellum (Berry 2000). Studies have also shown that the presence of hydrogen is required for motility and is an important factor for colonisation of the gut.

The *Arcobacter* species were able to grow well under all of the atmospheric conditions evaluated, including anaerobic (<1% oxygen), microaerophilic with or without hydrogen, and aerobically at both 25°C and 37°C. Previous studies (Lastovica 2000) have

shown that *Arcobacter* species grow well at 25°C in a microaerobic atmosphere but also tolerate grow in aerobic conditions; furthermore, this study has shown that these species can as also grow well at 37°C. A number of other studies have also reported the ability of *Arcobacter* species to grow under a range of incubation atmospheres (Lastovica and Skirrow 2000). This undemanding growth requirement is reflected by their prevalence and survival in food, environmental and clinical samples. There is now increasing evidence to show that *A. butzleri* is an emerging pathogen and it is likely to pose a significant risk to the consumer due to its survival and growth in aerobic conditions at room temperature (Vandenberg *et al.* 2004).

Future evaluation studies of the novel incubation atmospheres developed need to be applied to actual clinical and environmental samples to further validate the effectiveness of this atmosphere. It is possible that because only reference laboratory strains have been used, they may not represent the physiological state of stressed bacteria recovered from environmental and processed samples, which may exibit different atmospheric requirements. Since many previous studies have only used conventional microaerobic atmospheres for isolation of *Campylobacter* species, it is likely that the species which prefer much lower oxygen concentrations, or have a hydrogen requirement, as demonstrated in this study would have been underdetected, and therefore their clinical role underestimated.

This is the first study of its kind to attempt to quantify atmosphere requirements for such a large range of *Campylobactereaceae* of which for many non-*jejuni/coli* species little or no data exist. Previous studies have shown that there is extensive metabolic diversity between and within *Campylobacter* species, thought to play a significant role in physiology, epidemiology and pathogenicity of these organisms (Mohammed *et al.* 2004). This study has demonstrated that although this is true to a certain extent with regard to hydrogen and oxygen requirements, it is possible for all *Campylobacteraceae* species to grow in one universal atmosphere (3% oxygen, 7% hydrogen, 10% carbon dioxide). It has shown that a number of species previously thought to be strict anaerobes were able to grow well in a microaerophilic environment, and a number of conventional microaerophilic species were able to grow in a severely oxygen limited atmosphere (1% oxygen). It would appear that the presence of hydrogen, presumably utilised as an electron donor is the key to growth of many *Campylobacter* species, a requirement thought to have evolved due to the ecological niche occupied in nature.

CHAPTER 5

DEVELOPMENT OF TESTS FOR THE IDENTIFICATION OF *CAMPYLOBACTERACEAE*

5.1. INTRODUCTION

In almost every area of microbiology (including clinical studies, epidemiology, population genetics and comparative genomics), the identification of strains to species, and sometimes subspecies or even variant-level is required. Identification involves the matching of data sets derived from the unknown to those of defined taxa. A variety of different approaches have been used to identify campylobacters to the species level, some of which have been described in Chapter 1. An isolate is usually identified when phenotypic (e.g. biochemical tests, fatty acid or protein profiles) or genotypic (e.g. DNA fingerprints, sequences, PCR primers) tests are carried out and matched to that of a defined taxon. These approaches are generally determined by the aim of the experiment and time, facilities and funds available. Routine diagnostic laboratories, for example will generally only be equipped with commercially available tests and possibly PCR machines to carry out simple confirmations of *Campylobacter*. However, research laboratories may carry out more elaborate tests not just for confirmation, but also further speciation and sub-typing to the strain level (using RFLP, DGGE. AFLP). What is clear is that despite recent progress in the development of a range of sub-typing methods, no standard methods exist and all methods used come with inherent advantages and disadvantages.

During this study a number of novel identification tests were evaluated for both the presumptive identification of *Campylobacteraceae* species and identification to the species level. A variety of novel technologies were investigated including Episcopic Differential Interference Contrast (EDIC) microscopy, a novel latex agglutination test (to differentiate between thermotolerant and non-thermotolerant *Campylobacter* species), novel phenotypic identification tests, fluorescence *in situ* hybridisation using 16S rRNA-targeted oligonucleotide peptide nucleic acid probes and finally, investigation of a two-dimensional comparative protein profiling technique. In addition, novel methods for identification of *Campylobacter* species to be exploited on a commercial level were evaluated in collaboration with two commercial companies.

5.2. METHODS

Detailed experimental procedures of identification tests performed during this study are described in the Materials and Methods chapter (Chapter 2). A number of existing and novel tests for the identification of *Campylobacteraceae* were initially developed and evaluated against the CAMPYCHECK reference strain collection. Some of the more

successful identification tests were then selected for use in identifying isolates recovered from sewage sludge and salad vegetables (Chapter 6).

5.3. RESULTS

5.3.1. Morphological Identification

5.3.1.1.1.1. Colony Morphology

The first step in the identification of a *Campylobacter* species is by visual inspection of colony morphology and an experienced laboratory technician will be able to spot a presumptive *Campylobacter* species purely from its colony morphology. Figure 17 shows the colony morphology of representative *Campylobacteraceae* isolates including *C. coli*, *C. helveticus*, *C. concisus*, *C. jejuni*, *C. lari*, *C. hominis*, *B. ureolyticus* and *A. skirrowii.*

Campylobacter, *Arcobacter* and *Bacteriodes* species were grown on ABA in the MACS cabinet (7% H_2, 10% CO_2, 3% O_2, 80% N_2) for 48 h. The results showed that the colony morphology of the *Campylobacteraceae* colonies was extremely variable between species and within a species (data not shown). Generally, colonies could be described as smooth, shiny, and convex with a defined edge, or flat, transparent or translucent, and spreading with an irregular edge. They were generally colourless to greyish or buff and sometimes pink. Colonies were 1 to 2 mm in diameter, although they ranged between pinpoint in size to several mm. *C. helveticus* was the only species not to form discreet colonies and displayed flat, film like growth spreading across the entire plate. *C. hominis* and *C. rectus* only showed thin growth with very small colonies <1 mm. It is also worth noting that the colony morphology of *Arcobacter* and *Bacteriodes* species was very similar to *Campylobacter* species, therefore the former could easily be mistaken for *Campylobacter* species based on their colony morphology. Colony morphology of the same strain would vary from day to day, thought to be due to the moisture content of the plate which was affected by the length of drying and age of the plate.

5.3.1.2. Differentiation of *Campylobacteraceae* by Cellular Morphology using EDIC Microscopy

Results showed that detailed cellular morphology of *Campylobacter* species could be clearly visualised using Episcopic Differential Interference Contrast (EDIC) microscopy of non-stained bacterial smears (Figure 18 and Table 23). The morphology of the reference strains analysed was extremely pleomorphic and included spirals, s-shaped , curved/

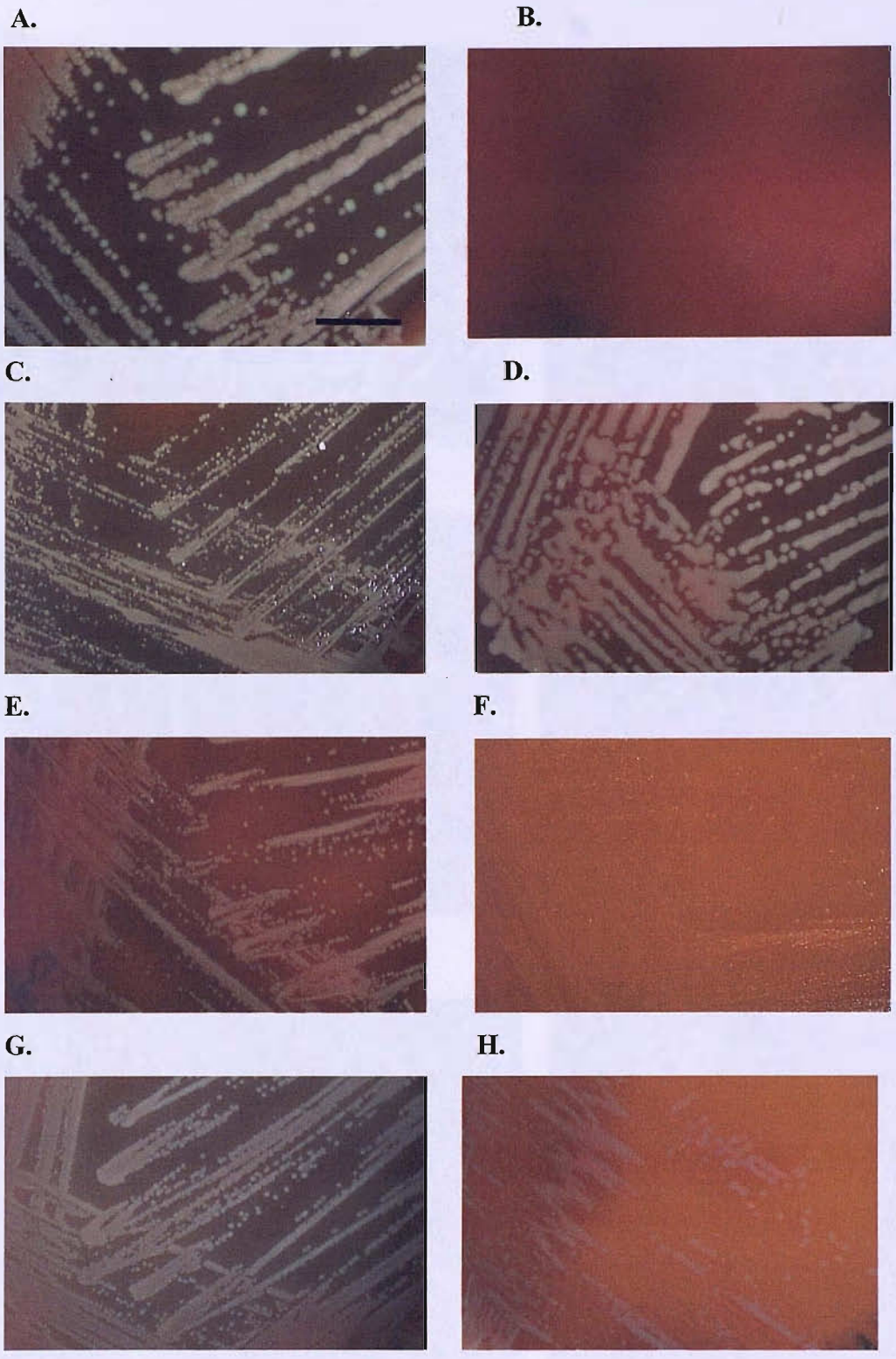


Figure 17. Colony Morphology of *Campylobacteraceae* Species Streaked onto Anaerobe Basal Agar. The cultures were incubated at 37°C in a microaerobic atmosphere supplemented with hydrogen (7% H_2, 10% CO_2 and 3% O_2) for 48 h. A. *C. coli*, B. *C. helveticus*, C. *C. concisus*, D. *C. jejuni*, E. *C. lari*, F. *C. hominis*, G. *B. ureolyticus*, H. *A. skirrowii*. Bar represents 1 cm.

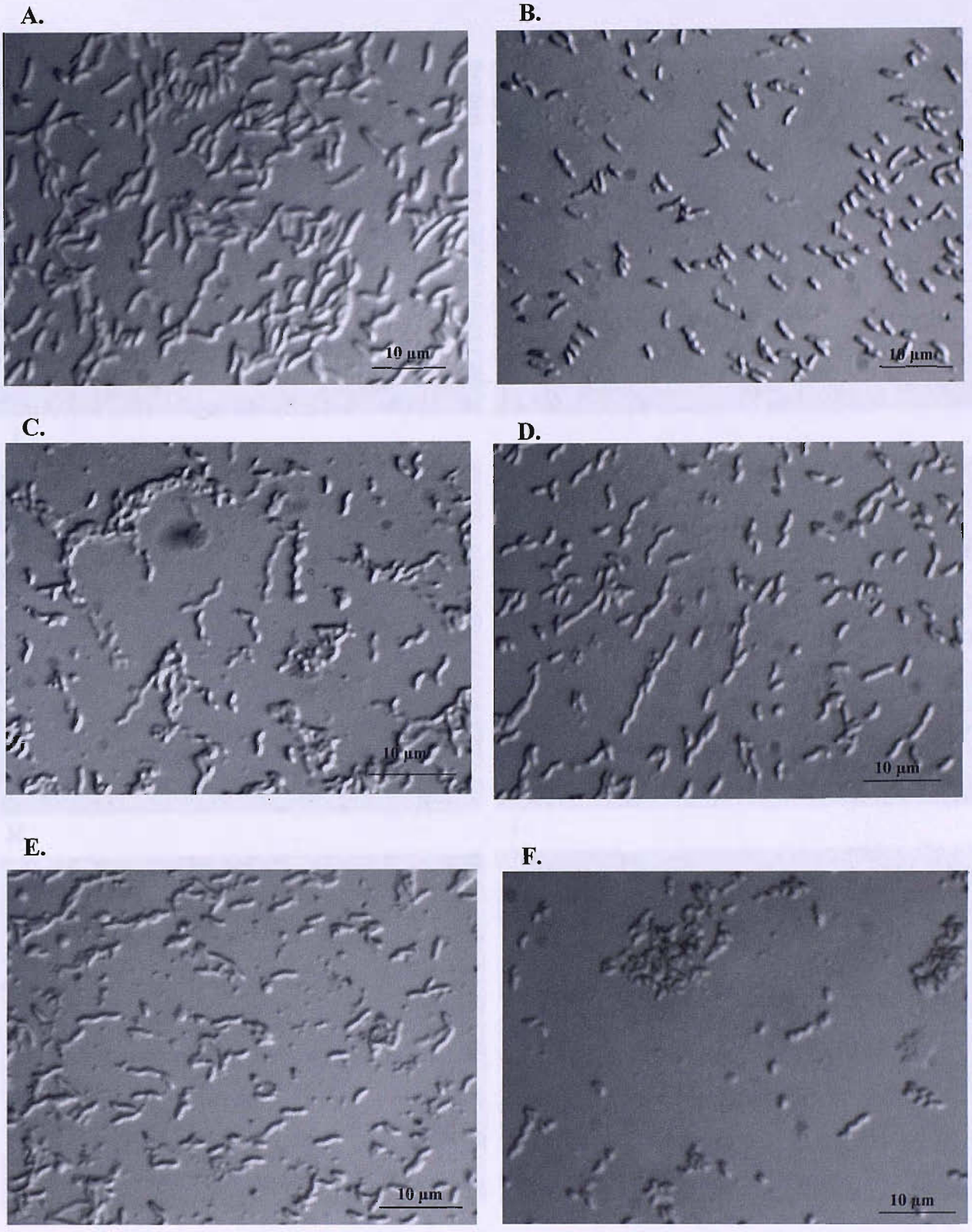


Figure 18. Morphology of *Campylobacteraceae* species from the CAMPYCHECK reference strain collection. Images taken using EDIC microscopy (X 2000 magnification). A. *C. coli* 2, B. *C. concisus* 4, C. *C. fetus* 12, D. *C. fetus* sub.sp *venerealis,* E. *C. hyointestinalis* subsp. *hyointestinalis*, F. *C. jejuni* 31, G. C. *lanienae* 37, H. *C. upsaliensis* 49, I. *C. curvus*, J. *C. rectus* 43, K. *B. ureolyticus* 52, L. *A. butzleri* 54. The cultures were incubated at 37°C in the MACS cabinet (7% H_2, 10% CO_2 and 3% O_2) for 48 h.

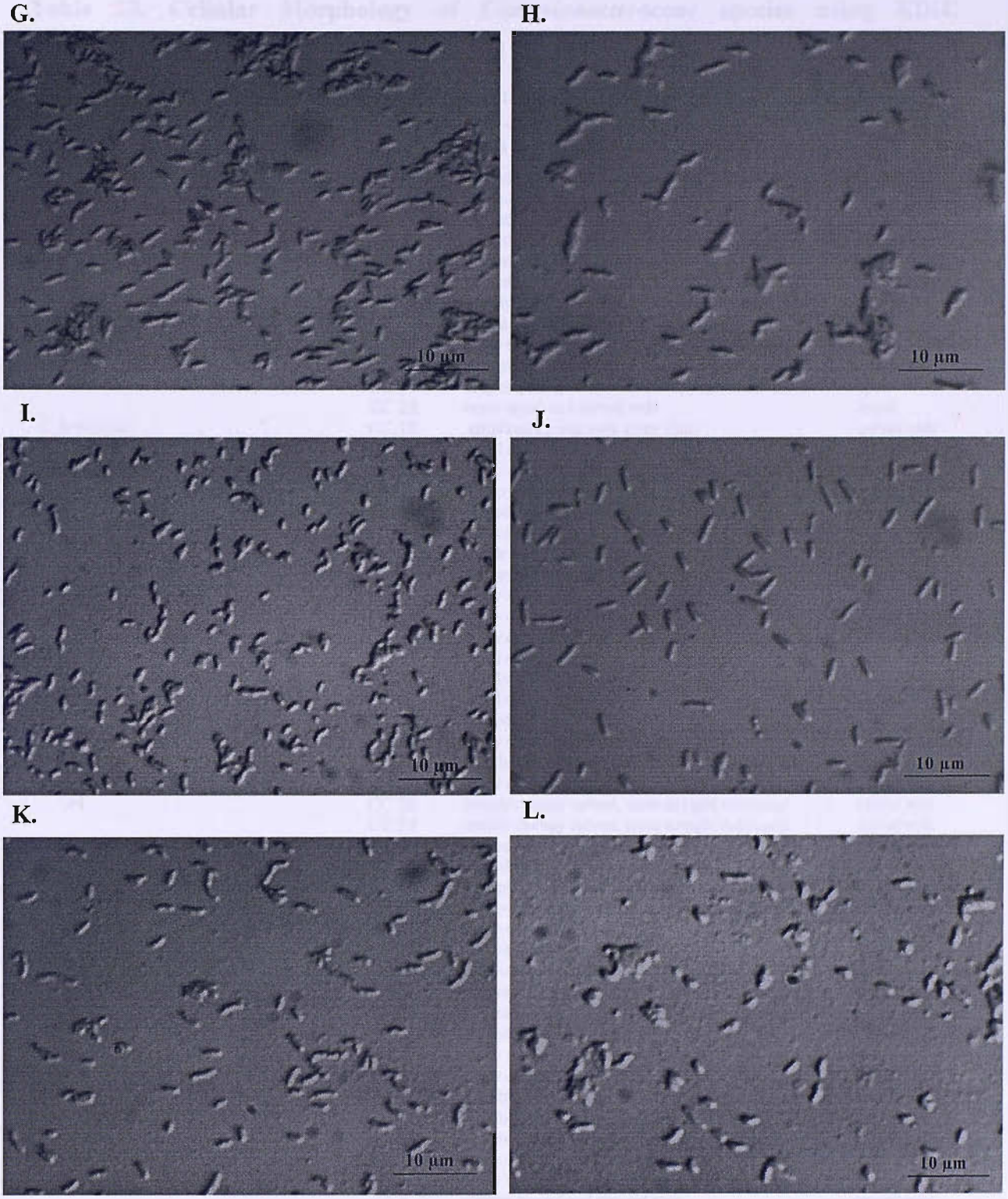


Figure 18 Continued.

Table 23. Cellular Morphology of *Campylobacteraceae* species using EDIC Microscopy. Reference strains were cultured for 48 h on ABA in a hydrogen enriched atmosphere. Heat-fixed smears were analysed using EDIC microscopy.

CAMPYCHECK reference strain	CC No.	Types of morphology observed	Predominant morphology
C. coli	CC 1	curved rods, straight rods, some spiral, some cocci	curved rods
	CC 2	curved rods, straight rods, some spiral, some cocci	curved rods
	CC 3	curved rods, some cocci	curved rods
C. concisus	CC 4	curved rods, some spiral, some cocci	curved rods
	CC 5	curved rods, some spiral	curved rods
	CC 6	curved rods, some spiral	curved rods
C. fetus subsp. fetus	CC 10	mainly spiral and curved, some straight rods	spiral
	CC 11	mixed, spiral/curved/straight rods	spiral
	CC 12	many slender spirals, curved/straight rods/ cocci	spiral
C. fetus subsp. venerealis	CC 13	curved rods	curved rods
	CC 14	long slender spirals, some stumpy spiral and curved	spiral
	CC 15	many spiral and curved rods	spiral
C. helveticus	CC 18	spiral and curved rods, some cocci	curved rods
	CC 19	spiral and curved rods, some cocci	curved rods
C. hyointestinalis subsp. hyointestinalis	CC 22	many curved stumpy rods, some slender spirals	curved rods
	CC 23	stumpy curved rods	curved rods
	CC 24	curved and straight rods	curved rods
	CC 25	curved rods	curved rods
C. hyointestinalis subsp. lawsonii	CC 26	slender curved rods	curved rods
	CC 27	stumpy curved rods	curved rods
C. jejuni subsp. doylei	CC 28	many stumpy curved rods, some cocci	curved rods
	CC 29	many curved rods, some straight	curved rods
	CC 30	many comma shaped, some cocci	curved rods
C. jejuni subsp. jejuni	CC 31	small curved rods, many cocci, few spiral	curved rods
	CC 32	small curved rods, many cocci, few spiral	curved rods
	CC 33	small curved rods	curved rods
	CC 34	curved rods, some straight	curved rods
	CC 35	many curved rods, some stumpy spirals	curved rods
C. lanienae	CC 36	mainly curved rods, cocci	curved rods
	CC 37	many stumpy curved rods	curved rods
C. lari	CC 38	mainly stumpy curved, some straight rods/cocci	curved rods
	CC 39	mainly stumpy curved, some straight rods/cocci	curved rods
C. mucosalis	CC 40	curved rods, some spiral, some cocci	curved rods
	CC 41	curved rods, some spiral	curved rods
C. sputorum	CC 46	stumpy curved, comma shaped rods, stumpy spirals	curved rods
	CC 47	stumpy curved, comma shaped rods, stumpy spirals	curved rods
	Cc 48	many curved rods	curved rods
C. upsaliensis	CC 49	curved, straight and spiral rods	curved rods
	CC 50	curved rods and spirals	spiral
	CC 51	curved/straight/some spirals	curved rods
B. ureolyticus	CC 52	curved and straight rods, some cocci	straight rods
	CC 53	curved and straight rods	curved rods
A. butzleri	CC 54	many spirals and curved rods	spiral
	CC 55	many curved rods	curved rods
	CC 56	mainly stumpy curved rods, some spirals	curved rods
A. cryaerophilus	CC 57	mainly stumpy curved rods, some spirals	curved rods
	CC 58	mainly stumpy curved rods, some straight	curved rods
A. skirrowii	CC59	many curved rods, some spirals	curved rods
C. curvus	CC7	many stumpy curved rods, some straight, s-shaped	curved rods
	CC8	stumpy curved and straight rods	curved rods
	CC9	stumpy curved and straight rods	straight rods
C. gracilis	CC 16	stumpy curved and straight rods, some cocci	straight rods
	CC 17	stumpy curved and straight rods, some cocci	straight rods
C. hominis	CC 20	stumpy curved and straight rods	straight rods
	CC 21	stumpy curved and straight rods	straight rods
C. rectus	CC 42	stumpy straight and curved rods	straight rods
	CC 43	stumpy straight and curved rods	straight rods
C. showae	CC 44	curved and straight rods	straight rods
	CC 45	stumpy straight and curved rods	straight rods

gull/comma-shaped rods, stumpy/long thin, or straight rods and coccoid shaped bacteria. This variability was also observed within a sample smear which could include cells that were cocci, long and short rods which were spiral or comma shaped. Generally, the *Campylobacter* cells were approximately 0.5-1.0 μm wide and 8.0-10 μm long. The coccoid shaped bacteria were approximately 1.0-1.5 μm wide and 1.0-1.5 μm long.

5.3.1.2.1.Immunological Identification: The Latex Agglutination Test

Initial experiments evaluating the existing commercially available latex agglutination test reagent (Microscreen *Campylobacter,* M46, Microgen Bioproducts, Camberley, UK) showed that it was unable to detect many emerging species (Table 24). The results showed that the only strains to display a positive reaction against this antibody latex specificity included C. *helveticus* (18,19), *C. jejuni subsp. doylei* (28-30), *C. jejuni subsp. jejuni* (31-35) and *C. coli* (1-3). Strains which did not react with the antibody included *C. concisus* (4-6), *C. fetus* subsp. *fetus* (10-12), *C. fetus* subsp. *venerealis* (13-15), *C. hyointestinalis* subsp. *hyointestinalis* (22-25), *C. mucosalis* (40,41) and *C. sputorum* (46,47). Some isolates showed strain specific differences within a species. Two isolates of *C. upsaliensis,* for example, showed a strongly positive reaction, the other strain tested showed a negative reaction. Two isolates of *C. lari* showed a strong positive reaction, but the other a weak, non specific agglutination. Therefore the results from this evaluation demonstrated that the original latex agglutination test was ineffective for detecting many of the emerging species included in the CAMPYCHECK strains set (Lastovica *et al.* 2004). As a result of the previous findings, a prototype latex agglutination test was developed by Microgen Bioproducts, which included the old latex reagent (white latex particles) (to detect the thermophiles) and a second latex reagent (yellow latex particles) with specificity for all *Campylobacteraceae.*

The novel pan-*Campylobacteraceae* latex reagent strongly reacted with all 59 strains from the CAMPYCHECK reference strain set.

From the results of reactions with both of the antisera reagents, it was possible to loosely classify the *Campylobacteraceae* isolates into two groups according to whether they were thermotolerant-like species (those that reacted to both the thermotolerant and species reagent) and the non-thermotolerant-like (or emerging) species (those that only reacted with the non-thermotoleant latex reagent). According to the prototype Microgen latex agglutination test, *Campylobacter* species classed as thermotolerant included *C.coli*, *C. helveticus*, *C. jejuni* subsp. *doylei*, *C. jejuni, C. lanienae*, *C. lari* and *C. upsaliensis.*

Table 24. Specificity of the Latex Agglutination Test. CAMPYCHECK strain number (CC No.) refers to culture collection number. Ctrl = control reagent; Campy = *Campylobacteraceae* latex reagent; Therm = thermotolerant *Campylobacter* latex reagent. Agglutination was assessed on a three point scale as per manufacturer's instructions. Results were scored as follows: - = no reaction; + = slightly grainy on a milky background; ++ = some agglutination clumps formed, +++ = strong agglutination, -/+ = weakly positive but inconclusive. N/T = not tested.

		Latex Reagent						Latex Reagent			
***Campylobacter* spp.**	**CC No.**	**Ctrl**	**Campy**	**Therm**	**Presumptive ID**	***Campylobacter* spp.**	**CC No.**	**Ctrl**	**Campy**	**Therm**	**Presumptive ID**
C. coli	CC 1	-	++	+++	Therm	*C. doylei*	*CC 30*	-	+++	+++	Therm
C. coli	CC 2	+	+++	+++	Therm	*C. jejuni*	*CC 31*	-	+++	++	Therm
C. coli	CC 3	+	+++	+++	Therm	*C. jejuni*	*CC 32*	+	+++	+++	Therm
C. concisus	*CC 4*	+	+++	+	Emerging	*C. jejuni*	*CC 33*	-	+	++	Therm
C. concisus	*CC 5*	-	+++	+	Emerging	*C. jejuni*	*CC 34*	-	++	+++	Therm
C. concisus	*CC 6*	-	+++	-	Emerging	*C. jejuni*	*CC 35*	-	++	++	Therm
C. curvus	*CC 7*	+	+++	+	Emerging	*C. lanienae*	*CC 36*	+	++ (+)	++ (+)	Therm
C. curvus	*CC 8*	-	++	-	Emerging	*C. lanienae*	*CC 37*	-	+++	++	Therm
C. curvus	*CC 9*	-	+++	-	Emerging	*C. lari*	*CC 38*	+	+++	++	Therm
C. fetus	*CC 10*	-	+++	-	Emerging	*C. lari*	*CC 39*	+	+++	++	Therm
C. fetus	*CC 11*	-	++	-	Emerging	*C. mucosalis*	*CC 40*	-	+++	-	Emerging
C. fetus	*CC 12*	-	+++	-	Emerging	*C. mucosalis*	*CC 41*	+	+++	-	Emerging
C. venerealis	*CC 13*	-	+++	-	Emerging	*C. rectus*	*CC 42*	+/-	+++	+/-	Emerging
C. venerealis	*CC 14*	-	+++	-	Emerging	*C. rectus*	*CC 43*	-	+ (+)	-	Emerging
C. venerealis	*CC 15*	-	+++	-	Emerging	*C. showae*	*CC 44*	+	++ (+)	+	Emerging
C. gracilis	*CC 16*	-	+++	-/+	Emerging	*C. showae*	*CC 45*	+	++ (+)	+	Emerging
C. gracilis	*CC 17*	-	++	-	Emerging	*C. sputorum*	*CC 46*	-	+++	-	Emerging
C. helveticus	*CC 18*	-	+++	+++	Therm	*C. sputorum*	*CC 47*	+/-	++	-	Emerging
C. helveticus	*CC 19*	+	+++	++	Therm	*C. sputorum*	*Cc 48*	+	++	++	Therm?
C. hominis	*CC 20*	-	++	-	Emerging	*C. upsaliensis*	*CC 49*	+	++	++	Therm?
C. hominis	*CC 21*	-	++	-	Emerging	*C. upsaliensis*	*CC 50*	-	+++	+ (+)	Therm?
C. hyointestinalis	*CC 22*	+	+++	+	Emerging	*C. upsaliensis*	*CC 51*	-/+	+++	++	Therm?
C. hyointestinalis	*CC 23*	-	++	-	Emerging	*B. ureolyticus*	*CC 52*	+/-	+++	+	Emerging
C. hyointestinalis	*CC 24*	-	+++	-	Emerging	*B. ureolyticus*	*CC 53*	+/-	+++	+	Emerging
C. hyointestinalis	*CC 25*	+	++	+	Emerging	*A. butzleri*	*CC 54*	-	++	-	Emerging
C. lawsonii	*CC 26*	-	++	-	Emerging	*A. butzleri*	*CC 55*	-	+++	-	Emerging
C. lawsonii	*CC 27*	+	++	+	Emerging	*A. butzleri*	*CC 56*	-	+++	-	Emerging
C. doylei	*CC 28*	-	+++	+++	Therm	*A. cryaerophilus*	*CC 57*	-	+++	-	Emerging
C. doylei	*CC 29*	+/-	+++	+++	Therm	*A. skirrowii*	*CC 59*	-	+++	-	Emerging

Species classed as emerging *Campylobacter* species included *C. concisus*, *C. curvus*, *C. fetus*, *C. venerealis*, *C. gracilis*, *C. hominis*, *C. hyointestinalis*, *C. lawsonii*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *Bacteriodes ureolyticus* and all of the *Arcobacter* species.

Further evaluation of the novel latex agglutination test was carried out on presumptive isolates recovered from sewage and salad vegetable samples (described in Chapter 6).

5.3.2. Biochemical Identification

A number of simple biochemical ID tests were carried out on the whole of the CAMPYCHECK reference strain set. Some of these tests were routinely used to confirm the purity of the strains including oxidase, KOH and L-ala tests and test for growth in aerobic conditions. Other tests including the urease, catalase, indoxyl acetate and hippurate tests were carried out to differentiate between *Campylobacter* species.

The results (Table 25) showed that all *Campylobacter*, *Arcobacter* and *Bacteroides* species were confirmed as Gram negative according to the KOH string test, producing a 'string' of DNA when mixed with 3% KOH. However, only weak reactions were displayed by *C. hominis* (20,21). All species were negative for the enzyme L-alan aminopeptidase (L-ALA) using both the Oxoid and Fluka detection systems. The majority of species tested were positive for oxidase although only weak reactions were shown by *C. concisus* species (4,5,6), *C. curvus* (9), *C. gracilis* (16), *C. hyointestinalis* (24), *C. lari* (39) and *C. mucosalis* (40,41). The only species capable of aerobic growth were *A. butzleri*, *A. cryaerophilus* and *A. skirrowii*. There were variable results for the catalase test, indoxyl acetate and the urease test which were dependent on both species and strain type. All strains were negative for the urease test except the two isolates of *B. ureolyticus* that were positive and *C. gracilis* which also displayed a weak reaction. Surprisingly, the urease positive *C. lari* (39) strain was urease negative. Species that were catalase positive included all strains of *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. doylei*, *C. jejuni*, *C. lanienae*, *C. lari*, *C. mucosalis*, *C. rectus*, *C. showae*, *A. cryaerophilus* and *A. skirrowii*. Species that displayed catalase variable reactions included *C. venerealis*, *C. gracilis*, *C. sputorum*, *B. ureolyticus* and *A. butzleri*. Species negative for catalase included *C. concisus*, *C. curvus*, *C. helveticus*, *C. hominis*, *C. mucosalis* and C. *upsaliensis*.

Approximately half of the species in the CAMPYCHECK reference strain collection were positive for indoxyl acid hydrolysis. Species which tested positive included

Table 25. Biochemical Identification Tests Screened Against the CAMPYCHECK Reference Strain Collection. + = positive reaction, - = Negative reaction, -/+ = weakly positive but inconclusive, N/T = Not tested, ? = test result did not agree with the result in the literature.

Campylobacter spp.	CC No.	KOH	L-ala	Oxidase	Catalase	Indoxyl Acetate	Urease	Hippurate Hydrolysis	Aerobic growth (25°C)
C. coli	CC 1	+	-	+	+	+	-	-	-
	CC 2	+	-	+	+	+	-	-	-
	CC 3	+	-	+	+	+	-	-/+	-
C. concisus	*CC 4*	+	-	+/-	-/+	+/-	-	-	-
	CC 5	+	-	+/-	-	-	-	-	-
	CC 6	+	-	+/-	-	-	-	-	-
C. curvus	*CC 7*	+	-	+	-	-/+	-	-	-
	CC 8	+	-	+	-	+	-	+	-
	CC 9	+	-	+/-	-	+	-	-	-
C. fetus subsp. fetus	*CC 10*	+	-	+	+	-	-	+?	-
	CC 11	+	-	+	+	-	-	-	-
	CC 12	+	-	+	+	-	-	-	-
C. fetus subsp. venerealis	*CC 13*	+	-	+	-	-	-	-	-
	CC 14	+	-	+	+	-	-	-	-
	CC 15	+	-	+	+	-	-	+?	-
C. gracilis	*CC 16*	+	-	-/+	-	-	-/+	+?	-
	CC 17	+	-	-/+	+	+	-/+	-	-
C. helveticus	*CC 18*	+	-	+	-	+	-	+?	-
	CC 19	+	-	+	-	+	-	-	-
C. hominis	*CC 20*	+/-	-	+/-	-	-	-	-	-
	CC 21	+/-	-	+	-	-	-	-	-
C. hyointestinalis subsp. hyointestinalis	*CC 22*	+	-	+	+	-	-	-	-
	CC 23	+	-	+	+	-	-	-	-
	CC 24	+	-	-/+	+	-/+	-	-/+	-
	CC 25	+	-	+	+	-	-	-	-
C. hyointestinalis subsp. lawsonii	*CC 26*	+	-	+	+	-	-	-	-
	CC 27	+	-	+	+	-	-	+ ?	-
C. jejuni subsp. doylei	*CC 28*	+	-	+	+	+	-	+	-
	CC 29	+	-	+	+/-	+/-	-	-?	-
	CC 30	+	-	+	+	+	-	+	-

Table 25 Continued.

Campylobacter spp.	CC. no.	KOH	L-ala	Oxidase	Catalase	Indoxyl Acetate	Urease	Hippurate Hydrolysis	Aerobic growth (25°C)
C. jejuni subsp. jejuni	*CC 31*	+	-	+	+	+	-	+	-/+
	CC 32	+	-	+	+	+	-	+	-
	CC 33	+	-	+	+	+	-	-/+	-
	CC 34	+	-	+	+	+	-	+	-
	CC 35	+	-	+	+	+	-	- ?	-
C. lanienae	*CC 36*	+	-	+	+	-	-	-	-
	CC 37	+	-	+	+	-	-	-	-
C. lari	*CC 38*	+	-	+	+	+	-	-/+	-
	CC 39	+	-	+/-	+	-/+	-?	-/+	-
C. mucosalis	*CC 40*	+	-	-/+	-	-	-	+?	-
	CC 41	+	-	-/+	-	-	-	-/+	-
C. rectus	*CC 42*	+	-	+	+	+	-	-/+	-
	CC 43	+	-	+	-/+	+	-	-/+	-
C. showae	*CC 44*	+	-	-/+	+	+	-	-	-
	CC 45	+	-	-/+	+	+	-	+	-
C. sputorum	*CC 46*	+	-	+	+	-	-	-/+	-
	CC 47	+	-	+	-	-	-	-	-
	Cc 48	+	-	+	+	-	-	-/+	-
C. upsaliensis	*CC 49*	+	-	+	-	+	-	-	-
	CC 50	+	-	+	-	+	-	-/+	-
	CC 51	+	-	+	-	+	-	-/+	-
B. ureolyticus	*CC 52*	+	-	+	-	-	+	-/+	-
	CC 53	+	-	+	+	-	+	-	-
A. butzleri	*CC 54*	+	-	+	+	+	-	-/+	+
	CC 55	+	-	+	+	+	-	-	+
	CC 56	+	-	+	-	+	-	-/+	+
A. cryaerophilus	*CC 57*	+	-	+	+	+	-	-/+	+
	CC 58	+	-	+	+	+	-	-/+	+
A. skirrowii	*CC 59*	+	-	+	+	+	-	-/+	+

all strains of *C. coli*, *C. curvus*, *C. helveticus C. doylei*, *C. jejuni*, *C. lari*, *C. rectus*, *C.showae* and *C. upsaliensis*. Species negative for indoxyl acetate hydrolysis included *C. concisus*, *C. fetus*, C. *venerealis*, *C. hominis*, *C. hyointestinalis*, *C. lawsonii*, *C. lanienae*, *C. mucosalis*, *C. sputorum* and *B. ureolyticus*. *C. gracilis* showed strain dependent results with one isolate testing positive and the other negative. No *Arcobacter* species were assessed for their ability to hydrolyse indoxyl acetate.

5.3.2.1. Development of a Biochemical Identification Test in a Miniaturised Multi-well Format for Potential Commercial Use

A prototype miniaturised biochemical identification test strip was developed by Microgen Bioproducts. The test incorporated 12 substrates on the strip for biochemical analysis and was largely based on biochemical identification tests used as part of the Cape Town protocol along with results from other studies in the literature (On *et al.* 1996; Lastovica 2006). During this study the test was analysed at various stages of product development for the identification of a range of *Campylobacter* species. The most recent prototype test evaluated comprised three off-strip tests including oxidase, catalase and indoxyl acetate plus a twelve well substrate strip. The twelve biochemical on-strip tests included lysine, ornithine, arginine dihydrolase, aryl sulphatase, hippurate, nalidixic acid, pyrazinamidase, ONPG (o-nitrophenyl-β-D-galactopyranoside), nitrate, urease, citrate and glucose (Figure 19 and Table 8). The system was therefore composed of 15 tests producing a five digit identification code, which could be subsequently analysed using a probabilistic method using the dedicated prototype Microgen software database and programme.

Evaluation of the Microgen prototype identification test was carried out at various stages of development of the product. Factors investigated included various substrate concentrations using different methods of substrate preparation, length of incubation time and incubation atmospheres of the test strips. The results showed that identical results were achieved at 24 h compared to 48 h. Furthermore, strips incubated in an aerobic atmosphere were comparable to those achieved in the MACS cabinet (7% H_2, 10% CO_2, 3% O_2) provided that certain wells were overlaid with mineral oil and a tight seal with the plastic cover over the wells was achieved. During the development of the 12 on-strip biochemical substrate tests, it was noted that a number of the expected reactions were not occurring as predicted with the various emerging *Campylobacter* species and that certain tests were not reproducible. Preliminary evaluation of the latest prototype biochemical test strip showed that many of the identification tests conflicted with results obtained using the Cape Town

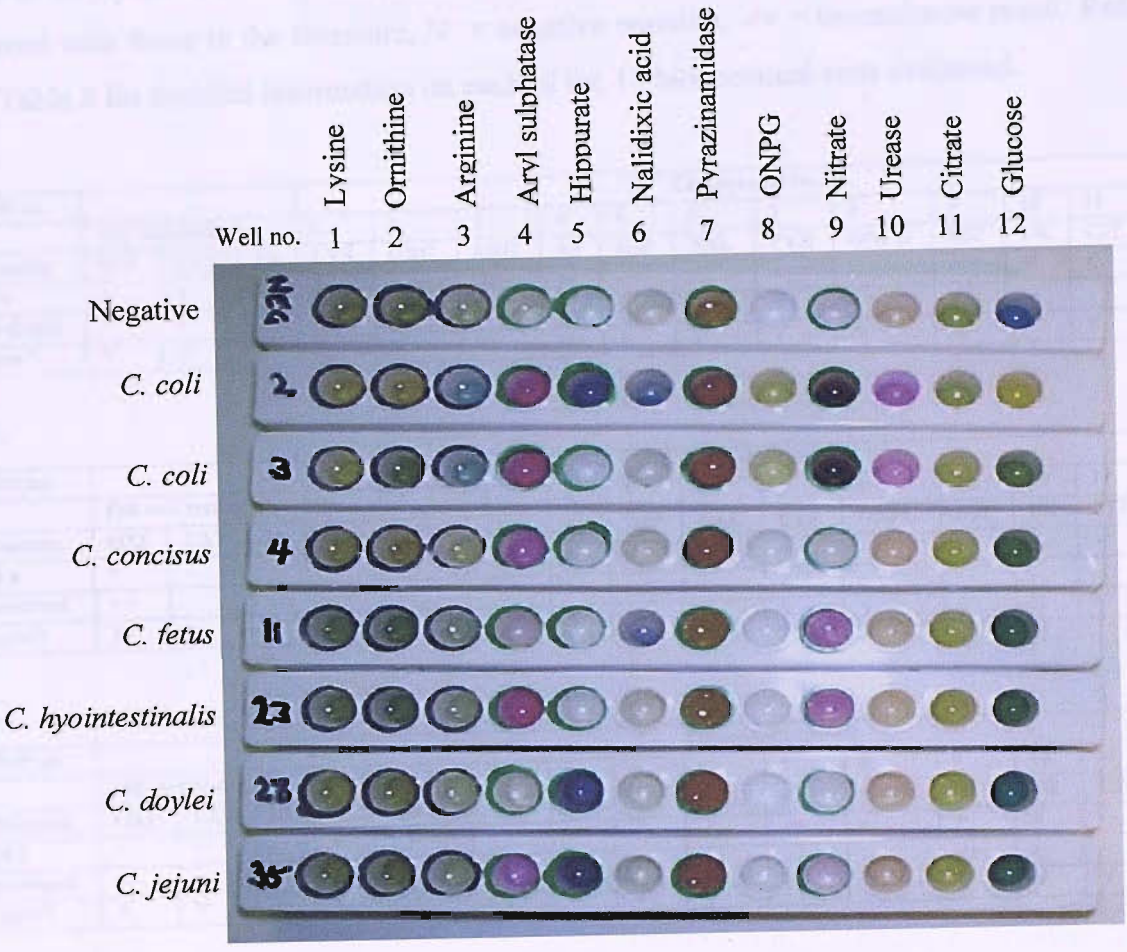


Figure 19. Example of the Microgen Bioproducts Miniaturised Biochemical Test strip. Refer to Table 8 for explanations of biochemical reactions for each test and colour change interpretation.

Table 26. Results of Microgen Bioproducts *Campylobacter* Biochemical ID System Tested Against Various *Campylobacter* species. Results were compared to those expected using data from the Cape Town protocol (Lastovica 2006) and studies described by On *et al.* ((On *et al.* 1996). A. *C. coli* 3, B. *C. concisus* 4, C. *C. fetus* 11, D. *C. doylei* 28, E. *C. jejuni* 34. + = positive reaction, - = negative reaction, Y = observed reactions agreed with those in the literature, N = negative reaction, -/+ = inconclusive result. Refer to Table 8 for detailed information on each of the 12 biochemical tests evaluated.

A.

Well no.				Campylobacter ID											
	Off well testing			1	2	3	4	5	6	7	8	9	10	11	12
Reaction	OXI	CAT	IA	LYS	ORG	ARG	AS	HIP	NAL	PYZ	ONPG	NIT	UR	CIT	GLU
24 h	+	+	+	-/+	+	-/+	+	-	-	+	-	-	+	-	-
predicted	+	+	+	+	+	+	-	-	-	+	-	+	-	-	-
Agree?	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	N	N	N	Y	Y

B.

Well no.				Campylobacter ID											
	Off well testing			1	2	3	4	5	6	7	8	9	10	11	12
Reaction	OXI	CAT	IA	LYS	ORG	ARG	AS	HIP	NAL	PYZ	ONPG	NIT	UR	CIT	GLU
24 h	+	-	-	-/+	+	-	+	-	-	+	-	-	+	-	-
predicted	+/-	-	-	-	-	-	+	-	+	+	-	-	-	-	-
Agree?	Y	Y	Y	?	N	Y	Y	Y	N	Y	Y	Y	N	Y	Y

C.

Well no.				Campylobacter ID											
	Off well testing			1	2	3	4	5	6	7	8	9	10	11	12
Reaction	OXI	CAT	IA	LYS	ORG	ARG	AS	HIP	NAL	PYZ	ONPG	NIT	UR	CIT	GLU
24 h	+	+	-	+	+	+	+	-	-/+	-/+	-	+	+	-	-
predicted	+	+	-	+	+	+	-	-	+	-	-	-/+	-	-	-
Agree?	Y	Y	Y	Y	Y	Y	N	Y	Y	?	Y	Y	N	Y	Y

D.

Well no.				Campylobacter ID											
	Off well testing			1	2	3	4	5	6	7	8	9	10	11	12
Reaction	OXI	CAT	IA	LYS	ORG	ARG	AS	HIP	NAL	PYZ	ONPG	NIT	UR	CIT	GLU
24 h	+	+	-/+	+	+	-/+	+	-	-/+	+	-	-	+	+	-
predicted	+	+	+	+	+	+	-	+	-	+	-	-/+	-	-	-
Agree?	Y	Y	Y	Y	Y	Y	N	N	?	Y	Y	Y?	N	N	Y

E.

Well no.				Campylobacter ID											
	Off well testing			1	2	3	4	5	6	7	8	9	10	11	12
Reaction	OXI	CAT	IA	LYS	ORG	ARG	AS	HIP	NAL	PYZ	ONPG	NIT	UR	CIT	GLU
24 h	+	+	+	-	-/+	-	+	-	-	+	-	-	-	-	+
predicted	+	+	+	+	+/-	+/-	?*	+	-	+	-	-	-	-	-
Agree?	Y	Y	Y	N	Y	Y	?	N	Y	Y	Y	Y	Y	Y	N

+ if biotype 2, - if

identification protocol and from results reported by other workers (Table 26). These discrepancies mainly included false positive reactions with urease and aryl sulphatase and false negative reactions with hippurate and lysine. Furthermore, there were problems with certain strains which were falsely positive for glucose and showed both false positive and negative results for nalidixic acid resistance.

5.3.3. Molecular Identification

5.3.3.1. FISH using 16S PNA Oligonucleotide probes

5.3.3.1.1. Thermophilic Probe

A 16S rRNA targeted oligonucleotide PNA probe, previously developed for the identification of thermotolerant *Campylobacter* species (*C. coli*, *C. jejuni* and C. *lari*) (Lehtola *et al.* 2005) was re-evaluated against strains within the CAMPYCHECK reference collection. Results showed that the thermotolerant probe detected all of the thermophilic species including *C. coli*, *C. jejuni* and C. *lari* (Figure 20 and Table 27). However the probe also bound to a lesser extent to other non-thermophilic species including *C. fetus* (12), *C. hyointestinalis* (22), *C. doylei* (29), *C. upsaliensis* (50) and *A. cryaerophilus* (58). There was also some degree of autofluorescence (on samples without hybridisation probe) for many species, however in most cases fluorescence was much higher when the probe was used.

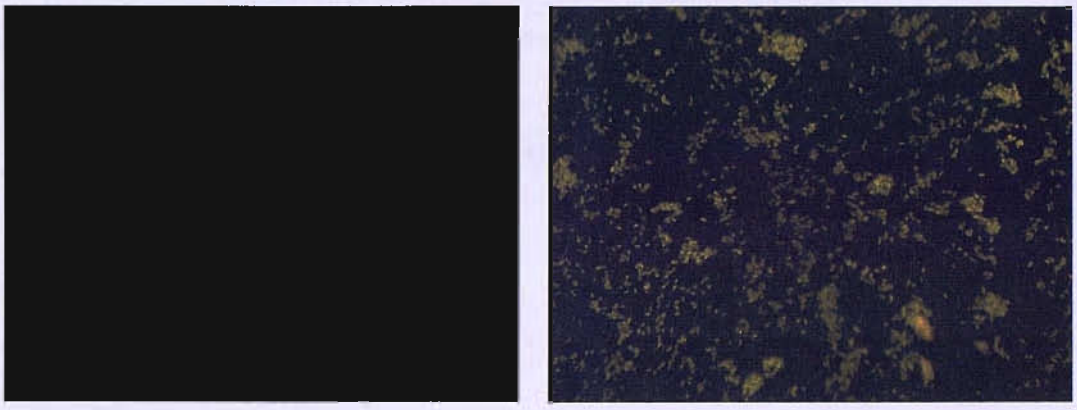
5.3.3.1.2. Development of *Campylobacteraceae* PNA Probe

5.3.3.1.2.1. Preliminary Evaluation

Preliminary evaluation of the newly developed *Campylobacteraceae* PNA probe showed that for the selected strains tested fluorescence levels of the control slide (no probe) were just as high as the slide where the probe was used. It was thought that the reason the probe was not working was due to the incompatibility of the PNA probe with the linked Alexa fluor dye (Brown 2006). Therefore, instead of spending time optimising the hybridisation conditions, another fluorophore, TAMRA was used. This was chosen due to its previous proven success with the thermophilic PNA probe.

Initial results using the *Campylobacteraceae* probe linked to the TAMRA fluorophore showed high fluorescence intensity when tested against selected *Campylobacteraceae* reference strains. However, subsequent testing of the probe with a number of non-*Campylobacteraceae* bacteria showed that fluorescence intensity was just as strong with 10/11 strains tested (Table 28 and Figure 21). The only non-*Campylobacteraceae* species

A.



B.

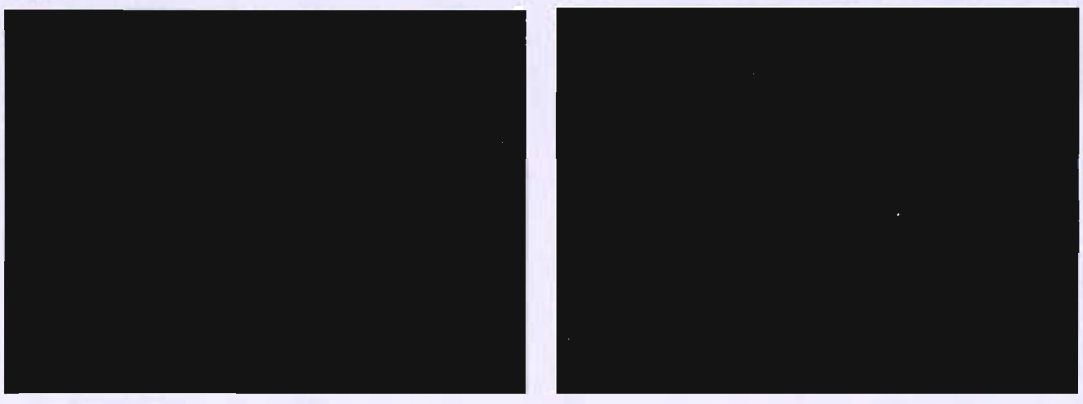


Figure 20. Example of Fluorescence *In Situ* Hybridisation of *C. jejuni* and *C. showae* using the Thermotolerant PNA Probe. *C. jejuni* (35) (A) and *C. showae* (B.) Control slide using hydridisation buffer without probe (left); Hybridised slide using thermotolerant probe (right). Fluorescence was much higher when the thermotolerant *C. jejuni* was evaluated.

Table 27. Identification of Selected *Campylobacteraceae* Using the TAMRA-Labelled Thermotolerant PNA Oligonucleotide Probe. CC no. refers to CAMPYCHECK reference strain. Hybridisation was measured by making a subjective estimate of signal intensity in 4 arbitrary classes: +, ++, +++, ++++. + = low signal, ++++ = high signal.

Species	CC no.	Control	Thermotolerant Probe
C. coli	1	+	++
C. coli	2	-/+	++
C. coli	3	+	+++
C. concisus	6	-	-
C. curvus	7	-	-
C. curvus	8	-/+	-/+
C. fetus	11	+	+
C. fetus	12	-/+	+
C. gracilis	16	+	+
C. hominis	20	-/+	-/+
C. fetus	22	+	+
C. doylei	29	-/+	+
C. jejuni	31	-/+	++
C. jejuni	32	+	+++
C. jejuni	35	-/+	+++
C. lari	38	+	++
C. lari	39	-/+	++
C. rectus	42	-/+	+
C. showae	44	+	+
C. showae	45	+	+
C. upsaliensis	49	-/+	-/+
C. upsaliensis	50	-/+	+
B. ureolyticus	53	-/+	+
A. butzleri	54	-/+	-/+
A. butzleri	55	-/+	-/+
A. butzleri	56	-/+	+
A. cryaerophilus	58	+	+
A. skirrowii	59	-/+	-/+
Salmonella spp.		-	-
Pseudomonas spp.		-	-

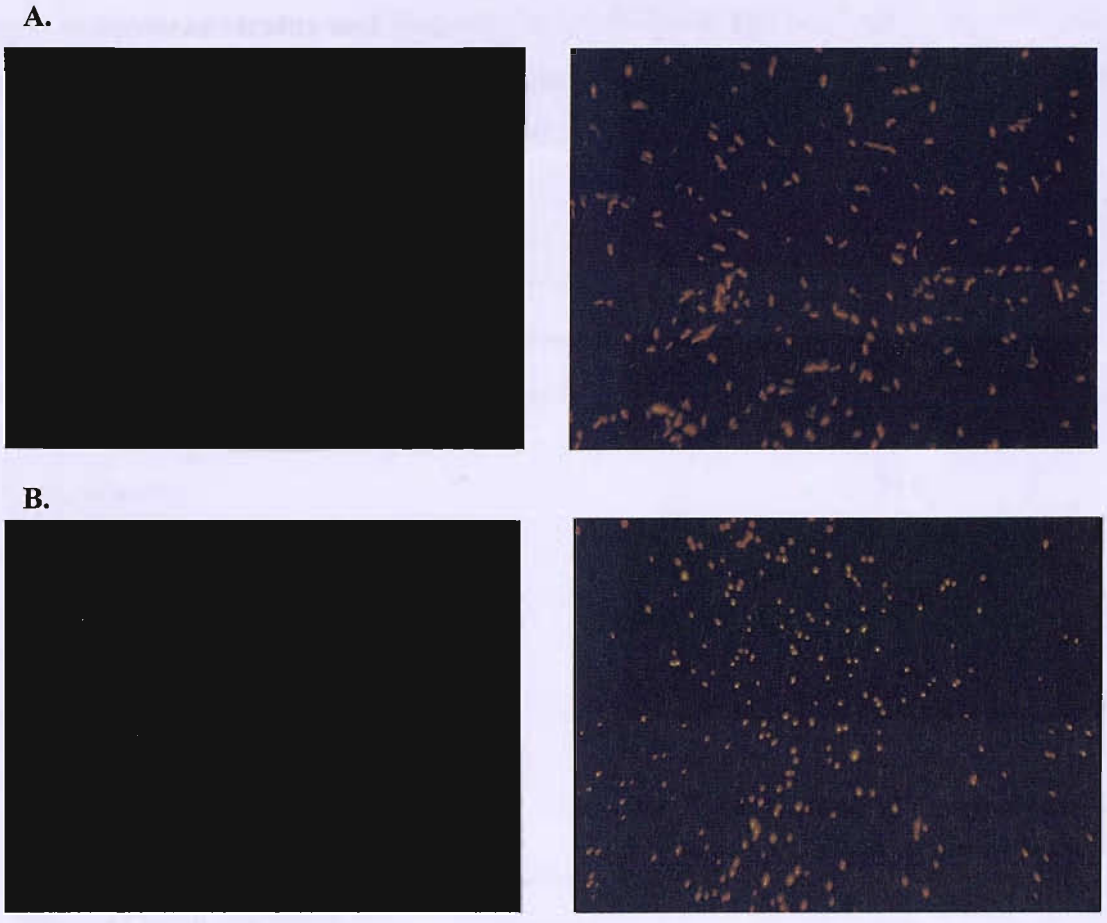


Figure 21. Examples of Fluorescence *In Situ* Hybridisation of *C. upsaliensis* and *Acinetobacter baumannii* using the Novel *Campylobacteraceae* PNA Probe. *C. upsaliensis* (49) (A.) and *Acinetobacter baumannii* (38) (B.) Control slide using hydridisation buffer without probe (left); Hydridised slide using probe (right). The fluorescence is just as bright for the negative control (*A. baumanii*) as *C. upsaliensis.*

Table 28. Evaluation of *Campylobacteraceae* Specific PNA probe against *Campylobacteraceae* strains and Negative Control Bacteria. Numbers in brackets refer to the CAMPYCHECK reference strain number. Hybridisation was measured by making a subjective estimate of signal intensity in 4 arbitrary classes: +, ++, +++, ++++. + = low signal, ++++ = high signal.

Initial Test of *Campylobacteraceae* PNA probe		
Positive Test Strains	Ctrl	Probe
C. coli (2)	-	++++
C. concisus (4)	-	+++
C. curvus (8)	-	++++
C. fetus (10)	+	++++
C. doylei (28)	+	+++
C. jejuni (31)	-	+++
C. lari (39)	-	+++
C. upsaliensis (51)	-	+++
A. butzleri (56)	+	+++
Negative Control Test Strains		
Acinetobacter baumannii	-	++++
Shigella sonnei	-	++++
Staphylococcus aureus	-	+++
Klebsiella oxytoca	+	+
Enterobacter cloacae	-	+++
Serratia marcescens	-	++++
Citrobacter freundii	+	++++
Bacillus cereus	+	++++
Proteus mirabilis	-	++++
Pseudomonas aeruginosa	-	++++
Salmonella typhimurium	-	++++

tested that did not to bind with the probe was *Klebsiella oxytoca.*

Due to the high levels of non-specific binding to non-*Campylobacteraceae* species, a database search (Ribosomal Database Project II) was carried out, allowing for one and two base mismatches in the target sequence. With one nucleotide base mismatch there were no probe sequence matches to the bacteria that had been binding non-specifically in laboratory experiments. Allowing for two base mismatches however led to the probe matching, with four hits for *Pseudomonas* species and one hit for *Shigella* species. In addition to the bacterial strains that had been tested, there was also a high number of theoretical matches to many other bacterial species in the database if one or two mismatches were allowed in the target sequence, with 354 and 5489 non-*Campylobacteraceae* hits respectively.

5.3.3.1.2.2. Optimisation of *Campylobacteraceae* Probe Conditions

5.3.3.1.2.2.1. Media and Auto-fluorescence

There were problems with auto-fluorescence, especially in the green UV filter channel. Therefore, a number of media were tested for their effects on auto-fluorescence. Bacteria were sub-cultured from ABA (supplemented with blood) onto CCDA, ABA (no blood) and TBA (no blood), incubated for 48 h and then hybridisations carried out. Results showed that high auto-fluorescence still remained on the control slides (without probe). Therefore, cultures were resuscitated from frozen stocks directly onto CCDA and with repeated sub-cultures onto CCDA (from blood plates). Results showed that auto-fluorescence was reduced by repeated subcultures onto CCDA and therefore CCDA was selected as the growth medium prior to carrying out hybridisations.

5.3.3.1.2.2.2. Hybridisation Temperature

Preliminary experiments showed that there was strong non-specific binding with other non-target bacteria. Therefore, a number of different hybridisation temperatures were assessed in an attempt to increase the stringency of hybridisation (Table 29). The temperatures were evaluated at 5°C increments from 55°C to 70°C. Results showed that increasing the hybridisation temperature from 55°C to 65°C decreased the fluorescence intensity of the non-*Campylobacteraceae* species, but also of the tested *Campylobacter* species. At 70°C binding to non-*Campylobacteraceae* species was significantly reduced, however binding to the target bacteria was also reduced. Increasing the temperature of the washing step to 60°C after carrying out hybridisation at 55°C had little effect on the level

Table 29. Investigations to Increase the Stringency of Hybridisation for the Detection of *Campylobacteraceae* Using a 16S rRNA Targeted PNA Oligonucleotide Probe. Hybridisation was measured by making a subjective estimate of signal intensity in 4 arbitrary classes: +, ++, +++, ++++. + = low signal, ++++ = high signal.

A.

Temperature	55 °C		60 °C		65 °C		70 °C	
Positive Control spp.	Ctl	Probe	Ctl	Probe	Ctl	Probe	Ctl	Probe
C. jejuni	+	+++	+	++	+	+	-	+
C. upsaliensis	+	+++	+	++	+	+	-	+
Negative control spp.								
Pseudomonas spp.	-	+++	-	++	-	+	-	-
Shigella spp.	-	+++	-	+++	-	+	-	-

B.

Spp.		15 min	30 min	60 min	90 min
C. jejuni	Ctrl	-	-	-	-
	Probe	+	++	+++	++++
C. upsaliensis	Ctrl	-	-	-	-
	Probe	-/+	+	+++	+++
Pseudomonas spp.	Ctrl	-	-	-	-
	Probe	++	++	++++	+++
Shigella spp.	Ctrl	-	-	-	-
	Probe	+	+	+++	+++

C.

Spp.		Control	60°C wash	1% BSA	Rnase
C. jejuni	Ctrl	-	-	-	-/+
	Probe	+++	+++	+++	++
C. upsaliensis	Ctrl	-	-	-	-
	Probe	+++	+++	++	++
Negative control spp.					
Pseudomonas spp.	Ctrl	-	-	-	-
	Probe	+++	+++	++	++
Shigella spp.	Ctrl	-	-	-	-
	Probe	+++	+++	+	++

Table 30. Optimisation of Formamide Concentration in Hybridisation Buffer and Sodium Chloride and Detergent Concentration in the Washing Buffer to Reduce Non Specific Binding. A. Optimisation of formamide concentration in hybridisation buffer; B. Optimisation of Triton X-100 and sodium chloride concentration in wash buffer. Hybridisation was measured by making a subjective estimate of signal intensity in 4 arbitrary classes: +, ++, +++, ++++. + = low signal, ++++ = high signal. Ctrl = control, Pr = probe.

A.

	Formamide concentration (%) in Hybridisation buffer																					
	0%		5%		10%		15%		20%		25%		30%		35%		40%		45%		50%	
Positive Control spp.	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr	Ctl	Pr
C. jejuni	-/+	+	-/+	++	-	+++	+	+++	-	+++	-/+	+++	-	+++	-	+++	+	++	-/+	++	+	++
C. upsaliensis	-	++	-	++	-	+++	-	+++	-	+++	-	+++	-	+++	-	+++	-	++	-	++	-	+
Negative control spp.																						
Pseudomonas spp.	-	+	-	++	-	+++	-	+++	-	+++	-	+++	-	++	-	++	-	+	-	++	-	+
Shigella spp.	-	+	-	++	-	++	-	+++	-	+++	-	++	-	+++	-	+++	-	++	-	+	-	+

B.

Spp.	NaCl concentration	1% Triton X-100			2% Triton X-100			3% Triron X-100		
		15mM	50mM	150mM	15mM	50mM	150mM	15mM	50mM	150mM
C. jejuni	ctrl	-	-	-	-	-	-	-	-	-
	Probe	++++	+++	+++	+++	+++	++	+++	++	+++
Pseudomonas spp.	ctrl	-	-	-	-	-	-	-	-	-
	Probe	++++	+++	++	+++	++	++	+++	++	++

of fluorescence.

5.3.3.1.2.2.3. Length of Hybridisation

Reducing the length of hybridisation from 90 min to 60 min had no effect on binding (Table 29). However, reducing the hybridisation time to 30 min and below reduced both non-specific binding to *Pseudomonas* and *Shigella* species but also *C. jejuni* and C. *upsaliensis* to the same extent.

5.3.3.1.2.2.4. Formamide Concentration

Reducing the formamide concentration in the hybridisation buffer to below 10% reduced binding to non-*Campylobacter* strains, however it also had the same effect on the tested *Campylobacter* strains (Table 30). Binding was also reduced at the highest concentration tested, at levels of 50%. However, this effect was evident in both the target and non-target bacteria.

5.3.3.1.2.2.5. Washing Buffer (detergent and salt concentration)

There was no effect on fluorescence binding when changing the concentrations of Triton X-100 and sodium chloride in the hybridisation wash buffer within the ranges tested. The binding was equally as strong on the *Campylobacter* and non-*Campylobacteraceae* strains tested (Table 30).

5.3.3.1.2.2.6. Use of RNase to Test for Specific RNA Binding

To elucidate whether the probe was binding specifically to RNA within the cell, RNA was degraded using RNase prior to hybridisation. The results showed that binding to both *Campylobacter* and non-*Campylobacter* species decreased when the cells had been previously treated with RNase, suggesting that the probe was binding to RNA within the cell (Table 29). However, there was still some binding present, and the cells were slightly distorted after treatment with the RNase. Therefore, further studies into the effect of RNase are required before any sound assumptions can be made.

5.3.3.1.2.2.7. BSA as a Blocking Agent to Prevent Non-specific Binding

Including 1% (w/v) bovine serum albumin (BSA) in the hybridisation buffer slightly decreased the non-specific binding to the non-*Campylobacteraceae* species (Table 29). Binding to *C. jejuni* and *C. upsaliensis* was also reduced but to a lesser extent. Further studies are required into the effect of BSA as a blocking agent before any sound

conclusions can be made.

5.3.3.2. Final Molecular ID of Isolates using PCR

The PCR method used to confirm the identification of isolates recovered from samples was carried out by collaborator, Dr Kurt Houf, Ghent University, Belgium. Therefore, although PCR was used as an identification tool, no optimisation experiments were required. Results to these tests are described in Chapter 6 since the tests were only carried out on presumptive *Campylobacteraceae* sample isolates.

5.3.4. Proteomic Identification

A two dimensional electrophoresis approach was employed to separate whole-cell protein extracts by charge and molecular weight. The protein profiles of a number of *Campylobacter* species were then compared using PDQuest software (BioRad). Identification of selected proteins using tandem mass spectrometry was carried out as described in the materials and methods Chapter (Chapter 2).

5.3.4.1.1.Optimisation and Validation of 2D-PAGE Method

An investigation was carried out to determine the concentration of protein to initially load onto the gel. If a too low protein concentration is loaded, important proteins in low abundance could be missed; alternatively overloading gels could reduce the resolution. Loading 0.3 mg and 0.5 mg of total protein were compared (Figure 22) The results showed that loading 0.3 mg of protein produced gels with only faint spots and in limited number, however loading 0.5 mg gave more defined brighter protein spots in higher abundance, therefore this concentration was chosen.

Many previous studies have used both Colloidal Coomassie blue and SYPRO Ruby to stain protein gels. In many cases SYPRO Ruby is reported to be the method of choice due to its higher detection level and sensitivity of protein spots on the gels. An experiment was carried out to compare SYPRO Ruby with Colloidal Coomassie blue. The results showed that the protein spots were much brighter and clearer with SYPRO Ruby and a higher number of spots could be detected (Figure 22). However, a few different spots were detected when using Coomassie blue which were not detected using SYPRO Ruby stain. Due to the overall higher level of sensitivity, SYPRO Ruby was chosen for future experiments.

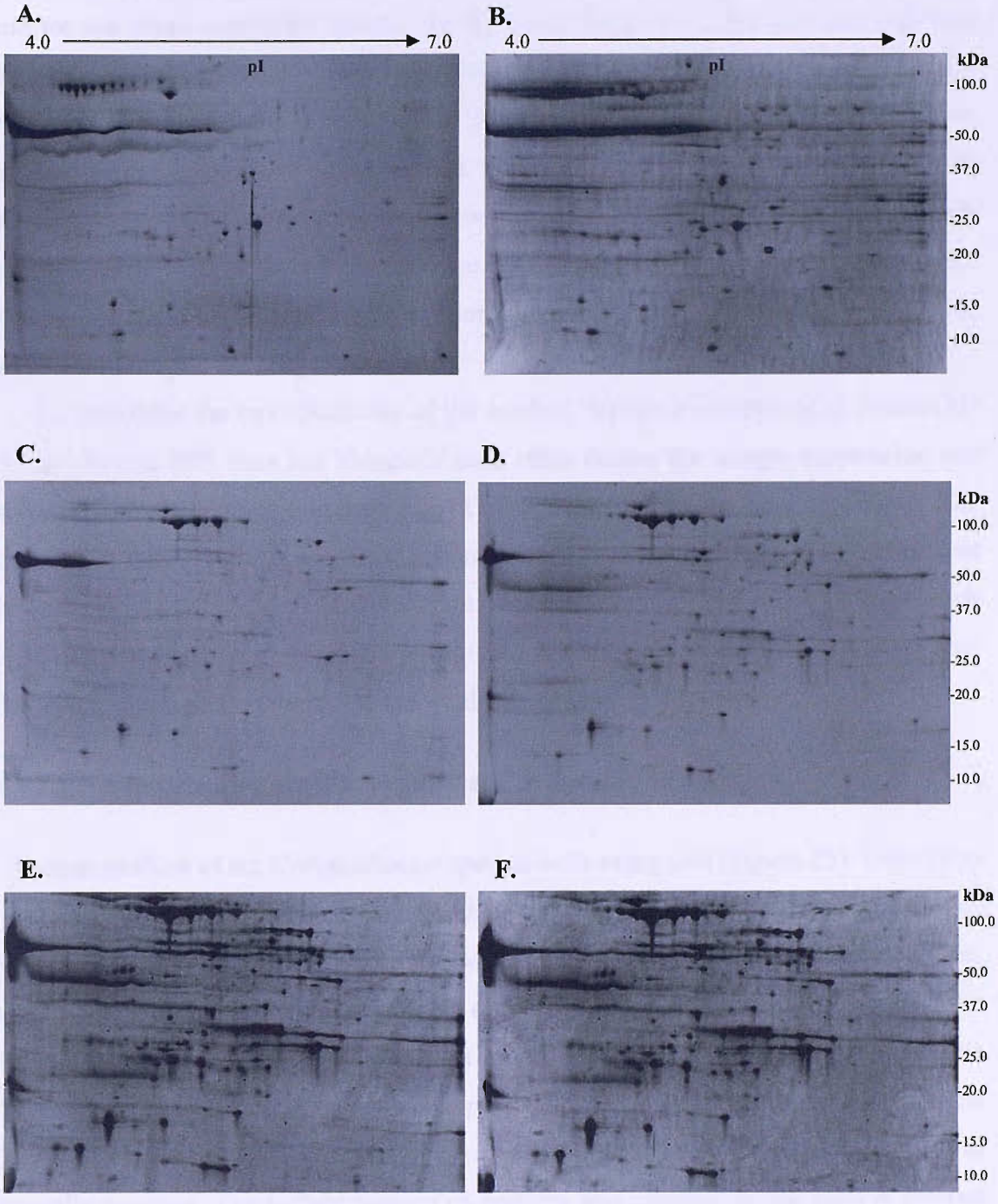


Figure 22. Optimisation of 2D-PAGE Conditions including the Amount of Protein Loaded onto the Gel, Evaluation of Protein gel stains and Demonstration of Reproducibility of the Method. *C. upsaliensis* protein profiles when 0.3 mg (A) and 0.5 mg (B) of total protein was loaded onto the Gel. *C. jejuni* when stained with Colloidal Coomassie brilliant blue (C) and SYPRO Ruby dye (D). *C. jejuni* NCTC11168 (31) to demonstrate reproducibility of method, (E). Replicate 1, (F) Replicate 2.

A preliminary experiment was carried out to determine which pH range would be optimal for use when separating proteins in the first dimension by their charge. Previous studies have shown that the majority of proteins can be isolated in the pH range of 4-7, but some experiments have also used pH 3-10 to isolate a wider range of proteins. Two dimensional protein profiles of *C. upsaliensis* were compared at a pH range of 4-7 and 3-10 (non linear). Results showed (data not shown) that some proteins were detected outside the pH 4-7 range within the pH 3-10 range. However, the majority of proteins were isolated within the pH 4-7 range and the separation and resolution of proteins spots was enhanced when the pH 4-7 range was used.

To determine the reproducibility of the method, replicate samples of *C. jejuni* (31) and *C. upsaliensis* (49) were run alongside each other during the sample preparation and electrophoresis steps. The protein profiles of *C. jejuni* were virtually identical (Figure 22). Analysis software (PDQuest, Bio-Rad Laboratories) revealed that there was 100% spot match between the two profiles (167/167 proteins). The replicate profile of *C. upsaliensis* 49, again demonstrated high reproducibility (data not shown) with a 97.4% protein spot match (156/160 proteins) between the two replicate protein profiles.

5.3.4.1.2. Comparative Protein Profiles of *Campylobacter* Species

Protein profiles of six *Campylobacter* species were compared (Figure 23). Only 50 to 167 protein spots were resolved on each gel depending on species. The protein profiles of the six species showed large discrepancies, which made quantitative comparative analysis of the gels a very challenging task. The large differences between the gels made it difficult to allocate 'landmark' proteins (protein spots found at conserved locations across all gels to be compared), therefore automatic protein profile alignment and spot matching using the PDQuest software (Bio Rad) was largely unsuccessful and in many cases the spots had to be manually matched. The highest number of proteins was resolved on the protein profiles of *C. jejuni* (167 spots) and *C. upsaliensis* (160 spots). These two species were also the most similar in terms of the number of protein spots found at seemingly conserved locations across the gels. The protein profiles of *C. lari* were also similar to *C. jejuni* and *C. upsaliensis*, however a lower number of proteins (70 spots) were isolated. *C. coli* (76 spots), *C. concisus* (50 spots) and C. *hominis* (115 spots) had very different protein profiles compared to *C. jejuni*, *C. upsaliensis* and *C. lari*. No quantitative comparisons were carried out on the differential expression of proteins of the *Campylobacter* species evaluated since the protein profiles of the six species were so different. Therefore, the probability of

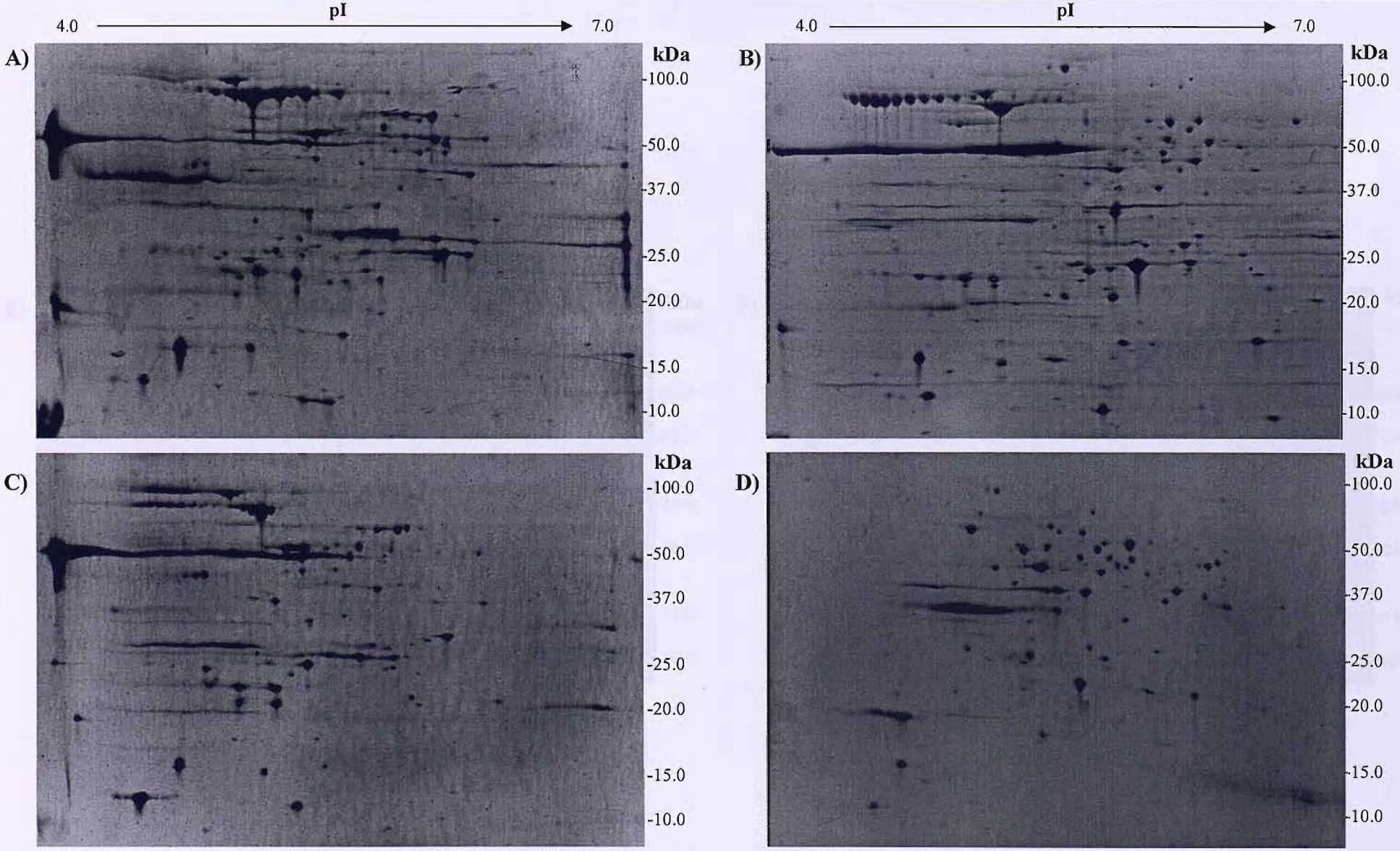


Figure 23. Comparative Two Dimensional Protein Profiles of *Campylobacter* species. A) *C. upsaliensis*; B) *C. jejuni*; C) *C. lari*. D) *C. concisus*; E) *C. hominis*; F) *C. coli*. SYPRO Ruby stained gel (inverted image), 22.5 second exposure.

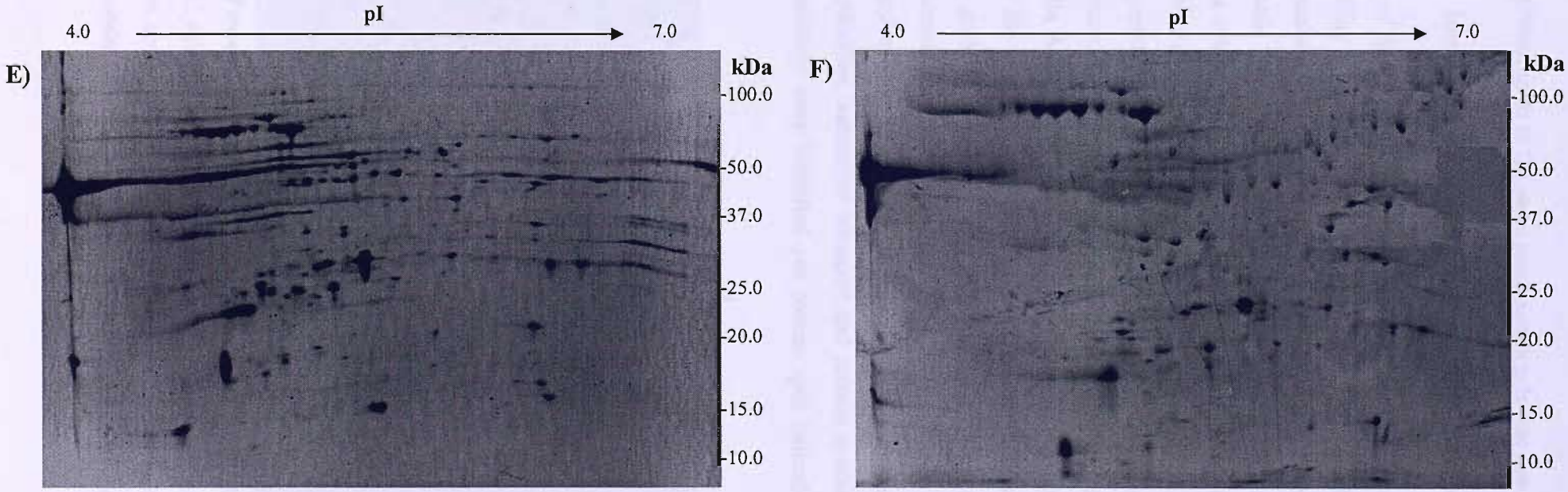


Figure 23 Continued.

matching protein spots to the same point across gels was low.

5.3.4.1.3. Identification of Protein Spots by MS/MS Mass Spectrometry

One of the advantages of using a two-dimensional gel electrophoresis approach was the possibility of carrying out further downstream identification of protein spots. A selected number of protein spots were identified using tandem mass spectrometry (Figure 24 and Table 31). The majority of proteins identified could be grouped into enzymes involved in oxidative defence. These proteins included alkyl hydroperoxide reductase, heat shock protein dnaK *Campylobacter*, 60 kDa chaperonin *Campylobacter*, trigger factor, (peptidyl-prolyl *cis/trans* isomerase), malate oxidoreductase *Campylobacter* and oxidoreductase. Structural proteins identified included the major outer membrane protein and flagellin A and B isoforms. A number of flagellin isoforms were found in different species by the observation of protein charge trains (protein spots with slightly different isoelectric points) (Figure 25). *C. coli* appeared to have a lower number of flagellin isoforms compared to the other species.

A small number of proteins were also identified which play a role in metabolism, signal transduction, membrane transport and protein synthesis. In many cases multiple protein sequences were identified per protein spot indicating incomplete separation of proteins.

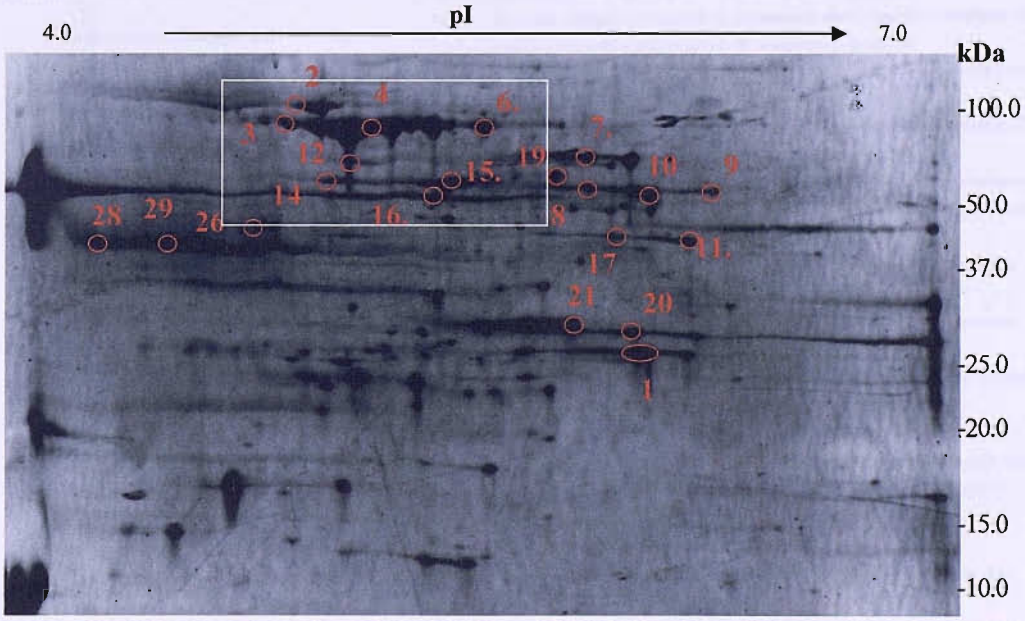


Figure 24. Protein Profile Map of Spots Identified Using Mass Spectrometry. Protein spots from C. *jejuni* protein profile were identified by tandem mass spectrometry. Protein spot numbers correspond to numbers in Table 31. White box indicates area of zoomed images of proteins identified as flagellin subunits shown in Figure 25.

Table 31. Protein spot Identification using Tandem Mass Spectrometry. Protein sequences were analysed using Protein Lynx software which automatically searched for database sequence matches. Detailed results including accession number, number of peptides identified and coverage are shown in the Appendix (Table III). The most probable proteins according to the score and percentage peptide coverage according to the Protein Lynx Software are shown.

Spot No.	Protein description	Protein function
1	**alkyl hydroperoxide reductase *Campylobacter***	Enzyme involved in in protection from oxidative stress via the breakdown of reactive oxygen species.
	hypothetical protein Pro0087	Unknown.
2	**alkyl hydroperoxide reductase *Campylobacter***	Enzyme involved in protection from oxidative stress via the breakdown of reactive oxygen species.
	heat shock protein dnaK *Campylobacter*	Important in the stress response. Acts as a chaperone induced by stress conditions such as heat shock.
	flagellin A *C. jejuni*	A heteropolymer of fla A. It is the subunit protein which polymerizes to form the filaments of bacterial flagella required for motility.
	putative methyl accepting chemotaxis protein	Membrane protein involved in signal transduction and chemotaxis.
3	**flagellin A *Campylobacter jejuni***	Flagellin A is a heteropolymer of fla A. It is the subunit protein which polymerizes to form the filaments of bacterial flagella required for motility.
	60 kDa chaperonin *Campylobacter jejuni*	Heat shock protein involved in stress responses. Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
	flagellin B *Campylobacter jejuni*	A heteropolymer of fla B. Flagellin B is required in combination with Flagellin A for a fully functional bacterial flagella required for motility.
4	**flagellin B *Campylobacter jejuni***	A heteropolymer of fla B. Flagellin B is required in combination with Flagellin A for a fully functional bacterial flagella required for motility.
	60 kDa chaperonin *Campylobacter*	Heat shock protein involved in stress responses. Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
	flagellin A *Campylobacter jejuni*	A heteropolymer of fla A. It is the subunit protein which polymerizes to form the filaments of bacterial flagella required for motility.
6	**flagellin A *Campylobacter jejuni***	A heteropolymer of fla A. It is the subunit protein which polymerizes to form the filaments of bacterial flagella required for motility.
7	**trigger factor (peptidyl-prolyl *cis/trans* Isomerase, chaperone**	Enzymes which catalyze a conformational change in a polypeptide chain accelerating the folding of proteins.
8	**putative MCP type signal transduction**	Membrane protein containing signal transduction activity as part of a two component response regulator important in chemotaxis.
	malate oxidoreductase *Campylobacter*	Involved in malate metabolism important in intermediate metabolism as a component of both the TCA cycle and the glyoxylate cycle important for many cellular processes.
9	**malate oxidoreductase *Campylobacter***	Involved in malate metabolism important in intermediate metabolism as a component of both the TCA cycle and the glyoxylate cycle important for many cellular processes.
	putative MCP type signal transduction	Membrane protein containing signal transduction activity as part of a two component response regulator important in chemotaxis.
10	**3 oxoacyl acyl carrier protein synthase**	Enzyme involved in fatty acid biosynthesis which catalyzes the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons from malonyl-ACP.
	alkyl hydroperoxide reductase *Campylobacter*	Enzyme involved in protection from oxidative stress via the breakdown of reactive oxyegen species.
	malate oxidoreductase *Campylobacter*	Involved in malate metabolism imortant in intermediate metabolism as a component of both the TCA cycle and the glyoxylate cycle important for many cellular processes.
11	**oxidoreductase *Campylobacter jejuni***	Enzyme important in oxidation and reduction reactions fundamental in metabolism and other processes.
12	**oxidoreductase *Campylobacter jejuni***	Enzyme important in oxidation and reduction reactions fundamental in metabolism and other processes.
	ATP synthase F1 sector beta subunit	Membrane protein which catalyzes ATP production from ADP in the presence of a proton gradient across the membrane.
14	**nifU protein homolog *Campylobacter***	Possible role in nitrogen metabolism.
15	**chemotaxis protein Campylobacter**	Involved in the transmission of sensory signals from the chemoreceptors to the flagellar motors.
16	**major outer membrane protein *Campylobacter***	Transmembrane porin important in cell signalling and cell permeability and antigenicity.
17	**predicted hypothetical protein X**	Unknown.
	COG1316 transcriptional regulator	Contains a DNA binding domain and controls transcription.
	major outer membrane protein *Campylobacter*	Transmembrane porin important in cell signalling and cell permeability and antigenicity
19	**alkyl hydroperoxide reductase *Campylobacter***	Important in Aerotolerance and Oxidative Stress Resistance
20	**COG1994 Zn dependant proteases**	Chaperone function.
	hypothetical transmembrane protein	Unknown.
	COG1316 transcriptional regulator	Contains a DNA binding domain and controls transcription of proteins.
	chemotaxis protein *Campylobacter*	Involved in the transmission of sensory signals from the chemoreceptors to the flagellar motors.
21	**inorganic pyrophosphatase *Campylobacter***	Enzyme which hydrolyzes diphosphate bonds to release energy during biosynthetic cellular reactions.
26	**ATP synthase F1 sector beta subunit**	Membrane protein which catalyzes ATP production from ADP in the presence of a proton gradient across the membrane.
28	**elongation factor TU *Campylobacter***	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
29	**tig smaller protein trigger factor *Campylobacter***	Involved in initiating transcription of proteins.

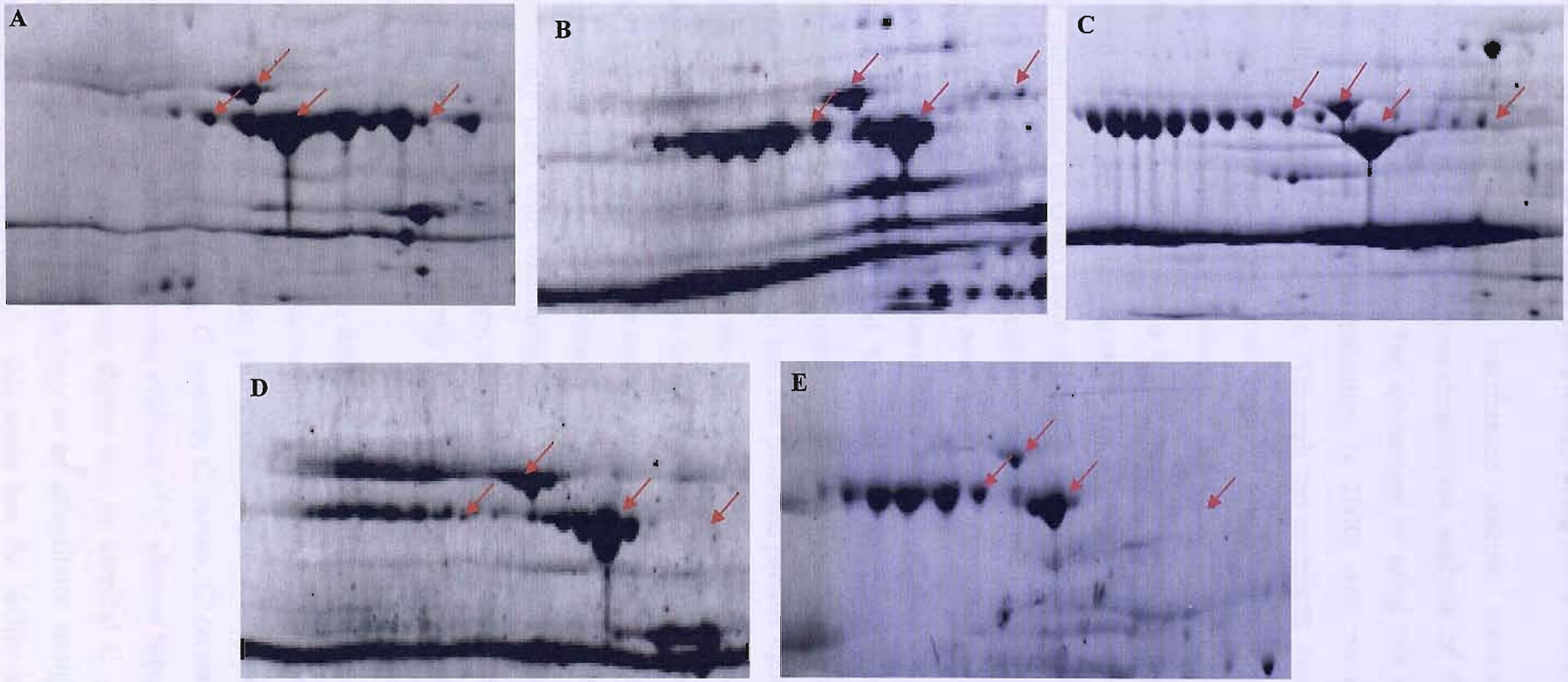


Figure 25. Zoomed Images of Flagellin Isomers in Various *Campylobacter* Species. Red arrows indicate protein spots that were identified as flagellin subunits using tandem mass spectrometry. *C. jejuni* (A), *C. hominis* (B); *C. upsaliensis* (C); *C. lari* (D); *C. coli* (E).

5.4. DISCUSSION

5.4.1. Analysis of Cellular Morphology using EDIC Microscopy

Episcopic differential interference contrast microscopy (EDIC) microscopy allowed rapid and detailed three-dimensional analysis of the cellular morphology of *Campylobacteraceae* species. The advantages of using this system is that cells can be viewed rapidly at high magnification (x 2000) with no need for complex staining procedures, oil and coverslips. Although the technique does not allow differentiation between Gram positive and Gram negative cells compared to the Gram stain, if used in conjunction with the KOH string test the Gram status can be quickly and accurately differentiated. Gram staining is highly labour intensive and unnecessarily complex with several staining and de-staining steps. The KOH test has been shown to be just as effective for determining the Gram status of bacteria (Buck 1982; Powers 1995).

Historically, EDIC microscopy has been used to visualise the surface morphology of bacterial biofilms *in situ* on opaque surfaces. The added advantage of this method is that it could be coupled to epi-fluorescence microscopy to view fluorescently labelled cells within biofilms (Rogers and Keevil 1992; Keevil 2003). Conventional differential interference contrast (DIC) microscopy exploits the destructive/constructive nature of light waves. The source light is split into two polarised parallel light beams before it reaches the specimen, having traversed the object, the wave paths alter in accordance with the thickness, slopes and refractive index. Variations in the sample cause interference between the two beams, and the details are visualised in a pseudo 3-dimensional appearance. EDIC microscopy is based on conventional Nomarski DIC microscopy, however, DIC is commonly used with transmitted light and is therefore unsuitable for viewing opaque materials. In EDIC microscopy, the prism is placed above the stage, and it is refracted light that is visualised, consequently 3D-type images can be generated (Rogers and Keevil 1992; Keevil 2003).

There was little or no difference between the cellular morphology of the majority of the 59 *Campylobacteraceae* strains tested. Morphology observed generally consisted of small curved or spiral rods. However, straight rods tended to predominate in the 'anaerobic' species including *C. gracilis*, *C. curvus*, *C. rectus*, *C. hominis* and *C. showae*. Previous studies have also shown evidence of *C. showae* being a 'straight rod' (Etoh *et al.* 1993). Another study has also shown that an atypical *C. coli* strain showed a stable spontaneous change in morphology to an aflagellated straight rod from its usual spiral flagellated form. Inrerestingly, this strain lost the ability to colonize the chick cecum

(Ziprin *et al.* 2005)

Although assessing cellular morphology is a helpful tool in the confirmation of presumptive *Campylobacteraceae* it cannot be definitively used since a number of other bacteria have similar morphological features.

There are very few studies in the literature evaluating the cellular morphology of the *Campylobacteraceae*, especially for a number of the emerging species. Therefore, this is the first study of its kind to investigate the cellular morphology of such a wide variety of *Campylobacteraceae* species. It is thought that the cellular morphology may vary, depending on the culture conditions used. In this experiment, for example, the morphology of *Campylobacter* species may have been different to that described previously in the literature due to the type of media used and the incubation environment. The majority of previous work has focussed on thermotolerant species such as *C. jejuni* and *C. coli,* which have collectively been described as 'spiral' or 'gull' shaped. Gram staining (with carbol fuchsin as the counterstain) and acridine orange staining are probably the most widely used microscopy methods in identifying *Campylobacter* species. Direct examination of faecal and blood specimens by dark-field or phase contrast microscopy has also been used as part of the routine diagnostic procedure, where the spiral shape and darting motility are used as characteristics to differentiate them from other organisms (Thorson *et al.* 1985; Wang and Murdoch 2004).

Several morphological forms of *Campylobacter* organisms have been reported in the literature, including spirals, S-shaped, gull shaped, commas, dimpled shaped, and coccoid. The spiral forms are thought to predominate in young cultures, while coccoid forms are found mainly in old cultures. The coccoid forms are generally believed to be degenerative and there is controversial evidence that these cellular forms may be in a viable but non-culturable (VBNC) state. The results in the present study showed that coccoid forms were almost always present among spiral or curved rod forms in the smears prepared from 48 h cultures. It is thought that the cells observed within a bacterial smear prepared from cells within a colony on the agar surface would have been heterogeneous in both age and physiological state depending on their location within the colony. A study by (Ng *et al.* 1985a) using electron microscopy to observe *C. jejuni*, *C. coli* and *C. lari* revealed that different morphological forms predominated in different parts of the colony. At the periphery cells were almost all spirals, where cells were actively growing due to the close proximity of nutrients. However, in the centre of the colony, cells were mainly coccoid shaped. These observations would explain the different morphological forms

observed in this study. Results from a previous study using *C. jejuni* have shown that cells from even a 48 h culture were in a coccoid form, especially if the cells were from crowded areas on the agar plate. However, isolated colonies tended to contain cells which were predominately spiral shaped (Buck *et al.* 1983).

Since the advent of microbiology, cell shape has been an important criterion in the description and classification of bacterial species. Many factors are thought to affect bacterial cell shape, but generally for *Campylobacter* species and other bacteria the molecular mechanisms underlying cell shape remain largely unresolved (Cabeen and Jacobs-Wagner 2005; Stewart 2005). The bacterial cell wall, with its peptidoglycan layer, has a primary role in maintaining cell shape. The penicillin-binding proteins (PBPs) carry out reactions for the synthesis and remodelling of peptidoglycan. Growth of the cell wall is not uniform, but localised to specific regions. Recent evidence has shown that internal structures such as the cytoskeletal protein actin and crescentin, an intermediate filament protein, are also important as in eukaryotic organisms (Stewart 2005). However, the molecular mechanisms that allow these bacterial cytoskeletal elements to affect the cell wall remain to be elucidated. One study using transmission electron microscopy has shown that in cells with spiral morphology the cytoplasm was close to the cell wall but in coccoid cells the cytoplasm was generally separated from the cell wall and was less dense (Buck *et al.* 1983).

5.4.2. Immunological Identification

The principle of the latex agglutination test is that latex particles are coated with rabbit immunoglobulin raised against antigen preparations from selected *Campylobacter* serotypes. When the sensitised latex particles are mixed with a solution containing *Campylobacter* antigens, a sensitive and specific immunochemical reaction takes place causing the finely dispersed latex particles to agglutinate into aggregates that are easily visible to the unaided eye. A previous study evaluating the Microscreen *Campylobacter* system showed that it reacted strongly with *C. jejuni* and *C. coli* strains (Sutcliffe *et al.* 1991). Furthermore, it was shown to react with a broader range of *Campylobacter* species (at high inoculum concentrations) than the other tests including *C. lari*, *C. upsaliensis* and *C. fetus*, did not cross-react with non-*Campylobacteraceae* taxa, and was shown to be easier to use than the Campyslide (BBL Microbiology Systems) (On 1996).

During this study, the specificity of the commercially available Microscreen *Campylobacter* latex test (Microgen Bioproducts, UK) was evaluated against the 59

reference strains within the CAMPYCHECK strain collection and further to this, a new prototype latex reagent with increased sensitivity was evaluated and found to be suitable for the detection of all *Campylobacteraceae* tested.

The results showed that the original latex test was unsuitable for the detection of many emerging *Campylobacteraceae* species tested (Lastovica *et al.* 2004). Due to the lack of specificity of the Microscreen latex test, a novel latex agglutination test was developed by Microgen to increase the sensitivity of the test to a wider range of *Campylobacteraceae*. This consisted of the old latex reagent (white latex particles) (to detect the thermotolerant species) and a second latex reagent (yellow latex particles), with specificity for all *Campylobacteraceae* (based on antiserum developed against one type of *C. concisus* by the University of Cape Town, South Africa). This new prototype latex kit was then evaluated, as before against the whole of the CAMPYCHECK strain set. This immunological test was initially intended to aid in the presumptive analysis of *Campylobacter* isolates rather than for the specific identification of *Campylobacteraceae*. However, the novel latex agglutination test (containing two latex reagents with different specificities) permitted the approximate discrimination between thermotolerant (those that react with both latex reagents) and non-thermotolerant *Campylobacteraceae* (those that reacted with the pan-*Campylobacteraceae* latex reagent). Further evaluation of this test was continued on isolates recovered from sewage and salad vegetable samples and so will be further discussed in Chapter 6. According to the novel Microgen latex agglutination test, *Campylobacter* species classed as thermotolerant included *C. helveticus*, *C. doylei*, *C. jejuni, C. lanienae*, *C. lari* and *C. upsaliensis*. Species classed as emerging *Campylobacter* species included *C. concisus*, *C. curvus*, *C. fetus*, *C. venerealis*, *C. gracilis*, *C. hominis*, *C. hyointestinalis*, *C. lawsonii*, *C. mucosalis*, *C. rectus*, *C. showae*, *C. sputorum*, *Bacteriodes ureolyticus* and all of the *Arcobacter* species.

All of the commercially available latex agglutination tests developed for the identification of *Campylobacter* species to date have been aimed at the identification of thermotolerant *Campylobacter* species and a number of studies have shown that none of these tests is suitable for identification of emerging *Campylobacter* species. Tests available include the Campyslide (BBL Microbiology Systems), Meritec-Campy (Meridian Diagnostics, Cincinnati, Ohio), Microscreen *Campylobacter* (Microgen Bioproducts Ltd, UK) and the Dry Spot Campylobacter test kit (Oxoid Ltd., Basingstoke, UK). Studies evaluating these tests have shown that all are unsuitable for the detection of non-thermotolerant species. The Campyslide was shown to react with 110 of the *C. jejuni* and *C. coli* isolates tested but

also with some *Pseudomonas aeroginosa* strains (Hodinka and Gilligan 1988). The Meritec-Campy latex test was shown to have 100% sensitivity in detecting *C. jejuni* and *C. coli* species but low sensitivity with *C. lari* isolates and complete lack of reactivity with strains of *C. fetus* and *A. cryaerophilus*. Some *C. upsaliensis* strains reacted with the test which also had 100% specificity for 101 non-*Campylobacter* organisms (Nachamkin and Barbagallo 1990). The Oxoid Dryspot Campylobacter test has been shown to give positive results with *C. jejuni, C. lari, C. coli, C. fetus* and *C. upsaliensis*, but isolates of other *Campylobacter* species such as *C. fetus* subsp. *fetus* will give variable results (Bridson, 1998).

The limitations of the latex agglutination test include non-specific agglutination of the control latex reagent which does not preclude the presence of campylobacters and the result has to be reported as inconclusive. Furthermore, if very low numbers of campylobacters are present a false negative result could be reported; conversely, if the concentration of non-*Campylobacteraceae* species is too high it could give a false positive result. It must also be noted that although the latex test has been evaluated by other partners in the CAMPYCHECK project (Clark *et al.* 2005), the performance characteristics of the *Campylobacteraceae* latex reagent have not been fully established using a wide range *Campylobacteraceae* and closely related bacteria and it is possible that false positive or negative results could be obtained at this stage. The fact that polyclonal antisera are used for the latex reagents reduces specificity since the latex beads would be coated with a number of antibodies to a range of surface antigens. If there had been more time during this study, it would have been interesting to further characterise these antibodies and their targets as part of the proteomic studies (described later). It is thought that immunological tests such as the latex agglutination test should be regarded with a certain degree of caution since the outer structures of *Campylobacter* species are thought to be highly prone to phase variability. The completion of the genome sequencing of a number of *Campylobacter* species has revealed that a number of genes encoding the biosynthesis or modification of surface structures are prone to phase variation via a slipped-strand mispairing mechanism during replication (Parkhill *et al.* 2000; Fouts *et al.* 2005). *C. upsaliensis* has been shown to contain nearly three times as many homopolymeric repeats as *C. jejuni*, seven times as many as *C. lari* and 22 times as many as *C. coli* (Fouts *et al.* 2005). Length variation of poly G/C tracts results in translational frameshifting and has been shown to be responsible for phase variation whereby bacteria randomly vary surface properties or antigenicity (Parkhill *et al.* 2000). Many of the genes

responsible for these hypervariable regions are clustered within regions involved in the formation of three major cell surface structures: capsular polysaccharide, lipooligosaccharide and flagella (Wren *et al.* 2001). It is thought that these mechanisms are present to allow the organism to adapt to the varied environment encountered during its life cycle, as well as in the evasion of the host immune response (Karlyshev *et al.* 2002). Although the immunochemical reaction is thought to be sensitive and specific for *Campylobacter* antigens, it is possible that due to phase variability of surface antigens strains could be misidentified.

5.4.3. Biochemical Identification

During this study, a number of phenotypic tests were investigated for the identification of *Campylobacter* species. These included the KOH test (Gram differentiation test), L-alanine aminopeptidase, oxidase, catalase, indoxyl acetate, urease, hippurate and growth at 25°C. The majority of the results gained were in agreement with results shown in previous literature (On *et al.* 1996; Steinbrueckner *et al.* 1999; Lastovica and Le Roux 2003; Lastovica 2006).

Campylobacter, *Helicobacter*, and *Arcobacter* strains could easily be differentiated from other Gram negative organisms using the L-alanine aminopeptidase test. L-alanine aminopeptidase is found in most Gram negative bacteria but not in Gram positive bacteria or in *Campylobacter*, *Arcobacter* or *Helicobacter* species (Carlone *et al.* 1983; Smith *et al.* 2006a). The prototype Oxoid Biochemical Identification System (O.B.I.S., Oxoid, UK) uses L-alanyl-7-amino-4-methylcoumarin as a substrate and the Fluka detection system uses L-alanine-4-nitroanilide. Although results from both types of test were identical in this study, the O.B.I.S. test was much quicker and easier to perform. The KOH test, which lyses the cell wall of Gram-negative bacteria but not Gram positive bacteria was used as a rapid alternative to the Gram differentiation technique. It was demonstrated that both the test for absence of L-alanine aminopeptidase and the KOH 'string' test used in combination provided a simple and rapid means of differentiating *Campylobacter*, *Arcobacter* and *Helicobacter* from other Gram-negative and Gram positive bacteria. Therefore, these tests were used as an initial pre-screen for the identification of presumptive *Campylobactereaceae* isolates recovered from sewage sludge and salad vegetables (Chapter 6). An additional test to which all members of the *Campylobacteraceae* strains were positive was the oxidase test (although *C. gracilis*, *C. concisus* and *C. showae* were only weakly positive). The oxidase test was used as an

additional test for the confirmation of *Campylobacteraceae* isolates recovered from samples (Chapter 6) as the majority of strains were oxidase positive.

It has been previously suggested that while *Campylobacter* and *Helicobacter* strains require microaerophilic or hydrogen-enriched microaerophilic atmospheres, strains of *Arcobacter* will grow under these conditions, as well as aerobically (Lastovica and Skirrow 2000; Lastovica 2006). Testing of *Campylobacter* and *Arcobacter* reference strains showed that aerobic growth at room temperature could be used as a simple test to readily differentiate *Campylobacter* from *Arcobacter* species.

The majority of *Campylobacter* and *Arcobacter* species were urease negative. *C. gracilis* strains were the only *Campylobacter* strains to be weakly positive and *B. ureolyticus* strains were positive for urease. However, this test is not a reliable differentiating factor as results from previous studies have shown that a number of atypical *Campylobacter* strains are urease positive (Moore and Matsuda 2004). More specifically, C. *lari* is urease variable and a group of strains which are urease positive are referred to as the urease-positive thermophilic *Campylobacter* (UPTC) group. Previous literature has also shown *B. ureolyticus* to be urease positive, however there is no evidence of *C. gracilis* being urease positive (On *et al.* 1996).

The most widely used phenotypic tests for preliminary identification and differentiation of *C. jejuni* and *C. coli* species are achieved using the hippurate and catalase tests (On 1996). However, in this study a number of *C. jejuni* strains were negative for hippurate hydrolysis and a number of other species which should have been negative were weakly positive. Previous studies have also shown that hippurate hydrolysis does not always correlate with genetic classification (Totten *et al.* 1987) and that differentiation between *C. jejuni* and *C. coli* is problematic since there are often atypical hippurate-negative strains of *C. jejuni* (Morris *et al.* 1985).

Although the phenotypic tests evaluated in this study (KOH test, L-ala, oxidase, catalase, indoxyl acetate, hippurate and test for aerobic growth at room temperature) were useful for the conformational identification of *Campylobacteraceae* and from this data presumptive identification of strains into groups of species could be made, it was clear that for the accurate speciation of *Campylobacter* using phenotypic analysis alone, a wider range of tests would be required. This would have been highly labour intensive and time consuming if tests were carried out in the traditional manner. Moreover, some of the results showed discrepancies with results from previous studies indicating that complete standardisation of the methods used to carry out the tests required. Due to the lack of

recommendations for identification test methods for this group of bacteria, most workers perform these tests in a manner specific to their laboratory. This may explain the frequent and notable discrepancies between workers who examine even the same strains yet obtain different results for the same test. Factors thought to influence the reproducibility of biochemical tests include preparation of substrate, basal medium used for growth, age and physiological state of the inoculum and the concentration of inoculum (On and Holmes 1991a; 1991b).

Due to the problems described with the identification of *Campylobacter* species using traditional phenotypic analysis, a prototype biochemical identification test strip for the speciation of *Campylobacter* species was investigated in collaboration with Microgen Bioproducts (Camberley, UK). This test included a variety of biochemical tests in a miniaturised multi-well strip format. It was thought that this test would improve standardisation and the time required to achieve results, conserving the cost of reagents and labour. The Microgen *Campylobacter* ID test was based on the biochemical identification schematic data of the Cape Town protocol (Lastovica 2006) and the extensive phenotypic data described by On *et al.* (1996). The test was modified by Microgen to incorporate 12 substrates on the strip for biochemical analysis. The most recent prototype test evaluated also included three off strip tests including oxidase, catalase and indoxyl acetate. The twelve biochemical on-strip tests included lysine, ornithine, arginine dihydrolase, aryl sulphatase, hippurate, nalidixic acid, pyrazinamidase, ONPG, nitrate, urease, citrate and glucose. The system was therefore composed of 15 tests producing a five digit identification code, which could be subsequently analysed using a probabilistic method using the dedicated Microgen software database and programme.

Evaluation of the Microgen prototype biochemical identification test was carried out at various stages of development of the product. Factors investigated included various substrate concentrations using different methods of substrate preparation, length of incubation time and incubation atmospheres of the test strips. During the development of the 12 on-strip biochemical substrate tests, it was noted that a number of the expected reactions were not occurring as predicted with a number of emerging *Campylobacter* species and that certain tests were not reproducible. Unfortunately, despite efforts to optimise this test, it was clear that the substrate system as it was currently configured would not deliver robust consistent results and that further development of the individual substrates was required before this product could be considered for commercial exploitation. Microgen is currently reviewing the specifications of this prototype test with

a view to establishing an improved substrate set capable of producing reproducible results. It is possible that the discrepancies in the results compared to those results in the Cape Town protocol could be due to the variation in the incubation atmosphere, which could cause variations in metabolic reactions including the type of enzymes expressed. It is also possible that the different set up of the tests would cause differences in the results achieved. A wider range of tests may need to be included for identification of all species to be accurate. Using a higher number of phenotypic tests would increase the discriminatory power and accuracy, reducing the chance of misidentifying an isolate. An extensive numerical taxonomic study (in which 67 phenotypic characters were used to examine 347 strains) demonstrated that 35 of 40 species and subspecies of campylobacteria could be identified successfully by cluster analysis (On *et al.* 1996). A commercial identification kit for campylobacteria which comprises 21 tests is currently available (API Campy; API Biomérieux Ltd., Marcy l'Etoile, France). However, this test was developed for the identification of thermotolerant species only and misidentifications of thse species have been demonstrated. One study showed that two *C. coli* isolates were misidentified as *Arcobacter cryaerophilus* and *C. jejuni* and *C. lari* was misidentified as *A. cryaerophilus* (Huysmans *et al.* 1995), therefore this test was not included in analysis as identification of a wide range of *Campylobacteraceae* species was required.

Despite problems associated with using phenotypical analysis to identify *Campylobacter* species, it remains the most common method used in routine identification of *Campylobacter* species when molecular methods are not available, therefore improvements in this application would be beneficial. Studies using differential results of biochemical tests in dichotomous trees are common, where reactions to a single test lead the user to different branches in the scheme and ultimately result in a named taxon. This type of analysis is routinely and successfully used to identify many different members of the family *Campylobacteraceae* as part of the Cape Town protocol (Lastovica 2006). However, there are computer programmes based on cluster or probabilistic analysis which require a large number of phenotypic results, allowing a more objective and reliable approach (On 1996).

There are many problems encountered when attempting to identify *Campylobacter* species phenotypically because they are asaccharolytic and are generally biochemically unreactive. Therefore, many phenotypic tests used to differentiate other bacterial groups such as the members of the family *Enterobacteriaceae* have no discriminatory potential for *Campylobacter* species. The accuracy of biochemical identification is limited due

significant phenotypic diversity within species and also a lack of discriminatory criteria between certain closely related species. It has been suggested that for accurate species identification molecular identification methods are required. The problem of identification using phenotypic analysis is particularly acute in *Campylobacter* since a number of phylogenetically related species such as *Arcobacter* and *Helicobacter* species will often not be included in such phenotypic schemes but can give results that closely resemble, or even be identical, with those of *Campylobacter* species. Of special note are the species *H. canadensis* and *H. pullorum*, that in limited biochemical schema, cannot be distinguished from *C. coli* or *C. lari*, respectively (Steinbrueckner *et al.* 1998). Atypical strains are extensively described in the literature and results from such strains will invariably lead to misidentifications. In addition, the taxonomy of the campylobacteria is a complex and rapidly evolving field. Although most known species are well defined (thus facilitating their identification), the number of taxa that make up the group has increased dramatically in recent times; at least one new species or subspecies has been described every year since 1988. This constant state of taxonomic flux has made the construction of identification schemes problematic. Problems may be encountered if a routine laboratory encounters a 'rare' or newly described species or if clear differential features between taxa are absent.

5.4.4. Fluorescence *In Situ* Hybridisation

Fluorescence *in situ* hybridisation (FISH) with rRNA-targeted oligonucleotide probes is an alternative rapid and specific method for identification of bacteria, which is often less prone to inhibitory substances than PCR methods. Previous studies have shown that PNA probes are extremely specific and can differentiate between non-specific targets with only one nucleotide difference (Stender *et al.* 2002; Lehtola *et al.* 2005). However, results from this study have shown evidence to the contrary. The 16S targeted PNA oligonucleotide probe developed for the identification of *Campylobacteraceae* species bound to all tested *Campylobacteraceae* strains but also, non-specifically, to 10 out of the 11 non-*Campylobacteraceae* strains. Attempts to increase the stringency of hybridisation generally reduced non-specific hybridisation, however this also reduced binding to the target bacteria.

Due to the amount of non-specific binding found during the preliminary analysis of the probe, a database search to check for specificity of the probe against target and non-target species allowing for 1 or 2 nucleotide base mismatches on the target sequence was carried out. Results showed that with one nucleotide base mismatch there were no probe

sequence matches to the bacteria that had bound non-specifically in laboratory experiments. Allowing for two base mismatches, however led to the probe matching with four hits for *Pseudomonas* species and one hit for *Shigella* species. However, this does not explain the widespread non-specific binding of the probe to the other non-*Campylobacteraceae* species tested in the laboratory whose sequences found in the database did not match with the probe, even allowing for 1 and 2 base mismatches. It must be taken into consideration that the designed probe is only as reliable as the database from which it was constructed. Despite the recent influx of new rRNA sequences into databases such as the Ribosomal Database Project (RDP-II), there are a number bacterial strains whose rRNA sequences are not yet included in the database or for which only partial sequences are available (Maidak *et al.* 2001). The specificity of binding is also thought to be determined by the position of the base mismatches in the sequences of non-target bacteria. Mismatches towards either end of the target sequence will results in a higher chance of binding with non-specific bacteria than if the base mismatches were towards the middle.

Criteria for the sequence of PNA oligonucleotide probes are that they should optimally be around 15 bases long which is much shorter than that of DNA probes (Stender *et al.* 2002). The probe sequence designed for the detection of *Campylobacteraceae* species in this study was a shortened version of a probe previously used as an 18-oligomer DNA probe to detect *Campylobacter* species and which had previously been shown to successfully discriminate between *Campylobacter* species and other bacteria (Schmid *et al.* 2005). The oligomer was shortened by three bases at the 3' end from 18 bases to 15 nucleotide bases and it is possible that the probe sequence was simply too short to provide accurate discrimination from other bacteria. Furthermore, a characteristic of PNA probes is that they are more permeable across the cell membrane compared to DNA probes due to the lack of electrostatic repulsion typically encountered when negatively charged complementary DNA oligomers hybridise (Stender *et al.* 2002). Therefore, it is likely that the target sequence would have been more accessible to the PNA probe than a DNA probe in both target and non-specific bacteria. It was considered that the non-specific binding could have been due to the position of the target sequence which could have been more accessible to the probe in non-target bacteria than in *Campylobacteraceae* species since in comparison to DNA probes PNA probes have the capability of reaching even inaccessible low affinity 16S rRNA binding sites within the cell (Wilks and Keevil 2006). The binding position of the chosen probe in *Campylobacter*

species was 653-668 (Schmid *et al.* 2005). This binding position has been shown to be relatively inaccessible (17% of brightest score) with low levels of fluorescence intensity (6-20% of total score) in *E. coli* with DNA probes (Fuchs *et al.* 1998). However, the non-target bacteria that showed matches to the probe by both database searching (with two base mismatches) and laboratory studies showed that the binding positions and fluorescence intensity scores of the target sequence provided no explanation for higher non-specific binding in these species. The sequence in *Pseudomonas* species was located at binding position 818, which in *E. coli* was shown to be a relatively inaccessible position (10% of brightest score) and of low fluorescence intensity (20-60% of brightest score), similar to that of *Campylobacter* species. However, in contrast, the location of the target sequence in *Shigella* species (binding position 1039) was highly accessible (41-60% of brightest score) with a high fluorescence intensity score (52%) compared to *Campylobacter* species (Fuchs *et al.* 1998). Another possible reason for the lack of specificity could have been due to the presence of the 'Y' nucleotide in the probe sequence, included to allow probe detection of a range of *Campylobacter* species that differed at this position. The fact that the 'Y' nucleotide codes for an unspecified pyrimidine nucleoside (uracil, thymine or cytosine) (Anonymous 1970) may have contributed to a reduction in specificity and increased the chances of binding to non-target bacteria. The GC content of an oligonucleotide is thought to influence duplex stability therefore making it difficult to achieve sufficiently stringent hybridisation conditions (Pernthaler *et al.* 2001). The GC content of the oligonucleotide probe developed was 60% and although this has been shown to be within the acceptable range it is still relatively high.

Despite the possible explanations for the lack of specificity of the probe, investigations were carried out to optimise the method in an attempt to increase the stringency of hybridisation. There are several strategies to determine stringent hybridisation conditions for a newly designed probe. The melting behaviour of oligonucleotide probes depends on temperature, the composition of the hybridisation buffer, oligonucleotide sequence (GC content), and on probe length (Pernthaler *et al.* 2001). It is possible for hybridisation stringency to be adjusted by both the temperature and the chemical composition of the buffers used.

A range of temperatures were evaluated at 5°C increments from 55°C to 70°C. The optimal temperature for the hybridisation of both *Campylobacter* species and non-target bacteria was 55°-60°C. Increasing the hybridisation temperature to 65°C to 70°C reduced the binding to non-target bacteria but also severely reduced the fluorescence signal of the

Campylobacter species. The wash temperature was increased by 5°C to 60°C in an attempt to more efficiently rinse off any excess or weakly-bound probe molecules that might have been contributing to the non-specific binding but unfortunately this had no effect. It is possible that increasing the wash temperature further may have reduced non-specific binding. The results showed that altering the salt (NaCl) and detergent (Triton X-100) concentration in the washing buffer had virtually no effect on fluorescence hybridisation. It was thought that this may have increased the stringency by improving the efficiency of rinsing off the excess unbound or weakly bound probe molecules from the cell. It is possible that testing a wider range of concentrations may have increased the stringency.

It has been previously reported that increasing the formamide concentration will increase the hybridisation stringency and therefore restrict non-specific binding of non-related bacteria. Formamide was included in the hybridisation buffer at concentrations of 0% to 50%. Fluorescence binding of both *Campylobacter* and non-target species was reduced below 10% and at 50% formamide. It has been reported that low levels of formamide are essential to increase the permeability of the cell membrane and allow access of the probe. This is likely to be the reason for a reduction in fluorescence binding below 10% formamide. At higher concentrations, as an organic solvent, formamide is thought to reduce the thermal stability of double-stranded polynucleotides and therefore reduce the weaker, non-specific bonds between the probe and mismatched hybrids. Unfortunately, although increasing the formamide concentration to 50% reduced non-specific binding, it also reduced binding to *Campylobacter* species. It is likely that increasing the formamide concentration further would simply reduce the fluorescence binding of the target bacteria.

Reducing the length of hybridisation was investigated as it was thought that specific binding would be much quicker than non-specific binding. The length of hybridisation was reduced from 90 min to 60 min, 30 min and 15 min. Reducing the hybridisation time reduced both binding to the target and non-target bacteria.

It is possible that the lack of specificity of the probe was simply due to binding to closely related targets which differ by 1-2 nucleotides, especially if the mismatch sequence was in greater abundance than the target molecule. Studies have shown that the specificity of probes that show one mismatch with non-target sequences can be increased by including one or several competitor oligonucleotides or 'blocker probes' in the hybridisation buffer. These competitor sequences are designed to perfectly match with the non-target sequence at homologous sites (but are not labelled) and therefore mask non-target sequences from

hybridisation with the labelled probe (Pernthaler *et al.* 2001).

It is possible that the probe was binding non-specifically to structures other than RNA either within the cell or on the cell surface including DNA sequences, peptides or proteins. The binding of the PNA probe to proteins is not an unlikely scenario since although PNA behaves as a nucleotide base by obeying Watson-Crick base pairing, its chemical structure shows that it is more correctly a protein and peptide-like molecule (Nielsen and Egholm 1999). Therefore, an investigation was carried out to assess the effect of including Bovine Serum Albumin (BSA) (1%) in the hybridisation buffer. It was thought that this may have reduced non-specific binding (formation of protein-PNA interactions) by blocking potential binding sites of proteins and peptides within the cell. Although including 1% BSA in the hybridisation buffer slightly reduced the non-specific fluorescence binding of the non-target bacteria it also reduced fluorescence binding of *Campylobacter* species but to a lesser extent. It is possible that increasing the BSA concentration may have further reduced non-specific binding without inhibiting the specific binding to *Campylobacter* species. Additionally, it is possible that a number of alternative blocking agents including casein, albumin and gelatine could be investigated.

An experiment was carried out to determine whether the non-specific binding of non-target bacteria was due to the probe sequence binding with mismatched RNA sequences or other structures within the cell such as peptides and proteins. During this experiment, cells were incubated with RNase prior to hybridisation. There was no difference in the hybridisation of both *Campylobacter* and non-target bacteria. It is possible that the RNA was not fully degraded by the RNase during this experiment as the cells remained fully intact. To further address this issue, RNA extractions could be carried out and then the probe tested on the RNA extracts of both *Campylobacter* and non-target bacteria.

Initially, autofluoresence was a problem when the first *Campylobacteraceae* probe (linked to Alexa fluor 488) was evaluated. The background green fluorescence was as high on the control samples (without probe) as when the probe was included meaning any fluorescence could not be attributed to the detection of the PNA-labelled probe. Further studies showed that cell autofluoresence was reduced when the cells were grown on CCDA agar (without cefoperazone) as opposed to blood agar, as suggested in a previous study (Lehtola *et al.* 2005). An important finding was that the growth media had a strong effect on the autofluorescence of bacteria. If *Campylobacter* species were maintained on tryptone agar supplemented with laked horse blood, strong autofluorescence of the bacteria

made it impossible to differentiate reference bacteria grown on tryptone blood agar and hybridised bacteria. These bacteria fluoresced brightly when observed by epifluorescence microscopy even without addition of probe during hybridisation. Fluorescence of the bacteria was seen in a wide wavelength range, so hybridisation would not work even by using other fluorochromes bound to PNA-probes. By contrast there was no autofluorescence observed in bacteria grown on blood-free CCDA agar. The effect of growth media on autofluorescence was not investigated systematically so the exact reason for this phenomenon is not known. Autofluorescence of a range of microorganisms, such as moulds, yeasts, *Legionella*, *Pseudomonas*, *Rhodospirillum* and *Cyanobacteria* has also been reported (Moter and Gobel 2000).

A 16S rRNA targeted PNA probe has previously been used to successfully detect thermophilic campylobacters in water samples and was shown to be superior to DNA probes (Lehtola *et al.* 2005; Wilks and Keevil 2006). This PNA probe was re-analysed during this study against a number of the *Campylobacteraceae* in the CAMPYCHECK reference strain collection. It strongly bound to the three thermotolerant *Campylobacter* species, however it also weakly bound to non-thermophilic *Campylobacter* species but not to the non-related bacteria tested including *Salmonella* and *Pseudomonas* species. This probe was used as a tool for the identification of presumptive *Campylobacteraceae* isolates which were recovered from sewage and vegetable samples and so will be further discussed in Chapter 6. A number of other studies have used FISH technology for the detection of *Campylobacter* species although to date, the only study to use PNA FISH technology for the identification of *Campylobacter* species was the study described by Lehtola *et al.* (2005). A number of studies have used DNA probes for the identification of *Campylobacteraceae* species. Both 16S- and 23S-rRNA targeted probes were successfully used for the detection of members of the genus *Campylobacter* and thermotolerant species in chicken faecal, liver and food samples (Moreno *et al.* 2001; Schmid *et al.* 2005). FISH has also been used to detect both thermophilic *Campylobacter* species and *Arcobacter* species in water and sewage sludge samples (Moreno *et al.* 2003). Recently, since the development of PNA technology there has been a general increase in the use of PNA oligonucleotide probes in place of DNA probes for the identification of a number of bacterial species due to the numerous of advantages over DNA probes as previously described. PNA FISH has been successfully used for the identification of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in blood cultures (Hartmann *et al.* 2005; Sogaard *et al.* 2005), *E. coli* and *Mycobacterium* in water samples (Lehtola *et al.* 2006b), *Listeria*

species in food samples (Sogaard *et al.* 2005) and for the *in situ* detection of *Legionella* and *Helicobacter* biofilms in water pipes (Wilks and Keevil 2006).

5.4.5. Evaluation of a Proteomic Technique for Sub-Typing of *Campylobacter* Species

5.4.5.1. Use of Two Dimensional Comparative Protein Profiling as a Sub-Typing Method

Many previous studies have focussed on the use of one-dimensional protein electrophoresis for the identification of *Campylobacter* species. However, although this method has been extremely effective, issues remain with the discriminatory power. The method can not always clearly discriminate between *C. jejuni* and *C. coli* and other closely related species (On 1996). Therefore, it was thought that a two-dimensional approach would resolve these issues.

The results showed that use of a two-dimensional comparative protein profiling approach was ineffective for use as a sub-typing method of *Campylobacter* to the species level. The method was almost too discriminatory between species, making it difficult to use the software available to quantitatively analyse differential protein expression profiles of different *Campylobacter* species. It was clear that considering whole-cell protein extracts were prepared, the proteins isolated using the two-dimensional electrophoresis approach were largely under represented on the gels and were a poor reflection on the actual number of proteins thought to be expressed from genome predictions. Only 13% of the potential cell proteins thought to be expressed in *C. upsaliensis* were isolated on the two dimensional protein profile. *C. upsaliensis* is thought to contain 1,261 proteins and only 160 protein spots were isolated using the present method (Fouts *et al.* 2005).

The high level of variation observed in the protein expression patterns of different species was thought to be due to small modifications in protein charge or molecular weight due to post-translational modifications of the protein, which would lead to large changes in the position of the spot. The generation of proteins with multiple isoelectric points or masses was likely to be due to post-translational modifications including phosphorylation, methylation, deamination or glycosylation and it has been reported that species diversity is observed more often in the proteome profiles of bacteria compared to DNA fingerprinting methods because of these differences (Cash 2000). Any isoforms of a protein resulting from modifications that cannot be predicted from DNA sequences are evident using 2D gel

separation as they result in alterations of the charge or molecular weight of the protein.

When interpreting the results it is generally assumed that the co-migrating spots are functionally identical proteins with amino acid homology. However it must be considered that even a single charged amino acid substitution can alter the position of a protein to what may seem a unique protein spot on a gel (Cash 2000). This, along with differences in migration of proteins due to post-translational modifications, can lead to a greater degree of variability between species on 2D protein profiles compared to when DNA-DNA hybridization techniques are used (Rodwell and Rodwell 1978).

The publication of *C. jejuni* 11168 genome sequence and recent closure of *C. jejuni* (RM2228), *C. coli*, *C. upsaliensis* and *C. lari* genomes has revealed much information regarding the structure and similarity of predicted coding sequences (Parkhill *et al.* 2000). Of the 1084 protein sequences shared by all the *Campylobacter* species, 46 of them had no match to any other organism in the database. *C. jejuni* NCTC1168, not surprisingly had the highest average protein percent identity (1,468 proteins averaging 98.41% identity) with *C. jejuni* RM1221 proteins. *C. coli* RM2228 was second, with 1,399 proteins averaging 85.81% identity. Surprisingly, *C. upsaliensis* RM3195 had the third highest average protein percent identity with *C. jejuni* RM1221 (1,261 proteins; 74.72% average identity), followed by *C. lari* RM2100 with 1,251 proteins having 68.91% average identity. Results from this experiment, however demonstrated that there were substantial differences between the protein profiles of all *Campylobacter* species tested. *C. jejuni* was most similar to *C. upsaliensis* and *C. lari* which, according to their predicted protein sequences showed 74.72% and 68.91% average identities. Unfortunately, quantitative comparative analysis of the protein profiles was not carried out due to the high level of heterogeneity between species, making analyses using the available software difficult.

Previous studies have shown that the extent of protein conservation between gels (specifically in the electrophoretic mobility) of different bacterial species is extremely wide ranging and is dependent on the bacteria in question. Many studies with other bacteria however have revealed strong links between identification using genetic and protein profile analysis. For example, the comparison of protein profiles of *Neisseria* species showed differences observed in their 2D GE profiles followed their genetically recognised taxonomic classification (Jackson *et al.* 1984). An extensive study of *Listeria* species including 29 isolates representing six different species readily distinguished the *Listeria* species, as well as managed to separate the *L. monocytogenes* strains in two distinct clusters by 2D gel electrophoresis, which strongly agreed with that obtained using

molecular methods (Gormon 1995) as did a study with *H. influenzae* clonal relationships (Cash *et al.* 1995). The study with *Listeria* showed that 600 protein spots were compared and only fourteen were conserved amongst the six species and 79 between the isolates of *L. monocytogenes* (Gormon 1995). In a similar manner the comparison of *H. influenzae* revealed 24 spots absolutely conserved between all isolates and an even bigger variation was observed between the different species and of the same genus (Cash *et al.* 1995). However other species such as *Mycoplasma* showed much lower degree of variation. When comparing six strains of *M. arthritidis*, only 25 strain variable proteins out of 340 proteins separated were reported and although more extensive variation is observed between *Mycoplasma* spp. there were still six common genus specific proteins detected (Andersen *et al.* 1984). A study comparing 2D gel protein profiles has also been able to discriminate between closely related species of *Ferroplasma*, which could not be typed using DNA-DNA similarities (Dopson *et al.* 2004). In addition, a 2D electrophoresis approach has been successfully used to characterise strains of *Helicobacter pylori*, a close relative of *Campylobacteraceae*. One study showed that even protein profiles of strains within a species were extremely variable with only a few proteins spots in common between the three *H. pylori* strains tested (Jungblut *et al.* 2000). In addition, the method was used to compare proteome maps of *H. pylori* strains from different geographical locations, where variability of some *H. pyori* proteins and the quantitative analysis of their level of expression were successfully evaluated (Govorun *et al.* 2003). It is possible that during this study the levels of protein expression between different *Campylobacter* species were too divergent for 2D PAGE to be useful a species identification tool and that a this approach would have been more effective as a *Campylobacter* strain typing method, due to its ability to resolve minor differences in protein expression, likely to exist within strains of a species. Unfortunately, no experiments were undertaken to compare protein profiles of different strains within a species during this study.

Experiments carried out in this study have shown that considerable heterogeneity between protein profiles of *Campylobacter* exist despite their apparent genetic relatedness, which could be due to post translational modifications or even single amino acid substitutions which could lead to big modifications to charge and therefore the isoelectric point, altering the position on the gel. The most likely reason for such observed differences could be due to the post-translational glycosylation of proteins. The identification of a gene locus in *C. jejuni* (strain 81-176), containing genes required for glycosylation of multiple proteins (including flagellin) provides evidence for this (Linton *et al.* 2005). *C. jejuni* is, as

yet the only bacterium known to have such a system of general protein glycosylation (Linton *et al.* 2002). It is possible that slightly different glycosylation systems exist in the different *Campylobacter* species, which is the reason why the protein profiles of the species were so divergent. *Campylobacter* are known to display variability in the length of polyG:C tracts produced by slipped strand mispairing during replication which can affect translation and has been shown to be responsible for phase variation of surface proteins (Parkhill *et al.* 2000). It is possible that slightly different glycosylation systems in different *Campylobacter* species and phase variation caused by strand mispairing is responsible for such divergent protein profiles between species and to those predicted by genome sequences. It has been suggested that glycosylation of non-flagellar proteins in *C. jejuni* might influence pathogenesis by contributing, for example, to immune evasion or adhesion (Logan *et al.* 2002).

5.4.5.2. Identification of Protein Spots using Tandem Mass Spectrometry

Ideally, protein identification would have been carried out on all of the protein spots from all of the protein profiles. However this was not practical or cost effective due to the expense of the identification procedure. Only a limited number of protein spots were identified from the *C. jejuni* protein profile. At the time of study it was decided to temporarily cease investigation in this area of research and focus on another, more applied aspect of the project until a later date if there was more time available.

Although identification of only a fraction of the protein spots from the *C. jejuni* protein profile was carried out, some valuable information regarding the type of proteins isolated on the 2D protein profiles was gained. Certain proteins found at conserved locations across gels and could be mapped across and identified with some degree of certainty. Many of the protein spots were identified as enzymes involved in oxidative stress including alkyl hydroperoxide reductase, heat shock protein dnaK *Campylobacter*, 60 kDa chaperonin *Campylobacter*, trigger factor (peptidyl-prolyl *cis/trans* isomerase), malate oxidoreductase *Campylobacter* and oxidoreductase. A previous study compared the 2D protein profiles of *C. jejuni* when cells were grown in broth or in agar (immobilised). Results showed that immobilised bacteria (grown on agar media) undergo a shift in cellular priorities away from metabolic, motility and protein synthesis capabilities towards emphasis on iron uptake, oxidative stress defence and membrane transport. Many oxidative stress enzymes identified when *C. jejuni* was grown on agar media in the study were the same as those identified in the present experiment.

5.4.5.3. Multiple Flagellin Isoforms in *Campylobacter* Identification

There were high levels of heterogeneity in selected protein spots identified as flagellin proteins. These were evidenced by multiple charge trains (proteins with slightly different iso-electric points) which were all identified as flagellin proteins. These isoforms are thought to be due to post-translational glycosylation and may play a major role in pathogenicity during the intestinal colonisation stage in the host (Szymanski *et al.* 2002; Wosten *et al.* 2004). Despite protein spot identification only being carried out on *C. jejuni* species, it is likely that the protein spots which mapped across to the same locations on the proteins profiles of other species (*C. coli*, *C. upsaliensis*, *C. lari*, *C. hominis* and *C. concisus*) were also flagellin proteins. Both flagellin A and flagellin B isomers were identified on the protein profiles and it has been shown that both flagellin monomers are required for a fully active flagella filament (Guerry *et al.* 1991). A previous study which used two dimensional gel electrophoresis to characterise the cell envelope proteins of *C. jejuni*, *C. coli*, and *C. fetus* showed that one or several charge trains, depending upon the species, strain, and type of preparation studied were isolated. They postulated that this heterogeneity in charge on the flagellar subunits was due to posttranslational modifications (Dunn *et al.* 1987; Alm *et al.* 1992). A genetic study on *C. jejuni* has also shown that flagellar antigenic variation was due to post-translational modification rather than differences in the primary amino acid sequence (Alm *et al.* 1992).

It was suspected for some time that *Campylobacter* flagellins were post-tanslationally modified (Logan *et al.* 1989), possibly glycosylated. Although a number of lines of evidence indicated that the flagellin protein was modified with sialic acid (N-acetylneuraminic acid (NANA)) (Linton *et al.* 2000), recent evidence has shown that the glycosyl component of the flagellin glycoprotein consists of pseudaminic acid, an unusual sugar related to sialic acid (Thibault *et al.* 2001; Goon *et al.* 2003). Genes involved in flagellin glycosylation are located in the same gene cluster as the flagellin structural subunits (Fla A and Fla B), potentially defining a flagellin biosynthesis and modification (glycosylation) locus (Linton *et al.* 2002). There is evidence to show that there are two distinct loci involved in *C. jejuni* protein glycosylation: the flagellin modification locus, involved in glycosylation of flagellin subunit proteins, and the *pgl* (for protein glycosylation) gene locus involved in systems of general protein (including flagellin) glycosylation (Linton *et al.* 2002). Studies have demonstrated that flagellin glycosylation is essential for flagella biogenesis in *Campylobacter* species and mutational studies have shown that strains lacking essential enzymes in the glycosylation pathway were non-motile

and non-flagellated, accumulating unglycosylated flagellin intracellularly (Young *et al.* 2002; Goon *et al.* 2003). Studies have shown that proteins can be glycosylated with multiple types of glycan groups. The most heavily glycosylated bacterial proteins described to date are the flagellins from *C. jejuni* and *C. coli* species. Flagellin from *C. jejuni* strain 81-176 has been shown to be glycosylated at 19 serine or threonine residues, and *C. coli* VC167 at approximately 16 sites (Guerry *et al.* 2006). It is thought that the variability in glycans can confer serospecificity among strains and that flagellin glycosylation plays a key role in virulence, likely to be important in primary intestinal adherence and colonisation of the gut (Guerry *et al.* 2006). It is thought that different strains of *Campylobacter* have different degrees of complexity in the genes involved in flagellin modification, which is reflected in the structural variability and serospecificity of the carbohydrate modifications present in flagellins (Logan *et al.* 2002). This is also demonstrated by the two dimensional protein profiles of different species in this study.

Flagellar expression in *Campylobacter* species is known to be subject to both phase and antigenic variation. Phase variation refers to the ability of some *Campylobacter* strains to exibit a bidirectional transition between flagellated and non-flagellated phenotypes. Antigenic variation refers to the ability of strains to reversibly express flagella of different antigenic specificities. Although it is thought that a molecular mechanism including programmed DNA rearrangement exists. Other studies have shown that the antigenic variation in *Campylobacter* species is also likely to be due to post-translational modifications such as glycosylation. Presumably, the ability of *Campylobacter* species to undergo surface changes confers advantages to the pathogen as it encounters multifarious environments. Phase and antigenic variation of surface antigens has also been described for other pathogenic bacteria including antigenic variation in of outer membrane proteins of *Neiseria* and *Borrelia* species, and antigenic variation in *Salmonella typhimurium.*

It is thought that flagellin is also the immunodominant protein recognised during infection, so flagella are clearly important virulence determinants of *Campylobacter* species (Guerry *et al.* 1990).

5.4.5.4. Limitations and Optimisation of 2D-PAGE Method

Although, historically 2D electrophoresis has been the method of choice for the separation of multiple proteins over a wide dynamic range and which has also been a successful tool for the identification of certain other bacterial species, it is clear that this method was unsuitable for use as a tool to identify *Campylobacter* species. It is likely that

this was due to complications arising due to the global glycosylation of *Campylobacter* proteins and the under representation of many cellular and membrane proteins. Proteins known to be problematic when carrying out 2D electrophoresis include low abundance proteins, hydrophobic and very basic proteins as well as very small or very large proteins (Molloy 2000). The method is also relatively labour-intensive, and has a low throughput (O'Connor *et al.* 2000). However, it is possible that if the method was further optimised to overcome these issues a higher level of success might have been achieved.

If time had permitted a plethora of ways to develop and improve the currently used method could have been investigated. The sample preparation step is probably the most important part of the method when carrying out 2D electrophoresis. There are many ways to modify the method to try and improve protein recovery. It was clear that a large amount of method optimisation was required to improve the recovery of certain proteins including proteins in low abundance and non-soluble membrane proteins. However, a number of proteins with pI's outside the pH 4-7 range would not be isolated. There are so many steps involved in the 2D gel electrophoresis technique that it would be impossible to compare and optimise each step in turn. Therefore, some confidence has to be put into the results of previous studies and an educated judgement made as to the best way to carry out the method. Recently there have been vast improvements with the method due to the introduction of new reagents (including chaotropes, surfactants and reducing agents) that allow for better solubilisation and protein separation approaches (Molloy 2000). It is possible that if alternative methods and reagents to those used in this study had been used they might or might not have led to an improvement in the method. Another aspect of the method that could have been optimised is the length of the rehydration, isoelectric focussing and electrophoris steps, along with the amount of current and power to supply to each separation step.

Difficulties with the use of PDQuest software to comparatively analyse two-dimensional gels have also been reported by previous workers with regards to detecting false spots and problems with spot matching (Rabilloud 2000; Rosengren *et al.* 2003).

A comparison between two types of gel stain, SYPRO ruby and Colloidal Coomassie brilliant blue showed that SYPRO ruby detected a slightly larger number of protein spots than Colloidal Coomassie blue. Colloidal Coomassie blue complexes with basic amino acids, such as arginine, tyrosine, lysine, and histidine and is thought to have a detection range of 8-50 ng of protein within a spot. SYPRO ruby is a sensitive ruthenium-based fluorescent dye that binds with basic amino acids including lysine, arginine, and

histidine (Lopez *et al.* 2000).

Horizontal streaking was also a problem along with the low number of proteins resolved on the gels. It is possible that horizontal streaking was occurring due to the use of the reducing agent DTT which is included to break disulphide bonds and is essential for protein solubilisation. However, because the mode of action of this reagent is an equilibrium reaction, loss of the reducing agent due to migration in the pH gradient can lead to reoxidation of the disulphides and contribute to horizontal streaking (Molloy 2000). It has been reported that this problem could be alleviated by increasing the concentration of DTT or use of alternative reducing agents (Rabilloud 2000). Furthermore, it has been suggested that horizontal streaking could be due to the high level of glycosylation of proteins interfering with the iso-electric focussing step. It could have also been due to contamination of the protein extract with interfering substances such as nucleic acids and salt contamination. The presence of nucleic acids such as RNA and DNA in a sample can result in poor focussing in the acidic region of the IEF gel. Therefore, steps to resolve these issues include the use of nucleases to remove nucleic acids.

PMSF (phenylmethylsulfonyl fluoride) was included in the sample preparation buffer to inhibit serine and cysteine proteases, released after cell lysis which can complicate 2D analysis. However, there are also a number of alternative protease inhibitors that may have been more suited. In addition, the introduction of a TCA precipitation step could have been included to purify the protein sample by removing interfering substances (Pridmore *et al.* 1999).

A large amount of optimisation of the method would be required at the sample preparation stage to decrease the streaking and increase the number of proteins resolved. It might have been more useful to focus on the isolation of outer membrane proteins only rather than whole-cell extracts. Studies could have then been carried out to characterise the specificity of the antibodies used in the latex agglutination tests. Further work could have been continued to optimise the method. Furthermore, other *Campylobacteraceae* and non-*Campylobacter* species could have been tested and also evaluation of numerous strain types within a species, as considerable heterogeneity is thought to exist within a species. However, the method is laborious, time consuming and not cost-effective, therefore it was decided to not carry on pursuing this method. The use of whole cell extracts only allows abundant proteins to be identified and therefore the use of pre-fractionation or use of 'zoom in' pH gradients may help in detecting the low copy number proteins and increase the overall proteome coverage achieved.

If time was not a limiting factor during this study, further work would have included continuing to optimise the number of proteins recovered on the 2D gels (i.e. try different pH ranges, deglycosylating enzymes, different methods of sample preparation to isolate more membrane proteins and identification of spots of interest). Work could also include comparisons of protein profiles between strains within a species. It would have been of interest to carry out western blots of the 2D protein profiles. This would have then permitted characterisation of a range of available *Campylobacter* specific polyclonal and monoclonal antibodies and protein spots by immunoblotting, therefore aiding in the development of immunological identification tools such as the latex agglutination test, developed by the collaborating industrial partner (Microgen Bioproducts). In fact preliminary work involving dot blots to optimise antibody and protein concentrations were carried out using a horse radish peroxidase detection method (HRP) but due to time limitations this work could not be completed. It is thought that the isolation and identification of both surface proteins and metabolic enzymes expressed could be complicated by the growth conditions, even though growth conditions were kept as standardised as possible during the experiments carried out. Although the reproducibility of the method from the sample preparation stage was compared, more detailed experiments could have been carried out to investigate the repeatability and reproducibility of the method. Further investigations are required before any conclusions on this work can be drawn. The identification of proteins from *C. jejuni* protein profile only was carried out, therefore it is also necessary to identify the proteins of other species so accurate comparisons between protein expression of different *Campylobacter* species can be drawn.

A 2D electrophoresis approach was used in this study due to its availability, its potential to carry out numerous quantitative inter-sample comparisons, its potential to carry out further down identification of proteins and its use providing previous success when used as an identification tool for other bacterial species. It is possible that with further optimisation of the method, improved results could have been achieved, however due to lack of time this was not possible.

The limitations of the use of 2D electrophoresis have inspired the development of a number of approaches to bypass 2D electrophoresis. However, no one type of proteomic method is thought to be optimal for the isolation and identification of all types of proteins and all approaches are thought to come with inherent disadvantages. An alternative electrophoresis method to the 2D approach is SDS-PAGE coupled with MS/MS, where complex samples are analysed by SDS-PAGE MS. Each band contains several proteins,

thus a list of the proteins present in the starting sample is made on a simplified fraction. Although this application could be very successful if a qualitative list of proteins expressed is required, this approach is not quantitative, yields are low (commonly below 1%) and some peptides, especially the large ones are lost during the extraction stage. Other alternatives include techniques whereby the entire sample is converted to peptides which are then purified before subjecting them to analysis by mass spectrometry. Various methods for peptide purification have been described including liquid chromatography (Link *et al.* 1997), capillary electrophoresis (Figeys *et al.* 1999), or a combination of cation-exchange chromatography or reversed phase chromatography (Coldham and Woodward 2004). Using these methods the limitations of two-dimensional electrophoresis are avoided and so a larger number of proteins can be detected. However, these methods take a large amount of time and computing power to deconvolute the data obtained and a large amount of time and effort can be expended in the analysis of uninteresting proteins (Graves and Haystead 2002). One example includes multi-dimensional protein identification technology (MUDPIT) (Washburn *et al.* 2001). Here, the total protein mixture is digested, sometimes by several enzymes, and the total digest is loaded on a multidimensional peptide chromatographic separation, interfaced in line with an MS/MS spectrometer. Using this method an extremely high proportion of the proteome representing all protein classes including minor proteins can be identified, in contrast to two-dimensional electrophoresis. However, the MUDPIT approach is far from simple to carry out and quantitative data are not available using this approach, which provides only a raw list of proteins in each sample. An approach which does allow quantitative inter-sample comparison is another MS approach, isotope coded affinity tags (ICAT) (Gygi *et al.* 1999). Here, two protein samples are compared which are differentially labelled (at cysteine residues) with probes of different mass and colour. The extracts are then pooled and digitised with trypsin and analysed by capillary electrophoresis coupled with mass spectrometry. However, this method is limited to the detection of proteins containing cystein residues and peptide losses during peptide analysis are not uncommon (Rabilloud 2002).

Unfortunately, membrane proteins are generally difficult to characterise due to their poor solubility during IEF and poor transfer into the second dimension (Molloy 2000) when whole-cell extracts are used. It is likely that the use of membrane-enriched extracts for the comparative analysis of the different species may have performed better. This approach would then have enabled further characterisation of antibodies (likely to be

specific for outer membrane proteins) and therefore aid in the development of immunological identification methods and also aid in the understanding of host-cell response.

Alternative protein separation methods for the identification of bacterial species include the use of a gel-free method called capillary isoelectric focussing electrophoresis/electrospray ionisation MS (CIEF-ESI-MS), which is analogous to the 2D gel electrophoresis in terms of the separation principles used. The advantage of this method is that it is extremely sensitive as it does not rely on silver or fluorescent stains to visualise proteins as the mass spectrometer itself identifies the proteins (O'Connor *et al.* 2000). Other proteomic methods which have been used for the identification of bacterial species include an alternative gel-free method called surface-enhanced laser desorption/ionisation mass spectrometry (SELDI-MS), often referred to a protein chip technology. Other technical advances involve the production of microarrays using antibodies, peptides or synthetic compounds (Herbert *et al.* 2001).

To date proteomic methods that have been routinely used to identify *Campylobacter* species are one-dimensional electrophoresis, however this method is not always successful at discriminating between closely related species. Another method that has been successfully applied to the identification of *Campylobacter* species is matrix assisted laser desorption/ionisation time of flight mass spectrometry (Mandrell *et al.* 2005). This method has been used to identify biomarker ions in the form of peptide fragments on mass spectra. The disadvantage of this method is that that it merely a comparative method and further characterisation and identification of proteins can not be carried out.

CHAPTER 6

ISOLATION OF CA*MPYLOBACTERACEAE* FROM SEWAGE SLUDGE AND SALAD VEGETABLES

6.1. INTRODUCTION

Fresh fruit and vegetable consumption has increased dramatically over recent years and is now seen as an essential part of a balanced diet. Increasingly, fresh fruit and vegetables are washed and packaged by the food industry and sold in a 'ready-to-eat' format, so the microbiological safety of such foods is very important (Buck *et al.* 2003). The potential source of contamination of these vegetables is likely to stem from insufficiently treated recycled wastewater used as fertiliser, run-off from nearby fields containing contaminated cattle faeces, from contact with wild birds and other animals, or even if handled by an infected person (during harvesting, preparation or packaging) (Jones and Heaton 2006). Watercress may pose a significantly greater risk than other common vegetables as it grows in waterways subject to waterborne contaminants (Edmonds and Hawke 2004).

Although, salad vegetables are often reported to be linked to outbreaks, very few studies have been able of recover *Campylobacter* species from this source (Evans *et al.* 2003). Studies in the literature have shown that cultural procedures used to isolate *Campylobacter* species from salad vegetables have been those solely developed for isolation of thermophilic *Campylobacter* species, using highly selective media and sub-optimal incubation conditions and, to date, *C. jejuni* is the only known *Campylobacteraceae* species to be isolated from salad vegetables. Therefore, this study aimed to investigate the prevalence of *Campylobacteraceae* on a range of salad vegetables when alternative cultural methods suitable for the isolation of all *Campylobacteraceae* are used.

The processing of human faeces as sewage is an important requisite for modern life in an urban environment of high population density. Waste water can contain a range of microbial pathogens, especially if not treated properly. It possesses a potential health risk due to its use as a fertiliser, which when recycled back to land could allow the potential ingress of bacteria from soil to crops, the farmer or cattle (Jones 2001b; Sahlstrom *et al.* 2004). These samples not only indirectly present a potential health risk, but also represent the overall health of the city and surrounding area (Jones *et al.* 1990a). Both unprocessed and processed (anaerobically digested) sewage has previously been shown to contain high numbers of *Campylobacter* and *Arcobacter* species and therefore digested sludge carries a high infection risk since it is recycled to land for use as fertiliser (Stampi *et al.* 1999). Generally, as described previously, culture methods used for the isolation of *Campylobacteraceae* from sewage have included use of selective media and incubation

atmospheres developed for thermophilic species only (Dousse *et al.* 1993).

During this part of the study, some of the cultural conditions (including incubation atmosphere, basal media and selective supplements) and identification tools that were optimised in previous studies using reference cultures were applied to recovery of *Campylobacteraceae* species from salad vegetables and sewage wastewater.

6.2. METHODS

A number of isolation methods for the recovery of *Campylobacteraceae* species from sewage and salad vegetable samples were investigated. These included development of a presence/absence method which included a 24 h broth enrichment step. A quantitative method also needed to be developed as enumeration data were required for statisticians at RIVM (Dutch National Institute for Public Health and the Environment) to formulate a Risk Assessment model for emerging *Campylobacter* species. Therefore, a vacuum filtration method was evaluated as it was thought that it would provide higher sensitivity as bigger volumes could be filtered (10 ml). Evaluation of a filter pad enrichment step was also investigated, as it was thought it would allow resuscitation of the sample and permit sample enumeration. Experiments also included the evaluation of ABA medium supplemented with the newly formulated VAT selective supplement (see Chapter 3). The methods evaluated in these experiments incorporated the novel plating medium and incubation atmosphere previously developed based on the results gained from the optimisation experiments carried out with the CAMPYCHECK reference strains using pure culture.

6.3. RESULTS

6.3.1. Method Optimisation

6.3.1.1. Salad Vegetables

6.3.1.1.1. Comparison of Recovery using a Presence/Absence Method with and without VAT Selective Supplements

Experiments were carried out to compare the recovery of *Campylobacter* species from watercress samples (spiked and unspiked) after enrichment in *Campylobacter* enrichment broth (CEB) with and without VAT selective supplement. Subsequently, the enriched samples were filtered (using a 0.6 μm pore size membrane as used as part of the Cape Town protocol) or directly plated onto ABA plates with and without VAT selective supplement (Table 32).

Results showed that when VAT selective supplement was included in the enrichment medium, there was no difference in recovery of *Campylobacteraceae* or the level of background flora suppressed, compared to when when CEB without selection was used (Table 32). When VAT selective supplement was included in the ABA plating medium (with or without VAT supplement included in the enrcichment broth), it reduced, but did not completely suppress the growth of non-*Campylobacteraceae* colonies. When the enriched samples were plated directly using the 10^{-1} and 10^{-2} serial dilutions onto ABA VAT, plates were overgrown with background flora (using both processed and unprocessed watercress). Direct plating of the 10^{-3} and 10^{-4} serial dilutions of enriched sample suppressed the majority of background flora and, in addition, recoveries of *Campylobacteraceae* colonies remained high. When the enriched samples were filtered (using a 0.6 μm membrane) onto ABA (with and without VAT), the levels of *Campylobacteraceae* were reduced by approximately $\log_{10}$ 2-3 cfu/25 g compared to when plating directly, although background was also reduced. Filtering the enriched sample through a 0.6 μm membrane onto ABA VAT using 10^{-3} and 10^{-4} dilutions did not recover any *Campylobacteraceae* colonies; however filtering the 10^{-1} and 10^{-2} dilutions onto ABA VAT permitted the recovery of high levels of CLM colonies. Following enrichment in *Campylobacter* enrichment broth (CEB) (without VAT), the highest level of background contamination was suppressed with the highest level of *Campylobacteraceae* recovered, when a combination of both filtration (using a 0.6 μm pore size membrane) of 10^{-1} and 10^{-2} dilutions onto ABA VAT and direct plating of 10^{-3} and 10^{-4} dilutions were used. Plates were completely overgrown with background flora when the enriched sample (in CEB with and without VAT selective supplement) was plated onto ABA without selection by filtration or VAT selective supplement (Table 32).

6.3.1.1.2. Evaluation of a Centrifugation Clarification Step

It was thought that the large amount of particulate matter contained in the enriched homogenated samples could be leading to clogging of the 0.6 μm membrane during filtration, preventing the passage of bacteria though the pores of the filter membrane, and contributing to the reduced level of sensitivity observed compared to direct plating (loss of $\log_{10}$ 2-3 cfu/25g). Therefore, including a low speed centrifugation step to clarify the enriched sample homogenate was evaluated. The results showed that the centrifugation step increased the number of *Campylobacter* recovered when using filtration, in fact *C. hominis* could not be recovered when using filtration if the centrifugation step was not

Table 32. Evaluation of VAT Selective Supplement for Recovery of *Campylobacteraceae* from Watercress Using an Enrichment Method. Both *Campylobacter* Enrichment Broth and ABA plating medium were evaluated with and without VAT selective supplement and were inoculated directly onto the plate or filtered though a 0.6 µm pore size membrane. Processed watercress was spiked with three *Campylobacteraceae* spp. at approximately $\log_{10}$ 4 cfu/25 g. Results expressed as $\log_{10}$ cfu (with CLM)/25 g, ± SEM (using the geometric mean of three replicate experiments). Five CLM colonies per plate were confirmed as *Campylobacteraceae* using the tests described in the method. CONT = contamination (plates overgrown with background flora).

Sample and spike	Plating technique	CEB (No selection)		CEB (with VAT)	
		ABA	ABA VAT	ABA	ABA VAT
Unprocessed (Control)	Direct	CONT	4.12 (±0.21)	CONT	3.93 (±0.56)
	Filtered	1.20 (±0.09)	2.02 (±0.14)	1.55 (±0.86)	1.28 (±0.34)
Processed (Control)	Direct	CONT	2.32 (±0.07)	CONT	2.12 (±0.10)
	Filtered	0	0	0	0
C. jejuni	Direct	CONT	6.63 (±0.18)	CONT	6.21 (±0.24)
	Filtered	3.31 (±0.39)	3.66 (±0.27)	3.12 (±0.41)	3.29 (±0.90)
C. concisus	Direct	CONT	5.44 (±0.3)	CONT	5.83 (±0.71)
	Filtered	3.33 (±0.61)	3.71 (±0.12)	3.22 (±0.56)	3.40 (±0.30)
C. hominis	Direct	CONT	4.03 (±0.44)	CONT	4.49 (±0.89)
	Filtered	2.09 (±0.16)	2.62 (±0.11)	2.22 (±0.04)	2.36 (±0.25)

Table 33. Evaluation of a Low Speed Centrifugation Step to Clarify the Watercress Sample After Enrichment. Results expressed as $\log_{10}$ cfu (CLM)/25 g ± SEM (using the geometric mean of three replicate experiments). Five CLM colonies per plate were confirmed as *Campylobacteraceae* using the tests described in the method.

Spike	Clarification step included	ABA		ABA VAT	
		Direct	Filter	Direct	Filter
C. jejuni	Centrifugation	CONT	3.54 (±0.16)	6.23 (±0.96)	3.71 (±0.16)
	No centrifugation	CONT	CONT	6.31 (±0.86)	2.82 (±0.26)
C. concisus	Centrifugation	CONT	3.91 (±0.53)	6.02 (±1.16)	3.72 (±0.08)
	No centrifugation	CONT	3.01 (±0.33)	6.60 (±0.27)	2.95 (±0.26)
C. hominis	Centrifugation	CONT	2.23 (±0.18)	4.06 (±0.42)	2.11 (±0.11)
	No centrifugation	CONT	0	4.13 (±0.57)	0

included (Table 33). Furthermore, the number of *Campylobacter* species recovered was not affected by centrifugation, as demonstrated by similar recovery rates from samples that were directly plated with and without a centrifugation step.

6.3.1.1.3. Development of a Quantitative Enumeration Method

A quantitative approach to enumerate *Campylobacter* recovery from salad vegetables was required as it was necessary to generate enumeration data so risk assessment models could be developed by RIVM collaborators. A number of approaches including direct plating and vacuum filtering onto a 0.1 μm pore size membrane were investigated. The advantage of vacuum filtering onto a 0.1 μm pore size membrane is that counts remain quantitative after filter pad enrichment. The method is also theoretically more sensitive as a higher sample volume can be analysed. The enumeration methods were directly compared to a presence/absence method.

Results showed that when the sample was vacuum filtered onto a 0.1 μm membrane, the volume was restricted to 10 ml, as filtering larger volumes quickly clogged the membrane and prevented the flow-though of larger volumes of the sample filtrate, although this was less of a problem at higher dilutions. There was no advantage of including a filter pad resuscitation step (with filter pad soaked in *Campylobacter* enrichment broth) compared to placing the 0.1 μm pore size membrane directly onto the ABA VAT for 48 h.

When direct plating of 200 μl of sample onto ABA VAT was compared to vacuum filtration of 10 ml sample onto a 0.1 μm pore size membrane and placing the membrane onto ABA VAT, direct plating was the most successful method in terms of recovery and reduction of background flora (Table 34). Furthermore, it was difficult to pick colonies with CLM, as *Campylobacter* colonies showed atypical morphology on the membrane surface. Furthermore, presumptive isolates had reduced viability and often died off when sub-cultured, were more prone to contamination on further subculture, and a higher number of colonies that were picked were subsequently identified as non-*Campylobacteraceae.*

The choice of appropriate serial dilutions to be plated was an important factor, as at the lower dilutions of 10^{-1} and 10^{-2}, the background contaminants were too high to obtain isolated *Campylobacteraceae* colonies suitable for generating accurate counts (despite the use of VAT selective supplement). Plating out dilutions of 10^{-3} and 10^{-4} allowed accurate counts of CLM colonies with the presence of only minimal background flora. However, the fact that the use of higher dilutions was required meant

Table 34. Evaluation of a Vacuum Filtration Method Compared to other Methods for Recovery of *Campylobacter* from Watercress. Recovery of *Campylobacteraceae* was assessed using a vacuum filtration method where the sample was captured by vacuum filtering onto a 0.1 μm membrane. This was compared to a direct plating method and filtering though a 0.6 μm membrane. Furthermore these experiments were run alongside a Presence/Absence enrichment method. VAT selective supplement was evaluated for each of the methods. Processed watercress was spiked with three *Campylobacteraceae* spp. at approximately $\log_{10}$ 4 cfu/25 g. CONT = contamination (plates overgrown with background flora). Results expressed as $\log_{10}$ cfu (CLM)/25 g ± SEM (using the geometric mean of three replicate experiments). Five CLM colonies per plate were confirmed as *Campylobacteraceae* using the tests described in the method.

	Enumeration Method					
	Vacuum Filtered		Direct Plating		0.6 μm filtration	
	No VAT	VAT	No VAT	VAT	No VAT	VAT
Unspiked (Processed)	CONT	0.65 (±0.19)	2.51 (±0.40)	2.47 (±0.16)	0	0
Unspiked (unprocessed)	CONT	TCTC	3.55 (±0.42)	3.66 (±0.42)	1.01 (±0.43)	1.37 (±0.06)
C. jejuni	CONT	0.58 (±0.06)	TNTC	TNTC	3.67 (±0.17)	3.74 (±0.43)
C. concisus	CONT	0.71 (±0.06)	5.55 (±0.31)	5.50 (±0.89)	2.52 (±0.52)	2.43 (±0.52)
C. hominis	CONT	0.85 (±0.04)	5.79 (±0.48)	5.49 (±0.52)	1.72 (±0.11)	1.56 (±0.09)

	24 h enrichment (presence/absence)			
	Direct plated		0.6 μm filtration	
	No VAT	VAT	No VAT	VAT
Unspiked (Processed)	CONT	3.86 (±0.17)	0 (0)	0 (0)
Unspiked (unprocessed)	CONT	5.64 (±0.75)	2.10 (±0.59)	2.65 (+0.26)
C. jejuni (Processed)	CONT	6.99 (±0.84)	3.30 (±0.52)	3.08 (±0.49)
C. concisus (Processed)	CONT	6.00 (±0.78)	3.77 (±0.18)	3.72 (±0.57
C. hominis (Processed)	CONT	5.32 (±0.80)	2.78 (±0.36)	2.44 (±0.35)

that the sensitivity of the method was reduced. Plating of the homogenised sample (without enrichment) though a 0.6 μm pore size membrane permitted counts at 10^{-1} and 10^{-2} dilutions, as background flora was reduced. However, this also reduced the sensitivity of recovery as the numbers of *Campylobacteraceae* were also reduced (Table 34).

6.3.1.2. Sewage Sludge Method Evaluation

Methods for the isolation of *Campylobacteraceae* from raw and digested sewage using direct plating (quantitative enumeration) and enrichment (presence/absence) methods were evaluated. During method optimisation, the samples to be spiked for method optimisation experiments were first pasteurised at 70°C for 1 h to reduce the number of background *Campylobacteraceae* species and increase the accuracy of enumeration by reducing the chance of counting bacteria which were not spiked. Pasteurising the sample was preferred to autoclaving so the changes to the properties of the sample matrix were minimised. A low speed centrifugation step was included after sample enrichment before serial dilutions were created. The results (data not shown) showed that including this clarification step increased the number of *Campylobacteraceae* recovered by approximately $\log_{10}$ 2 cfu/g when filtration through a 0.6 μm pore size membrane was used; this was thought to be due to the reduction in particulate matter leading to clogging of membrane pores. Furthermore, recovery of *Campylobacter* species when directly plating was not affected by the centrifugation step.

Evaluation of a vacuum filtration method was investigated to increase the sensitivity of the method and allow quantitative enumeration. The results showed that there was no difference in recovery when the vacuum filtered homogenised sample (i.e. cells captured on a 0.1 μm membrane) was placed directly on a VAT plate compared to when a filter pad resuscitation step (24 h) was used. Not including VAT in the plating medium resulted in plates which were highly contaminated with background flora (Table 35A and Figure 26).

Similar numbers of *Campylobacteraceae* were recovered when the samples were plated directly onto ABA VAT and when the vacuum filtration capture method was used (Table 35A and B). However, the level of background contamination was slightly higher, colonies appeared atypical on the membrane and the isolates recovered were less viable, when using the vacuum filtration method. The most suitable quantitative method, which cut back background flora and recovered optimal numbers of *Campylobacteraceae* was when plating out 200 μl of the 10^{-3} and 10^{-4} dilutions. *Campylobacteraceae* could also be

Table 35. Evaluation of Isolation Methods for the Recovery of *Campylobacter* Species from Sewage Sludge. Pasteurised MAD sewage sludge was spiked with three *Campylobacteraceae* spp. at approximately $\log_{10}$ 4 cfu/25 g. Results expressed as $\log_{10}$ cfu (CLM)/25 g ± SEM (using the geometric mean of three replicate experiments). Five colonies with CLM per plate were picked and subsequently identified as presumptive *Campylobacteraceae*. TCTC = too contaminated to count.

A.

Sample type (Spike level)	Vacuum Filtration			
	24 hr enrichment of 0.1 µm membrane on filter pad then transfer to ABA		Placement of 0.1 µm membrane onto ABA plate	
	No VAT	VAT	No VAT	VAT
Control (MAD sludge)	Lawn	3.66 (±0.46)	Lawn	4.43 (±0.52)
Pasteurised MAD sludge	TCTC	1.76 (± 0.07)	TCTC	1.48 (±0.06)
C. jejuni ($\log_{10}$ 4 cfu/25g)	TCTC	4.35 (±0.15)	TCTC	4.36 (±0.12)
C. concisus ($\log_{10}$ 5 cfu/25g)	TCTC	4.52 (±0.25)	TCTC	4.37 (±0.65)
C. hominis ($\log_{10}$ 5 cfu/25g)	TCTC	4.17 (±0.16)	TCTC	4.20 (±0.31)

B.

Sample type (Spike level)	Direct plating (no enrichment)			
	Direct plated		0.6 µm filtration	
	No VAT	VAT	No VAT	VAT
Control (MAD sludge)	4.48 (± 0.27)	4.02 (±0.67)	2.78 (±0.76)	2.56 (±0.64)
Pasteurised MAD sludge	2.03 (± 0.29)	2.30 (±0.49)	0	0
C. jejuni ($\log_{10}$ 5 cfu/25g)	4.42 (± 0.45)	4.39 (±0.54)	2.85 (±0.16)	2.67 (±0.07)
C. concisus ($\log_{10}$ 5 cfu/25g)	4.33 (± 0.23)	4.29 (±0.13)	2.61 (±0.76)	2.37 (±0.12)
C. hominis ($\log_{10}$ 5 cfu/25g)	3.92 (± 0.52)	3.95 (±0.36)	0	0

C.

Sample type (Spike level)	24 h Enrichment (Presence/absence)			
	Directly plated		0.6 µm filtration	
	ABA	ABAVAT	ABA	ABAVAT
Control (MAD sludge)	TCTC	TCTC	TCTC	4.70 (±0.25)
Pasteurised MAD sludge	TCTC	TCTC	TCTC	1.70 (±0.06)
C. jejuni ($\log_{10}$ 5 cfu/25g)	TCTC	TCTC	TCTC	5.30 (±0.14)
C. concisus ($\log_{10}$ 5 cfu/25g)	TCTC	TCTC	TCTC	5.24 (±0.20)
C. hominis ($\log_{10}$ 5 cfu/25g)	TCTC	TCTC	TCTC	3.65 (±0.06)

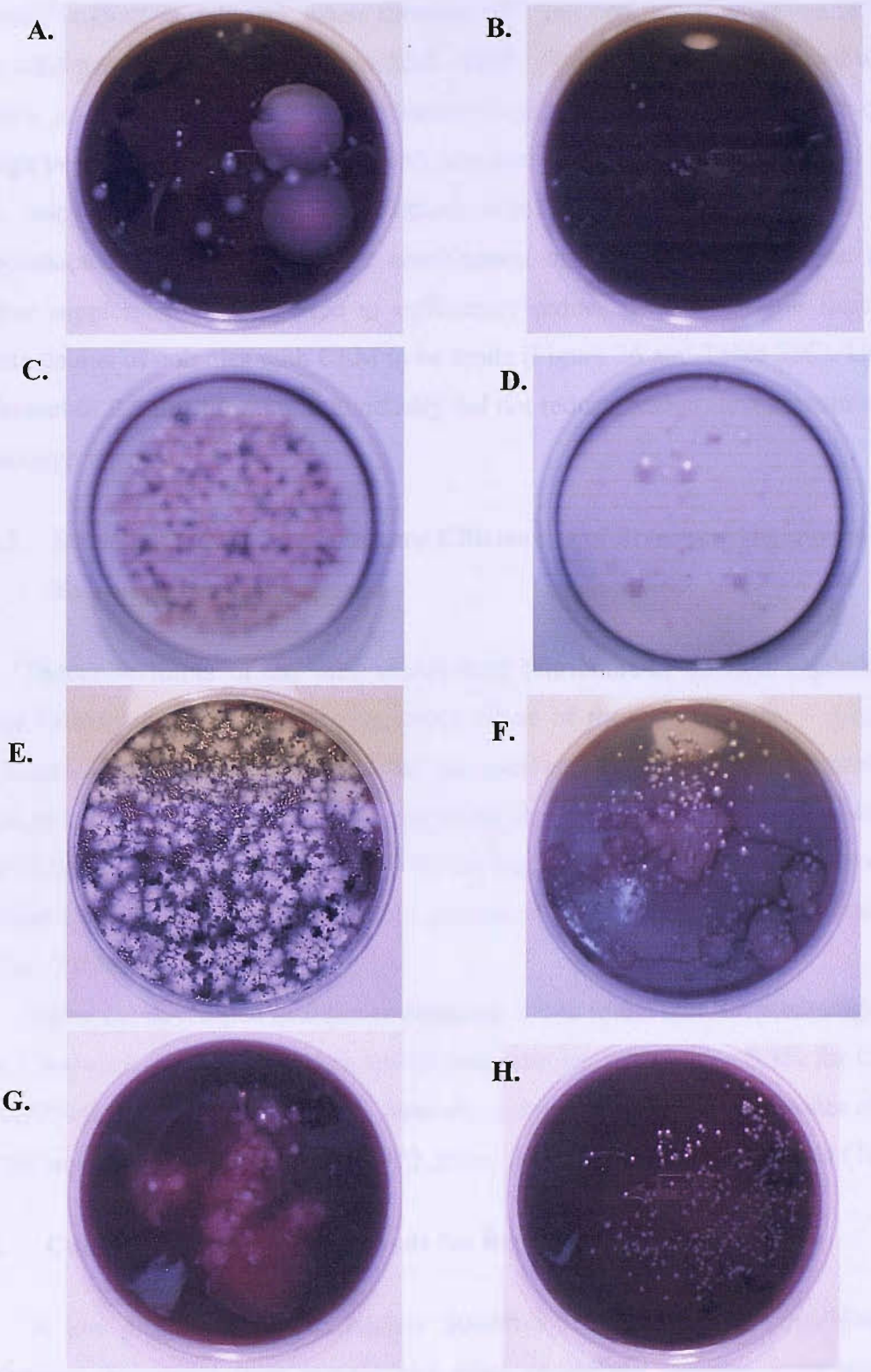


Figure 26. Method Comparison of Recovery of *Campylobacteraceae* species from Sewage Sludge. A. Direct Enumeration (10^{-3}) (no VAT); B. Direct Enumeration (with VAT (10^{-3}); C. Vacuum filtration Enumeration (no VAT (10^{-4}); D. Vacuum filtration Enumeration (with VAT (10^{-4}); E. ABA without VAT or filtration; F. ABA with VAT (no filtration); G. ABA with filtration, no VAT; H. ABA with filter and VAT. In E-H, samples were enriched in CEB.

recovered without enrichment when filtering 10^{-1} and 10^{-2} serial dilutions of a sample (using a 0.6 pore size membrane) onto ABA VAT (Table 35B). This method was highly selective, completely reducing the presence of contaminating flora on most occasions, although this method was also considerably less sensitive.

When using the presence/absence method, after enriching the sample for 24 h in *Campylobacter* Enrichment Broth, a combination of both 0.6 μm filtration and VAT selective supplement was required to sufficiently reduce the background flora to allow accurate counts of colonies with CLM to be made (Figure 26 and Table 35C). Using VAT supplement or 0.6 μm filtration individually did not reduce background contamination to a high enough level.

6.3.1.3. Detection Limits and Recovery Efficiencies of Presence/Absence and Enumeration Method

Detection limits of the presence/absence (enrichment) method depended on the type of *Campylobacter* species being tested. None of the *Campylobacter* species tested were detected at $\log_{10}$ 2 cfu/25g, therefore the numbers of *Campylobacter* species must be present at $\log_{10}$ 3 and above for detection using this isolation method. The number of *C. jejuni* could be detected at the lowest levels (at $\log_{10}$ 3 cfu/25g and above) on both sewage and salad samples. *C. hominis* had to be present at $\log_{10}$ 4 cfu/25g before detection was possible (Table 36).

Using the developed quantitative method, it was found that the percentage recovery of the *Campylobacter* species when spiked into samples varied from 8.3% for *C. jejuni* to only 0.72% for the more fastidious *C. hominis*. *A. butzleri* had the highest rates of recovery with the isolation protocol recovering >33.2% of the inoculum initially spiked (Table 37).

6.3.2. Culture Confirmatory Methods for Recovered Sample Isolates

A low proportion (approximately 20-40%) of isolates with *Campylobacter*-like morphology, recovered from sewage and salad vegetabele samples, were subsequently identified as *Campylobacteraceae* using the KOH and L-ala tests as an initial pre-screen. All of the isolates that were positive using the KOH test and negative for L-alanine aminopeptidase were all subsequently identified as *Campylobacter* or *Arcobacter* species using PCR analysis. All of these presumptive *Campylobacteraceae* isolates also displayed *Campylobacter*-like cellular morphology (observed using EDIC microscopy) and were oxidase positive. Using the CAMPYCHECK strains as a reference, the cellular

Table 36. Detection Limits of the Presence/Absence Method for Salad Vegetables and Sewage. Y =detected; N = Not detected.

		Vegetables (processed)		Sewage Sludge (MAD, pasteurised)	
Spp.	Spike level (cfu per 25 g)	Detected	Detection Limit	Detected	Detection Limit
C. jejuni	530000	Y		Y	
	53000	Y		Y	
	5300	Y		Y	$>\log_{10}$ 3 cfu/25 g
	530	Y/N	$>\log_{10}$ 2-3 cfu/25 g	N	
	53	N		N	
C. concisus	250000	Y		Y	
	25000	Y		Y	$>\log_{10}$ 4 cfu/25 g
	2500	Y/N	$>\log_{10}$ 3-4 cfu/25 g	N	
	250	N		N	
	25	N		N	
C. hominis	110000	Y		Y	
	11000	Y	$>\log_{10}$ 4 cfu/25 g	Y	$>\log_{10}$ 4 cfu/25 g
	1100	N		N	
	110	N		N	
	11	N		N	

Table 37. Mean % Recovery Efficiencies from the spiked Enumeration Data. (Recovery Efficiency = (cfu/25 g recovered) – ((cfu/25 g on recovered on unspiked sample) / (cfu/25g spiked) x100). Samples were spiked with positive control species at inoculums ca $\log_{10}$ 5 cfu/25 g. The enumeration counts were adjusted so only colonies that were subsequently identified as *Campylobacteraceae* were counted.

Spp.	Sewage Sludge	Salad Vegetables
C. jejuni	7.14%	8.30%
C. concisus	5.8%	8.30%
C. hominis	0.72%	0.83%
A. butzleri	43.4%	33.2%

morphology of isolates recovered from salad vegetables and sewage samples could be compared. The results revealed that the majority of the presumptive *Campylobacteraceae* isolates that had been pre-screened using the KOH/L-ala test resembled those of the CAMPYCHECK reference strains, being of similar size and either curved or spiral shaped rods (Appendix Table I and Table II). Isolates that were clearly not *Campylobacteraceae* or culture isolates that were not pure could be easily recognised by cell size and shape. The majority of isolates that were confirmed using PCR to be *Arcobacter* species grew aerobically at 25°C, however, 10% of these *Arcobacter* isolates failed to grow in these conditions. This could be attributed to certain strains having a lowered ability to grow aerobically, especially if they were sub-lethally stressed. The fact that this test could falsely identify *Arcobacter* species as *Campylobacter* species is concerning since many routine testing laboratories use this test as a means of differentiating these two species.

The thermotolerant latex test correctly detected all three isolates subsequently identified as *C. jejuni* species. However, the reagent also cross reacted with 20% of the isolates subsequently identified as *Arcobacter* species. This re-emphasises the requirement for molecular confirmation of species. The *Campylobacteraceae* latex reagent was able to detect 87% of the *Arcobacter* isolates (87 out of 100 isolates) and 89% (16 out of 18 isolates) of *Campylobacter* isolates. The lack of sensitivity of detection can be largely attributed to non-specific agglutination in which there was agglutination even with the control latex reagent which did not include antibody. However, the latex agglutination test was very useful at discriminating between isolates with 77% of presumptive *Campylobacteraceae* being accurately grouped in either the thermotolerant or non-thermotolerant (mostly *Arcobacter* species) groups.

The thermotolerant PNA probe used in conjunction with Fluorescence FISH detected all three *C. jejuni* isolates. However, it also reacted with 75% (9 out of 12) of non-*jejuni/coli Campylobacter* isolates and non-specifically with 15% (15 out of 100) of the isolates subsequently identified as *Arcobacter* species.

All of the presumptive *Campylobacteraceae* isolates (118 isolates in total) sent to collaborators at Ghent University, Belgium, for confirmatory molecular identification using multiplex PCR were identified as either *Arcobacter* or *Campylobacter* species. The isolates comprised mainly of arcobacters with a predominance of *A. butzleri* and some *A. cryaerophilus* isolates. No *A. skirrowii* or *A. cibarius* were identified. A minority of the isolates (n=18) were identified as campylobacters. Three of these were subsequently identified as *C. jejuni/coli* using the multiplex PCR test. The remaining fifteen isolates did

Table 38. Summary of Identification Tests Carried Out on Isolates Recovered from Sewage Sludge and Salad Vegetables. The performance of identification tests was compared to final identification of isolates using PCR analysis. Using PCR identification Isolates were grouped into *Arcobacter*, Non-*jejuni/coli* isolates, *C. jejuni* species or *Campylobacter* species (see Appendics Table I and Table II for ID profile of each isolate).

Final Molecular ID (PCR)	Number of isolates	KOH	L-ALA	EDIC Microscopy	Oxidase	Aerobic growth at RT	Thermotolerant latex	*Campylobacteraceae* Latex	Thermotolerant PNA Probe
***Arcobacter* spp.**	**100**	100 (100%)	0 (0%)	100 (100%)	100 (100%)	90 (90%)	20 (20%)	87 (87%)	15 (15%)
Non-*jejuni/coli* spp.	**12**	12 (100%)	0 (0%)	12 (100%)	12 (100%)	0 (0%)	4 (33.3%)	11 (91.7%)	9 (75%)
C. jejuni/coli	**3**	3 (100%)	0 (0%)	3 (100%)	3 (100%)	0 (0%)	2 (66.7%)	2 (66.7%)	3 (100%)
***Campylobacter* spp.**	**3**	3 (100%)	0 (0%)	3 (100%)	3 (100%)	0 (0%)	3 (100%)	3 (100%)	3 (100%)
Total	**118**	118	0	118	118	90	29	103	30

SampleType		***Campylobacteraceae* Recovered (Confirmatory ID by PCR)**
Salad Vegetables	Unprocessed	*A. butzleri*, Non-*jejuni/coli Campylobacter* spp.,
	Processed	*A. butzleri*, Non-*jejuni/coli Campylobacter* spp., *C. jejuni/coli*
Sewage waste	Raw	*A. butzleri*, *A. cryaerophilus*, Non-*jejuni/coli Campylobacter* spp., *C. jejuni*
	Digested	*A. butzleri*, *A. cryaerophilus*, Non-*jejuni/coli Campylobacter* spp.

Table 39. Identification of Non-*Campylobacteraceae* Isolates Recovered on ABA VAT Using the Microgen GN-ID Biochemical Identification Kit. Isolates were recovered from both sewage and salad vegetable samples using the direct plating enumeration method and presence/absence enrichment method.

Identity of Competitor Species Recovered	Colony Description on ABA VAT	Sample Source	Prevalence in sample
Enterobacter agglomerans	Large yellow colony, (~5 mm).	Salad vegetables and sewage (direct and enriched).	High
Aeromonas hydrophila	*Campylobacter*-like morphology but slightly bigger/browner (~1 mm).	Salad vegetables and sewage (direct and enriched).	High
Pseudomonas fluorescens	Brown spreading/swarming across plate.	Salad vegetables and sewage (enriched, usually on non-filtered plates).	Medium
Proteus mirabilis	Yellow/brown spreading/swarming across plate.	Salad and sewage samples (enriched, usually on non-filtered plates).	Medium
Salmonella spp.	Silver/brown thick shiny colony (~2-3 mm).	Salad vegetables (enriched).	Low
Hafnia alvei	*Campylobacter*-like morphology but slightly bigger/browner (~1 mm).	Salad vegetables (enriched).	Low
Pasteurella multocida	*Campylobacter*-like morphology but slightly bigger/browner (~1 mm).	Sewage (direct and enriched).	Low
Aeromonas caviae	Yellow, silver, grey (~2 mm).	Salad vegetables (enriched).	Low

not yield the expected *C. jejuni* or *C. coli* amplicons and therefore represent other *Campylobacter* species.

6.3.2.1. Biochemical Identification of Recovered Non-*Campylobacteraceae*

Despite the use of VAT as a selective supplement to reduce growth of background flora, a number of contaminants were still recovered on the ABA VAT plates, especially on enriched samples. Most of the background contaminants present on the medium could be easily differentiated from colonies with *Campylobacter*-like morphology, and were present at very low levels compared to those with *Campylobacter*-like morphology. However, there were a number of non-*Campylobacteraceae* bacteria that resembled *Campylobacteraceae* when recovered on primary isolation plates. Some of the most common contaminants were identified by a range of biochemical tests using the commercially available GN-ID test (Microgen Bioproducts; Table 39). The most prevalent of the background flora was *Enterobacter agglomerans* and *Aeromonas hydrophila* which were found on sewage and salad vegetable samples that had been enriched and directly plated. Species that were mainly isolated from samples that had been enriched included *Pseudomonas fluorescens* and *Proteus mirabilis.* Isolates recovered on enriched salad vegetable samples only included *Salmonella* species, *Hafnia alvei* and *Aeromonas caviae.* Enriched sewage samples often recovered *Pasteurella multocida.* The species which caused problems included *Aeromonas hydrophilia, Pasteurella multocida* and *Hafnia alvei* which, on occasions resembled *Campylobacter* colonies on primary isolation plates.

6.3.3. Recovery from Sewage and Salad Vegetables using Optimised Methods

6.3.3.1. Recovery of *Campylobacteraceae* from Salad Vegetables

A small survey trial was carried out to assess the prevalence of *Campylobacteraceae* species on a number of leafy salad vegetables using the previously optimised methods. Initially, only the presence/absence method was used, but further into the study a direct plating enumeration method was carried out alongside the presence/absence method. Although a range of baby leaf salads were analysed, the majority of samples evaluated were watercress samples (Table 40 and Table 41).

Using the presence/absence method, the prevalence of *Campylobacteraceae* species on unprocessed salad vegetables was 57% (8 out of 14 samples). All of these samples contained *A. butzleri* and, in addition, a *C. jejuni/coli* isolate was recovered on one sample. The prevalence of *Campylobacteraceae* on processed samples was 50% (7 out of 14 samples), of which *A. butzleri* was recovered from all of these samples. In addition, an isolate identified as *C. jejuni/coli* was recovered from one of the processed watercress samples and a non-*jejuni/coli* species present on another sample.

Enumeration data (Table 41) showed that CLM cfu counts of unprocessed samples were approximately $\log_{10}$ 5 cfu/ 25 g and in processed samples cfu counts were slightly lower at approximately $\log_{10}$ 4 cfu/ 25 g, but in may cases there were no differences in CLM cfu counts between the unprocessed and processed samples. These counts severely overestimated the true number of *Campylobacteraceae* species on the sample, since only a low proportion of these were subsequently identified as presumptive *Campylobacteraceae* species and further confirmed using PCR identification. A number of both processed and unprocessed samples analysed in January showed high CLM counts of between $\log_{10}$ 4-5 cfu/25 g, however, none of the colonies counted were subsequently identified as *Campylobacteraceae* species.

Table 40. Presence/Absence Sampling Data for Watercress and other Salad Vegetables. Method included 24 h enrichment followed by plating onto ABA VAT.

Date isolated	Sample Type	Pro/Un	Sample source, location	Presence in 25g	Presence confirmed by PCR
17/08/2005	Watercress	U	Alresford, Hampshire, UK (Vitacress)	Y	*A. butzleri*
17/08/2005	Watercress	U	local green grocers, Hampshire, UK	Y	*A. butzleri*
17/08/2005	Watercress	P	Alresford, Hampshire, UK (Vitacress)	Y	*A. butzleri*
10/09/2005	Watercress	U	Alresford, Hampshire, UK (Vitacress)	Y	Presumptive *Arcobacter* spp. (isolates lost viability before molecular ID)
10/09/2005	Watercress	P	local green grocers, Hampshire, UK	Y	Presumptive *Arcobacter* spp. (isolates lost viability before molecular ID)
28/09/2005	Watercress	P	Sainsburys supermarket, source unknown	Y	*A. butzleri*,, non-*jejuni/coli* spp.
07/11/2005	Watercress	U	Vitacress, Hampshire, UK	Y	*C. jejuni/coli* spp. , *A. butzleri*
07/11/2005	Watercress	P	Vitacress, Hampshire, UK	Y	*C. jejuni/coli* spp., *A. butzleri*
12/12/2006	Spinach	U	Vitacress, Spain	Y	*A. butzleri*
12/12/2006	Wild Rocket	U	Vitacress, source unknown	Y	*A. butzleri*
12/12/2006	Watercress	U	Vitacress, source unknown	N	
12/12/2006	Watercress	U	Local green grocer A, Portswood, UK	Y	*A. butzleri*
12/12/2006	Watercress	U	Local green grocer B, Portswood, UK	Y	*A. butzleri*
12/12/2006	Spinach	P	Sainsburys supermarket, Spain	Y	*A. butzleri*
12/12/2006	watercress/Spinach/Rocket	P	Sainsburys supermarket, source unknown	Y	Presumptive *Arcobacter* spp (isolates lost viability before molecular ID)
12/12/2006	Spinach	P	Morrisons supermarket, source unknown	N	
12/12/2006	Watercress	P	Morrisons supermarket, source unknown	N	
12/12/2006	Watercress	P	M and S supermarket, source unknown	Y	*A. butzleri*
16/01/2006	Spinach	U	Vitacress, Portugal	N	
16/01/2006	Watercress	U	Vitacress, Portugal	N	
16/01/2006	Wild Rocket	U	Vitacress, Portugal	N	
16/01/2006	Red Chard	U	Vitacress, USA	N	
16/01/2006	Watercress	U	Local green grocer A, Portswood, UK	N	
16/01/2006	Rocket	P	Portugal	N	
16/01/2006	Watercress	P	Portugal	N	
16/01/2006	Rocket	P	Portugal	N	
16/01/2006	Watercress	P	Morrisons supermarket, source unknown	N	
16/01/2006	Young leaf spinach	P	Sainsbury's supermarket, source unknown	N	

P, processed, U, unprocessed; Y, *Campylobacteraceae* present in 25g determined by presumptive ID tests (KOH, L-ala, Latex test) or confirmed by PCR; N, not present

Table 41. Enumeration Data for Sampling of Watercress and other Salad Vegetables. Samples were plated directly onto ABA VAT.

Date isolated	Sample Type	Pro/Un*	Sample source /location	Log_{10} CLM CFU/25g	% presumptive	% presumptives confirmed PCR
07/11/2005	Watercress	U	Vitacress, Hampshire, UK	4.51	20%	100% *C. jejuni, A. butzleri*
07/11/2005	Watercress	P	Vitacress, Hampshire, UK	3.78	10%	100% *C. jejuni, A. butzleri*
12/12/2006	Spinach	U	Vitacress, Spain	5.68	20 %	100% *A. butzleri*
12/12/2006	Wild Rocket	U	Vitacress, source unknown	6.08	20 %	100% *A. butzleri*
12/12/2006	Watercress	U	Vitacress, source unknown	5.41	0%	0
12/12/2006	Watercress	U	Local green grocer A, Portswood, UK	6.18	40%	100% *A. butzleri*
12/12/2006	Watercress	U	Local green grocer B, Portswood, UK	3.30	60~%	100% *A. butzleri*
12/12/2006	Spinach	P	Sainsburys supermarket, Spain	3.92	40%	100% *A. butzleri*
12/12/2006	Watercress/spinach/rocket	P	Sainsburys supermarket, source unknown	4.18	20%	Presumptive *Arcobacter* spp.
12/12/2006	Spinach	P	Morrisons supermarket, source unknown	5.66	0%	0
12/12/2006	Spinach	P	Morrisons supermarket, source unknown	5.26	0%	0
12/12/2006	Watercress	P	M and S supermarket, source unknown	5.32	20%	*A. butzleri*
16/01/2006	Spinach	U	Vitacress, Portugal	4.51	0%	0
16/01/2006	Watercress	U	Vitacress, Portugal	0	0%	0
16/01/2006	Wild Rocket	U	Vitacress, Portugal	4.28	0%	0
16/01/2006	Red Chard	U	Vitacress, USA	3.50	0%	0
16/01/2006	Watercress	U	Local green grocer A, Portswood,UK	4.14	0%	0
16/01/2006	Rocket	P	Portugal	4.20	0%	0
16/01/2006	Watercress	P	Portugal	4.91	0%	0
16/01/2006	Rocket	P	Portugal	4.28	0%	0
16/01/2006	Watercress	P	Morrisons supermarket, source unknown	4.71	0%	0
16/01/2006	Young leaf spinach	P	Sainsbury's supermarket, source unknown	3.30	0%	0

P, processed, U, unprocessed; % presumptives = percentage of *Campylobacter* like morphology (CLM) colonies that were picked and subsequently identified as presumptive *Campylobacteraceae* using confirmatory ID tests (i.e. KOH, L-ala, Latex); % presumptive *Campylobacteraceae* isolates confirmed as *Campylobacter* or *Arcobacter* spp. by PCR identification.

6.3.3.2. Recovery of *Campylobacteraceae* from Sewage Sludge

Initial studies investigated recovery from a range of processing stages at the sewage works site, including the raw inlet weir (which receive all waste from sewerage pipes), de-watered pre-digested sludge, mesophilic anaerobic digested sludge, pellets (dried digested sludge) and effluent (from final settlement tanks to return to the sea). No *Campylobacter* or *Arcobacter* species were recovered from the inlet weir, pellets or effluent samples. However, samples from both raw (de-watered, pre-digested) and digested sewage samples recovered *Campylobacteraceae*, therefore further recovery studies continued on these two sample types

The results showed, unquestionably, that the most prevalent species recovered from both raw (pre-digested) and MAD (digested) sewage sludge were *A. butzleri* and *A. cryaerophilus*, which were recovered from 89% of the samples evaluated (8 out of 9 samples) (Table 42 and Table 43). *A. butzleri*, *A. cryaerophilus* and non-*jejuni/coli Campylobacter* species were consistantly recovered from raw samples. In addition, an isolate identified as *C. jejuni/coli,* was recovered from one of the raw samples tested. The prevalence of *Campylobacter* species was reduced on digested sewage samples (only recovered on one sample out of five). However, the presence of *Arcobacter* species remained on all samples after the digestion process (apart from one digested sample tested where no *Arcobacter* or *Campylobacter* species were recovered).

Enumeration data for sewage samples showed that the levels of isolates with CLM recovered from raw sludge (pre-digested) samples were approximately $\log_{10}$ 6 cfu/25g and approximately $\log_{10}$ 4-5 cfu/ 25 g for digested sludge (with no CLM colonies on one digested sample). Although the enumeration of presumptive *Campylobacteraceae* according to CLM counts was high, only 40-80% of these colonies were subsequently confirmed as *Campylobacteraceae* species and, therefore, CLM counts are not a true reflection of the actual level of *Campylobacteraceae* present in the sewage sample. On a number of occasions, when using the quantitive method (direct plating), colonies picked for subsequent identification died off or became contaminated before identification tests could be carried out. When both enumeration and enrichment methods were run in parallel, a wider range and higher number of isolates were recovered when the presence/absence method was applied, with only *A. butzleri* and *A. cryaerophilus* being isolated on both sample types using the quantitative method. Therefore, it is likely that the quantitative data is not a true reflection of the actual levels of *Campylobacteraceae* in the sample.

Table 42. Presence/Absence Sampling Data for Waste Water and Treated Sewage Sludge. Method included 24 h enrichment followed by plating onto ABA VAT.

Date isolated	Sample Type	Sample source?	Presence in 25 g	Presumptive isolates confirmed by PCR
02/08/2005	1. Inlet waste	Milbrook Sewage Works	N	No
02/08/2005	2. Raw sludge (pre- digestion)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.
02/08/2005	3. Digested (MAD)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus*
02/08/2005	4.a Pellets (Dried digested)	Milbrook Sewage Works	N	No
02/08/2005	4.b Effluent (back to sea)	Milbrook Sewage Works	N	No
09/11/2005	Digested (MAD)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus*
23/11/2005	Raw sludge (pre-digestion)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.
23/11/2005	Digested (MAD)	Milbrook Sewage Works	N	No
19/12/2005	Raw sludge (pre-digestion)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus, C. jejuni/coli,* non-*jejuni/coli* spp.
19/12/2005	Digested (MAD)	Milbrook Sewage Works	Y	*A. butzleri, A. cryaerophilus*
16/01/2006	Raw sludge (pre-digestion)	Milbrook sewage works	Y	*A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.
16/01/2006	Digested (MAD)	Milbrook sewage works	Y	*A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.

MAD, Mesophilic Anaerobic Digestion

Table 43. Enumeration Sampling Data for Wastewater and Sewage Sludge. Method included plating samples directly onto ABA VAT.

Date isolated	Sample Type	Sample Source	Presumptive CLM colonies Log_{10} CFU/25 g	% colonies identified as presumptive *Campylobacteraceae*	% presumptive isolates confirmed by PCR
09/11/2005	Digested (MAD)	Milbrook sewage works	5.21	71%	0% identified due to loss of viability (P/A data recovered *A. butzleri, A. cryaerophilus*)
23/11/2005	Raw sludge (pre-digestion)	Milbrook sewage works	5.29	47%	34% *A. butzleri*, 66% *A. cryaerophilus* (P/A method also recovered non-*jejuni/coli* spp.)
23/11/2005	Digested (MAD)	Milbrook sewage works	0	0%	0%
19/12/2005	Raw sludge (pre-digestion)	Milbrook sewage works	6.31	40%	100% *A. butzleri* (P/A data also revealed *A. cryaerophilus, C. jejuni/coli,* non-*jejuni/coli* spp.)
19/12/2005	Digested (MAD)	Milbrook sewage works	4.32	0% *	0% (P/A data recovered *A. butzleri, A. cryaerophilus*)
16/01/2006	Raw sludge (pre-digestion)	Milbrook sewage works	5.85	80%	0% due to contamination & loss of viability (P/A data revealed *A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.)
16/01/2006	Digested (MAD)	Milbrook sewage works	4.36	60%	0% due to contamination & loss of viability (P/A data revealed, *A. butzleri, A. cryaerophilus,* non-*jejuni/coli* spp.)

*A number of colonies lost viability or became contaminated before presumptive identification could be carried out.

6.4. DISCUSSION

6.4.1. Method Optimisation for Sample Recovery

Studies were carried out to develop and evaluate protocols suitable for the recovery of *Campylobacteraceae* species from sewage sludge and salad vegetable samples. Both an enumeration method and enrichment (presence/absence) method were investigated. Optimisation studies focussed on evaluation of selection methods to allow reduction of background flora, while not affecting recovery of *Campylobacteraceae* species. This included selection using the novel VAT selective supplement and a 0.6 μm pore size membrane filtration method (used as part of the Cape Town protocol).

Although including VAT selective supplement in the enrichment broth was evaluated to reduce background contamination, it had little effect on the background flora and the number of *Campylobacter* species recovered, so was omitted from the *Campylobacter* enrichment broth in all further studies. It was considered that as the VAT selective supplement was not significantly reducing the levels of background flora; therefore, there was no need to include it, since its presence may potentially inhibit the growth of stressed and sub lethally injured cells, likely when recovering isolates from processed salad samples and treated sewage samples. Including the VAT selective supplement in the plating medium was considered acceptable since the cells would have had a 24 h period during enrichment in *Campylobacter* enrichment broth to recover. Previous studies have demonstrated that delayed addition of antibiotics after approximately 6 h incubation can give damaged cells a chance to recover, but this was not evaluated during this study (Corry *et al.* 1995a; 2002).

Including VAT selective supplement in the plating medium (ABA) reduced, but did not completely suppress the background flora, neither did it affect the recovery of any of the tested *Campylobacter* species. Enhancing media selectivity, while not affecting recovery of the target organism, is a common microbiological paradox, which is an especially frequent problem with *Campylobacter* species. Due to their fastidious nature they are prone to overgrowth by background contaminants. As the aim of the study was to recover those *Campylobacteraceae* species that are sensitive to many of the antibiotics commonly included in selective media, it was decided that rather than developing a highly specific medium, containing high concentrations of antibiotics to inhibit background flora, but also having a detrimental effect on the recovery of *Campylobacteraceae* species, a medium which was less specific but which would permit successful growth of all *Campylobacteraceae* species was developed. However, this meant that in many cases there

were problems with the accuracy of enumeration due to the presence of background flora masking *Campylobacteraceae* colonies.

Although using a combination of VAT selective supplement and filtering through a 0.6 μm pore size filter was adequate to obtain a suitable number of isolated CLM colonies on the plate surface, non-*Campylobacteraceae* species were consistently recovered from all sample types analysed. On average only approximately 20-40% of the presumptive *Campylobacteraceae* colonies picked were subsequently identified as *Campylobacteraceae*. However, many different types of colony, including atypical *Campylobacteraceae* colonies were selected for subsequent identification just in case these atypical colonies were subsequently identified as *Campylobacteraceae*. It was thought that this approach would reduce the chances of failing to detect *Campylobacteraceae* species with atypical colony morphology. The most commonly isolated background contaminants were identified as *Enterobacter agglomerans and Aeromonas hydrophila.* The high level of mis-identification of colonies with *Campylobacter*-like morphology made it especially difficult to carry out accurate enumeration of *Campylobacter* recovery, as evidenced by experiments which gave counts of CLM colonies (direct plating) of 10^4 cfu/25g, none of which were subsequently identified as *Campylobacteraceae* species. Although ABA VAT plating medium permitted the growth of background contaminants, it was also found that many of the same contaminating flora were also present when highly selective media (including Preston, CAT, CCDA and Skirrow) containing a range of antibiotics were used. Therefore, indicating that these plating media would not recover sensitive *Campylobacter* species, and offer no advantage in increasing selectivity.

Using direct plating by a combination of ABA VAT and filtation through a 0.6 μm pore size membrane was successful at reducing the background flora, but unfortunately also severely reduced the sensitivity of the method, reducing recovery of CLM colonies by approximately $\log_{10}$ 2-3 cfu/25 g compared to when plating directly. The use of this combination was less of an issue after enrichment, as cells would have had a chance to grow to detectable numbers. Indeed, the use of filtration through a 0.6 μm membrane onto ABA VAT after enrichment was essential to reduce background contaminants and permit the recovery of *Campylobacteraceae* from sewage samples.

If there was more time available for the development of media and recovery methods, increasing the specificity of the plating medium to increase the accuracy and likelihood of isolating and identifying *Campylobacteraceae* species, without affecting *Campylobacteraceae* recovery, would have been further investigated. Furthermore, detailed investigations would have been carried out to compare the novel methods

developed to the existing standard methods reported in previous studies (Corry *et al.* 1995a; 2002). The possibility of samples containing viable but non-culturable (VBNC) isolates is likely. This phenomenon has been widely reported in *Campylobacter* species, and isolates in the VBNC state would not be detected using cultural methods (Talibart *et al.* 2000; Cools *et al.* 2005). Therefore, parallel studies using both culture dependent and culture independent molecular methods should be considered. The development of a chromogenic medium would be exceptionally beneficial to help differentiate *Campylobacteraceae* from background flora, but unfortunately no such medium has been developed to date.

It is thought that to overcome some of the drawbacks of the developed method, such as sensitivity of detection and selectivity against non-*Campylobacteraceae* species without use of potentially inhibitory selective agents, incorporating an immunomagnetic separation (IMS) step could be investigated. Previous studies have shown that IMS can help improve recovery by providing an antibody-based concentration procedure that uses magnetic beads coated with antibody against the target species. There are a number of commercially available immuno paramagnetic beads available, mainly developed for *C. jejuni* recovery. It is possible that the antibodies used as part of the latex agglutination tests could be attached to immunomagnetic beads in order to increase the specificity to all *Campylobacteraceae* species. Although, this method has been applied to the recovery *Campylobacter* species, its use has only been reported on a limited scale (Bopp *et al.* 2003).

The detection limits of the presence/absence method used for both sewage and salad vegetables were evaluated for three *Campylobacter* species. The detection limits varied depending on species but, generally, were extremely high ($>10^3$ cfu/25 g). *C. jejuni* was detected at the lowest numbers ($\log_{10}$ 2-3 cfu/25 g), however *C. hominis* was not detected unless it was present at $\log_{10}$ 4 cfu/25 g and above. Therefore, the poor sensitivity of the method must be taken into consideration when analysing results, as the only species detected would be those present in high numbers. *Campylobacteraceae* species present below these levels would not have been detected. The lack of sensitivity is not so much of a problem for recovery from sewage samples, since *Campylobacteraceae* are usually found in large numbers; however, for salad vegetable samples it is important to use a method where even low numbers can be detected to evaluate their risk as food borne pathogens, as the infective dose of *C. jejuni* is thought to be as low as 500 cells (Robinson 1981). The species isolated using these methods were likely to be those in high abundance or those displaying fast growth kinetics, which could out compete the competitive background flora

within the sample. The lack of sensitivity of the enrichment (presence/absence) method could be due to the growth of *Campylobacter* species being out-competed by background flora during the enrichment step. Furthermore, the fact that samples plated after enrichment were either plated out at high dilutions or using membrane filtration, to reduce background flora, contributed to the lack of sensitivity of the method.

The recovery efficiencies of the quantitative method (direct plating onto ABA VAT) used to enumerate samples were evaluated for the recovery of three *Campylobacter* species and *A. butzleri* on both salad vegetable samples and sewage samples. It was found that the percentage recovery of the *Campylobacter* species when spiked into sewage samples varied from >30% for *A. butzleri to* only 0.7% for the more fastidious *C. hominis.* The low recovery rates, especially for some of the more fastidious species such as *C. hominis* and *C. concisus* could be due to die-off while executing the method, perhaps due to exposure to an aerobic environment at room temperature. Furthermore, it is possible that the bacteria would be retained on the sample rather than moving into suspension during homogenisation. These poor recovery efficiencies must be taken into consideration when evaluating disease risk.

6.4.2. Identification of Recovered Isolates

Following primary isolation, isolates were first identified based on their colony morphology and then sub-cultured for further phenotypic and molecular identification using tests that had been previously optimised (Figure 10).

All of the isolates with *Campylobacter*-like colony morphology that were positive using the KOH test and negative for L-alanine aminopeptidase as the initial pre-screen for *Campylobacteraceae* were all subsequently identified as *Campylobacter* or *Arcobacter* species using PCR analysis. All of these presumptive *Campylobacteraceae* isolates also displayed *Campylobacter*-like cellular morphology (using EDIC microscopy) and were oxidase positive. Using the CAMPYCHECK strains as a reference, the cellular morphology of isolates recovered from salad vegetable and sewage samples could be compared. The results revealed that the majority of the presumptive *Campylobacteraceae* isolates that had been pre-screened using the KOH/L-ALA test showed a resemblance to that of the CAMPYCHECK reference strains, being of similar size and either curved or spiral shaped rods (Appendix Table I). Isolates that were clearly not *Campylobacteraceae* or mixed cultures could be easily recognised by cell size and shape.

The majority of isolates that were confirmed to be *Arcobacter* species grew aerobically at 25°C, however, 10% of these *Arcobacter* isolates failed to grow in these

conditions. This could be attributed to certain strains having a lowered ability to grow aerobically, especially if they were sub-lethally stressed. The fact that this test could misidentify *Arcobacter* species as *Campylobacter* species is concerning, as this is often used as standard to phenotypically differentiate these species. This re-emphasises the requirement for molecular confirmation of species to be carried out.

The thermotolerant latex agglutination test correctly detected all three isolates subsequently identified by PCR as *C. jejuni* species. However, the latex reagent also cross reacted with 20% of isolates subsequently identified as *Arcobacter* species and also non-thermotolerant *Campylobacter* strains. This is not surprising since these taxa are so closely related, it is likely that surface antigens would be similar. Furthermore, the lack of discrimination could be due to the use of polyclonal antibodies as opposed to monoclonal antibodies in the production of the latex reagent. The *Campylobacteraceae* latex reagent was able to detect 87% of the *Arcobacter* isolates (87 out of 100 isolates) and 89% (16 out of 18 isolates) of *Campylobacter* isolates. The lack of sensitivity of detection can be largely attributed to non-specific agglutination, in which there was agglutination even with the control latex reagent which did not include antibody. However, the latex agglutination test was very useful at discriminating between isolates with 77% of presumptive *Campylobacteraceae* being accurately grouped into either thermotolerant or non-thermotolerant (mostly *Arcobacter* species) groups. The prototype latex test has so far only been tested on a small number of reference strains and futher optimisation and evaluation of the test is required. If time was not a limiting factor during this PhD study an interesting experiment would have been to further characterise and identify the target structures of these antibody reagents using immuno-pull down methods.

All of the presumptive *Campylobacteraceae* isolates (118 isolates in total) sent for confirmatory molecular identification using multiplex PCR were identified as either *Arcobacter* or *Campylobacter* species. The majority of the isolates (100/118) were identified as *A. butlzeri* and some *A. cryaerophilus* with a minority of the isolates (18/118) identified as campylobacters. Three of these were subsequently identified as *C. jejuni* using the multiplex PCR test. The remaining fifteen isolates did not yield the expected *C. jejuni* or *C. coli* amplicons, and therefore represent other *Campylobacter* species. The accurate species level identification of these fifteen isolates required either the availability of live cultures so that strains could be cultivated in standardised conditions for protein electrophoresis, or, alternatively, DNA based identification using AFLP (Duim *et al.*, 2001). Unfortunately, the molecular identification of *Campylobacter* and *Arcobacter* using these methods could not be performed as initially planned due to problems with obtaining

authorisation to send live cultures to the University of Ghent laboratory. However, Professor Peter Vandamme has expressed his interest to continue this collaboration if future work in this area should continue.

6.4.3. Recovery from Salad Vegetables

Results from the study demonstrated that the prevalence of *Campylobacteraceae* species on unprocessed salad vegetables was 57% (8 out of 14 samples). All of these samples contained *A. butzleri* and, in addition, a C. *jejuni/C. coli* isolate was recovered from one sample. The prevalence of *Campylobacteraceae* on processed samples was 50% (7 out of 14 samples). *A. butzleri* was recovered from all of these samples, in addition, a *C. jejuni/coli* isolate was recovered from one of the processed watercress samples and a non-*jejuni/coli* species present on another sample.

Although many epidemiology studies have demonstrated links between consumption of salad vegetables with food poisoning outbreaks caused by *Campylobacter* species, very few cases have recovered *Campylobacter* species from the associated food (Sobel *et al.* 2001; Evans *et al.* 2003; Cardinale *et al.* 2005). The prevalence of a number of pathogenic bacteria on both raw and ready-to-eat vegetables have shown that although a number of other bacteria (e.g. *Listeria* and *Salmonella*) have been isolated from ready-to-eat samples, *Campylobacter* species were not detected (Rasrinaul *et al.* 1988; Park and Sanders 1992; Odumeru *et al.* 1997; Little *et al.* 1999; Mosupye and von Holy 1999; McMahon and Wilson 2001; Sagoo *et al.* 2001; Moore *et al.* 2002; Thunberg *et al.* 2002; Sagoo *et al.* 2003). As far as the published data is concerned, all of the studies carried out to date have been concerned with the recovery of *C. jejuni* from salad vegetables, using culture methods developed for *C. jejuni* only, and there remains a lack of studies assessing the prevalence other members of the *Campylobacteraceae* including *Arcobacter* species.

The first published report of the isolation of *Campylobacter* from a 'produce-related' item may have been the isolation of *C. jejuni* from 3 of 200 (1.5%) retail fresh mushroom samples (Doyle and Schoeni 1986). In one of the most extensive studies from Ottawa, Canada, Park and Sanders (1992) tested 1564 fresh produce samples obtained from farmers outdoor markets (533 samples) and supermarkets (1031 samples). Of 10 different vegetables tested, *C. jejuni* was isolated from 2/60 (3.3%), 2/67 (3.1%), 2/74 (2.7%), 1/40 (2.5%), 1/42 (2.4%) and 1/63 (11.6%) samples of spinach, lettuce, radish (with leaves), green onion, parsley and potatoes, respectively, with all of the positive samples originating from farmers' markets. In addition, multiple strains were isolated from some samples and all *Campylobacter* strains isolated were obtained only after enrichment culture methods;

none were isolated from any sample by direct plating (Park and Sanders 1992). A later study revealed that *C. jejuni* was present on 3.57% of the samples from a local market in northern India which included spinach (1/9 samples) and fenugreek (1/9 samples) (Kumar *et al.* 2001).

A study investigating the presence of *Campylobacter* species in mixed salad vegetables packaged in a modified atmsophere from supermarkets showed that *Campylobacter* were isolated from 12/36 (33.3%) and 8/54 (14.8%) samples tested on the date of purchase or stored at 4°C and tested on the use by date, respectively. The average rate of isolation of *Campylobacter* from these packaged salad vegetables (22.2%), was much higher than that reported for any other study of fresh produce (Phillips 1998). The number of colony-forming units isolated from the packaged salad samples was low (80–170 cfu/g). These results suggest that the modified atmosphere used for packaging the salad vegetables (reported to be 5–8% CO_2, 2–5% O_2 and 87–93% N_2) might enhance the survival of *Campylobacter* in other produce or food samples, even though the viability of *Campylobacter* may decrease over time in this atmosphere (Phillips 1998). It is worth noting that the methods used to isolate these *Campylobacter* species included use of highly selective media (Preston enrichment broth and Preston agar) with incubation in a standard microaerobic atmsophere at 42°C, therefore it is likely that the study would have underestimated the prevalence of many strains of *Campylobacteraceae*.

In a study of 400 samples of ready-to-eat vegetable samples collected over a 2-year period in the Nantes region of France, 2/150 and 0/250 grated ready-to-eat vegetables and salad samples, respectively, were positive for *C. jejuni* (Federighi *et al.* 1999). Differences were noted in the efficiency of isolation of *Campylobacter* in two different selective enrichment broths (Preston broth and Park and Sanders) and three different plating media (Karmali, Butzler and Skirrow), indicating the importance of the media and culture methods for accurate estimates of *Campylobacter* incidence. This study constituted the first instance in which *C. jejuni* and one strain of *C. coli* was isolates in processed ready-to-eat salad vegetables (Federighi *et al.* 1999).

A recently reported surveillance study was carried out to determine the prevalence of *Campylobacter* in a range of food commodities in Pakistan (Hussain *et al.* 2007). Over a period of 3 years (January 2002-December 2004), a total of 1636 food samples of meat, milk and other food commodities were procured from three big cities in Pakistan. Of these, 22 vegetable and fruit salad samples were analysed, of which 9 samples (40.9%) contained *C. jejuni* or *C. coli* species. Unfortunately, the study did not specify whether these samples were raw or processed. Furthermore, the isolation methods used were unsuitable for the

recovery of emergent *Campylobacteraceae* (i.e. use of mCCDA and incubation in a standard microaerobic atmosphere at 42°C).

None of the studies previously described reported the evaluation of watercress samples in their findings, as this study did. It is thought that watercress may pose a significantly greater risk than other common salad vegetables (e.g. lettuce) as it grows in waterways (sometimes completely submerged) subject to waterborne contaminants. Sites where watercress grows usually have comparatively small volumetric flows, which do not allow for the dilution of contaminants they receive, and they are often either in urban or intensive agricultural areas. These areas may therefore be subject to faecal contamination from urban and rural run-off, which may introduce enteric pathogens, including *Campylobacter* into the receiving waters. The person gathering watercress could also be at risk of infection from contact with contaminated surface waters. The only published data found evaluating the recovery of a range of microoganisms from watercress was carried out in Wellington, New Zealand. The study showed that *Campylobacter* was detected in the watercress growing waters at all sites tested (80% of the samples) and in 11 % of the watercress samples (Edmonds and Hawke 2004).

This study along with evidence from previous studies (Federighi *et al.* 1999) has shown that *Campylobacter* species can be recovered from both raw and ready-to-eat processed salad vegetables. Generally, ready-to-eat vegetables go through very little processing before packaging and also prior to consumption. In-plant processing of these products generally includes a chlorinated-water (100 ppm) rinse prior to packaging in a modified atmosphere.

The main routes for contamination of vegetables are thought to be due to run-off from nearby fields containing contaminated cattle faeces, contact with wild birds and other animals or even through handling by an infected person (during harvesting, preparation or packaging) and due to lack of worker hygiene (Buck *et al.* 2003). In practice, when the hands of workers are contaminated, most cross-infection or auto-infection is likely to occur before the fingers are completely dry (Coates *et al.* 1987).

It is also possible that the potential source of contamination of salad vegetables could stem from insufficiently treated recycled animal wastes by wind blown aerosols formed during slurry spraying and from irrigation water contaminated by run-off from fields into streams and rivers. The potential of contamination by sewage sludge is unlikely because while large amounts of sewage sludge are disposed of onto farm land in the UK, it is not in areas where fruit and vegetables are grown (Sagoo *et al.* 2001). Wild animals, especially flocks of wild birds (which contain high levels of *Campylobacter* species), can

contaminate growing vegetables and fruits. This is likely due to wild birds contaminating pre-harvest crops while foraging for food. The possibility of crops becoming contaminated by faecal bombing on crops grown under bird migration routes has also been suggested (Jones and Heaton 2006).

A previous study has demonstrated that *C. jejuni* is able to utilise organic compounds available on roots and leaves of lettuce and spinach and persist in the rhizosphere of spinach for prolonged periods (Brandl *et al.* 2004). A similar study has also proved that *C. jejuni* may survive for sufficiently long periods on various fresh produce (including lettuce, carrot and cucumber) to pose a risk to the consumer (Karenlampi and Hanninen 2004). It is generally considered that 10% of enteropathogens are not removed by washing due to physiological attachment to plants (Jones and Heaton 2006). Furthermore, it is thought that if *Campylobacter* species exist as biofilms on the leaf surface, they will be much more resistant to disinfectants contained in the wash during processing (Joshua *et al.* 2006). Washing can also lead to the spread of bacteria. For example if only one leaf is contaminated, the washing process transfers bacteria to the other leaves. Although thermotolerant *Campylobacter* are quite sensitive to environmental stress, *C. jejuni* has been shown to survive for several days in a moist environment at low non-freezing temperatures.

Ready-to-eat vegetables in modified atmosphere packaging are kept in moist, refrigerated conditions with a relatively low level of oxygen (~5%). As survival of *Campylobacter* is longest at low oxygen concentrations, modified-atmosphere packaging may prolong the survival of this pathogen (Boysen *et al.* 2007).

In all of the studies described on the incidence of *Campylobacter* species in fresh produce, only *C. jejuni* has been isolated and this is likely to be due to the recovery methods used being inadequate for the isolation of emerging species. For example the methods used during all of the studies generally used only highly selective media such as Preston broth and agar, incubation at 42°C as apposed 37°C and in a microaerophilic atmosphere (without hydrogen). Therefore the prevalence of emerging *Campylobacteraceae* species would have been severely underestimated. It is possible that many of the emerging *Campylobacter* species, which have the capacity to cause disease, would not have been isolated due to inadequate use of culture conditions.

Surprisingly, to date no data have been published on the prevalence of *Arcobacter* species in fresh produce. Therefore, the present study has important public health implications due to increasing evidence that this organism is a clinically important human pathogen, combined with the high prevalence of *A. butzleri* in both unprocessed and ready-

to-eat salads (Vandenberg *et al.* 2004). Epidemiology studies have shown that eating salad vegetables is a major risk factor for *Campylobacter* infection, yet hardly anyone has actually isolated it from fresh produce. It is possible that the reasons for this are that *Campylobacter* and *Arcobacter* species are being confused symptomatically or by misidentification during cultural isolation. The most common symptoms of both species are acute watery diarrhoea accompanied by abdominal pain, nausea and vomiting. Furthermore, arcobacters are differentially sensitive to a number of common antimicrobical agents present in selective media used for thermophilic *Campylobacter* isolation and routine techniques used for *Campylobacter* species will not necessarily isolate arcobacters. Therefore, arcobacters present on fresh produce may be playing a significantly more important role in contributing to food borne illness than at first thought. However, due to lack of any epidemiological data on the prevalence of this organism in fresh produce, along with the lack of perceived importance of the arcobacters as pathogens, it is clear that further work on their prevalence in fresh produce and their association with disease is required to provide more evidence to support these assumptions. A PCR method for detection of *C. jejuni* and *Arcobacter* species from a number of salad vegetables has been developed, however recovery has only been applied to artificially inoculated samples (Winters and Slavik 2000).

To date, although there have been many associations of *Arcobacter* species with human illness, there have been very few studies associating food with *Arcobacter* infection. Therefore, the fact that during this study *A. butzleri* was so prevalent in many types of baby leaf salads, of the ready-to-eat variety, is a major public health concern. The fact that arcobacters are so prevelant in the environment may allow them to contaminate salad vegetable pre-harvest from run-off from near-by fields or from contaminated water sources or the use of contaminated fertilizer.

6.4.4. Recovery from Sewage Sludge

The results showed that by far the most prevalent species recovered from both raw (de-watered, pre-digested) and MAD (digested) sewage sludge were *A. butzleri* and *A. cryaerophilus* which were recovered from 89% of the samples evaluated (8 out of 9 samples). *A. butzleri*, *A. cryaerophilus* and non-*jejuni/coli Campylobacter* species were always present on raw samples. In addition, on one occasion a *C. jejuni/coli* isolate was recovered from one of the raw samples tested. The prevalence of *Campylobacter* species was reduced on digested sewage samples (only recovered on one digested sample out of five), although the presence of *Arcobacter* species remained after the digestion process

(apart from on one digested sample tested where no *Arcobacter* or *Campylobacter* species were recovered).

Enumeration data for sewage samples showed that CLM recovered from samples were in the range of $\log_{10}$ 5.29 to $\log_{10}$ 6.21 cfu/25g for raw sludge (pre-digested) and $\log_{10}$ 4.32 to $\log_{10}$ 5.21 for digested sludge (with no CLM colonies on one digested sample). Differences in these ranges within samples could be due to seasonality or at what stage of the digestion process the samples were taken from the digester tanks. The number of *Campylobacter* species in sewage is likely to be an underestimate of the number actually present, since *Campylobacter* species are unable to multiply outside of warm-blooded host animals. They also survive for a shorter time in these conditions and are susceptible to environmental stress, such as ambient temperatures, atmospheric oxygen concentrations and UV radiation (Jones 2001b). However, the counts recorded during this study may well overestimate the actual number of *Campylobacteraceae* isolates recovered on samples, since subsequent identification data showed that only 40-80% of the presumptive colonies counted and picked were subsequently identified as presumptive *Campylobacteraceae* species. Moreover, a number of colonies picked from samples that were directly plated died off or became contaminated before subsequent identification tests could be carried out. This made it difficult to ascertain what proportion of colonies counted were in fact *Campylobacteraceae* species. On many occasions when both enumeration and enrichment methods were run in parallel, a wider range and higher number of isolates were recovered when the presence/absence method was applied. Reasons for this could be that the cells plated directly onto ABA VAT may have been in a stressed state, so were likely to be more sensitive to antibiotics in VAT selective supplement. Furthermore, the levels of isolates present in the sample may well have been below the detection limit of the method when directly plated and so would not have been isolated. However, when the presence/absence method was used, the cells would have had a chance to recover (during the 24 h enrichment step) from their stressed state and grow to a detectable level. Previous studies have shown the levels of *Campylobacter* encountered in sewage to be as high as $\log_{10}$ 7 cfu/ml (Jones *et al.* 1990b).

A previous study detected the presence of *Campylobacter* in 43.9% of 116 raw sewage samples evaluated. The species distribution was 94.1% *C. jejuni* and 5.9% *C. coli* (Dousse *et al.* 1993). The method used to recover these species consisted of an enrichment step in Preston broth for 6 h at 42°C followed by plating onto Preston's selective agar and incubation in standard microaerophilic conditions; therefore it is likely that the emerging *Campylobacteraceae* species known to be susceptible to antibiotics contained in these

media (including arcobacters) and those which require hydrogen would not have been recovered in these conditions (Dousse *et al.* 1993). A study on four types of sewage sludge (primary, activated, thickened and anaerobically digested sludge) taken from a treatment plant in Bologna (Italy) also found high numbers of *Arcobacter* species (Stampi *et al.* 1999). *Arcobacter butzleri* isolates were found in all types of sludge with frequencies of 80% in activated and thickened sludges and 41% in digested sludges. *C. jejuni* and *C. coli* were more numerous during the March-September period and were found only in primary sludge (22.7%). It is not surprising that a higher number of *Arcobacter* species can be detected, due to their higher tolerance to environmental oxygen and ability to grow in these conditions at lowered temperatures compared to *Campylobacter* species. Previous studies have shown that treating raw sewage using digestion normally reduces *Campylobacter* numbers to zero, but occasional positive samples are obtained (Horan *et al.* 2004). In the present study, although *Campylobacter* species were rarely found in digested samples (except for the presence of non-*jejuni Campylobacter* species in one sample), *A. butzleri* and *A. cryaerophilus* generally persisted after digestion. A Swedish study investigating the incidences of bacterial pathogens in sewage sludge from sewage treatment plants showed that *C. jejuni* was isolated from 20% of raw sludge samples (13/64 samples) and 4% of treated sludge (2/69 samples). In addition *C. coli* was isolated from 9% and 2% of raw and treated sludge, respectively (Sahlstrom *et al.* 2004). Although sludge digestion has been reported to lower the number of campylobacters substantially, the findings from this and other studies show that campylobacters, or more likely *Arcobacter* species, are able to survive the sewage treatment process and have the potential to contaminate crops (Stampi *et al.* 1999). For example a previous experiment using experimental digesters showed that the mesophilic anaerobic digestion process reduced the numbers of other enteric bacteria such as *Salmonella*, *Listeria* and *E. coli* by around $\log_{10} 2$ but did not affect *C. jejuni* (Horan *et al.* 2004). Mesophilic anaerobic digestion is the primary process used to reduce the numbers of enteric pathogens in sewage sludge in the UK (Anonymous 1999) before recycling to land. It has also been suggested that the distribution of sewage sludge to land may be one of the routes by which campylobacters re-enter the human food chain and from these results it is clear that the anaerobic digestion procedure does not completely remove *Campylobacter* species and therefore transmission via this route seem very likely.

Arcobacter species have been found in sewage and activated sludge, with frequencies varying from 41 to 80% (Stampi *et al.* 1999). This has serious implications for animal and human health, since the role of these species as emerging pathogens is now

becoming appreciated. However, the prevalence of *Arcobacter* species is likely to have been largely underestimated in previous isolation studies, as they are frequently misidentified as atypical *Campylobacter* when relying on conventional plating methods and phenotypic tests due to their sensitivity to many selective antibiotics contained in *Campylobacter* isolation media.

Analysis of individual sewers in north-west England showed that *Campylobacter* numbers were related to the incidence of campylobacteriosis in the city. Campylobacters in sewage also showed identical seasonal patterns to that of the human population, namely with a large peaks in late May and June. This led to the hypothesis that annual peaks in human infections are related to increased amounts of *Campylobacter* in the sewage sludge (Jones *et al.* 1990b; Jones 2001b). Furthermore, it has been shown that in north-west England, the same strains of *C. jejuni* isolated from patients' stool samples are found in sewage, however the route of transfer of *Campylobacter* from the environment to the consumer is not known. Further molecular genotyping studies are required to match environmental and human strains to trace infection routes and understand health risks. However, this potentially means that the species isolated from the sewage samples during this study are a reflection of the prevalence of *Campylobacteraceae* species in the population of Southampton and surrounding areas.

In the absence of spores or any robust physiologically-based protective mechanism, the main survival strategy for *Campylobacter* appears to be the production of huge numbers, in the hope that once in the 'environment', enough will survive to infect another host. It has also been demonstrated that campylobacters can persist, but not grow, in biofilms, where they are protected from biocidal activity (Buswell *et al.* 1998). Since the viable but non-culturable (VBNC) form of *Campylobacter* was first reported, its presence has been demonstrated in a number of aquatic systems (Talibart *et al.* 2000). However, there remains much debate as to whether the virulence of these forms is equivalent to that of the cultural forms. If this was the case the VNBC forms contain added risk since they are not detected by cultural methods. Previous research appears to favour the view that VNBC forms have an important role in the transmission of the disease, at least for certain strains (Cappelier *et al.* 1997). A previous study compared a number of selective broths and agars (Karmali, CCDA, and Preston) for the detection of *Campylobacter* species in sewage sludge and concluded that there was no significant difference between the media used, but unfortunately identification to the species level was not attempted. It showed that despite using highly selective media (Karmali, CCDA, and Preston broth and agars) for the recovery of *Campylobacter* species from sewage, competitive flora were always present,

namely *E. coli*, *Proteus mirabilis* and *Bacillus* species, presenting problems with counting and picking suspect colonies (Koenraad *et al.* 1995). The authors concluded that at the time, a quantitative, unambiguous method with a high recovery rate, high sensitivity and general applicability for the detection of *Campylobacter* in sewage was not yet available and the selectivity of the isolation media needed to be further improved, as appears to still be the case to date. The study showed that using a PCR method on enriched sewage samples was more rapid and sensitive than conventional isolation. The problem with recovery of *Campylobacter* species from sewage is that generally the population of campylobacters in the environmental samples compared with competing bacteria such as coliforms, swarming *Proteus* and *Enterococcus* species is less. In addition, campylobacters have slow growth rate, so they can be easily overgrown by other competing organisms present in the environmental samples.

A previous study was carried out in an attempt to modify the Cape Town protocol to isolate a wider range of *Campylobacter* species from water samples and sewage samples (Diergaardt *et al*, 2003). Here, large volumes (5 ml, 10 ml and 50 ml) were first concentrated by vacuum filtration and trapped on a 0.1 μm pore size membrane before inverting onto a 0.65 μm pore size filter on Tryptose Blood Agar or for comparative purposes inverted directly onto CCDA selective agar. Results showed that the modified Cape Town Protocol performed poorly with 0.1% recovery rates compared to 10% directly onto CCDA. The study also showed no isolates were confirmed as *Campylobacter*, however four isolates were identified as *A. butzleri*. This was similar to the case in the present study where many isolates have been typed as *Arcobacter* species.

However many other studies, using mainly PCR and culture techniques have isolated *C. jejuni* and *C. coli* species in high numbers from many types of sewage samples (Lauria-Filgueiras and Hofer 1998; Alexandrino *et al.* 2004; Sahlstrom *et al.* 2004). A PCR approach was also used for the detection and accurate differentiation of *Campylobacter* and *Arcobacter* species, therefore reducing the chance of misidentifying *Arcobacter* as atypical *Campylobacter*, which is likely to occur using conventional biotyping schemes (Jacob *et al.* 1996). A study comparing FISH, PCR and culturing techniques for the recovery of *Campylobacter* and *Arcobacter* species from water and wastewater isolated *C. coli* from three river water and seven wastewater samples and *Arcobacter* species from four river water samples and 12 wastewater samples (Moreno *et al.* 2003). The authors concluded that culture methods were unsuccessful, mainly due to the overgrowth of background contaminants, causing the samples to be considered negative in most cases as characteristic colonies could not be observed. Culture methods

were also unsuccessful due to the *Campylobacter* species present in the samples being extremely stressed and in a viable but non culturable form (Moreno *et al.* 2003). Another study using PCR to detect *C. jejuni* and *C. coli* species showed that all samples analysed were negative (Alexandrino *et al.* 2004).

A study investigating the diversity of *Campylobacter* isolates from activated sludge systems in Brasil showed that both human and animal pathogenic biotypes could be isolated from activated sludge during initial processing steps (Lauria-Filgueiras and Hofer 1998). Laboratory analysis of 390 samples showed the presence of 169 thermophilic strains. *C. jejuni* (biotypes I and II) was the most prevalent species isolated (40.8%) followed by 16% identified as *C. coli*. A small percentage of the isolates were also identified as *C. lari* or untypable *Campylobacter* species. The isolation method included use of a medium containing a wide range of antibiotic supplements and incubation was in a microaerophilic atmosphere at 43°C, therefore the method would not have isolated emerging *Campylobacteraceae* including *Arcobacter* species. The authors commented that the isolation of *C. lari* could have been due to the presence of seagulls in the recycling plant. Indeed, during collection of samples from the treatment works site for the present study, it was observed that flocks of seagulls were feeding off particles in the inlet and sediment tanks, and it is possible that seagulls could play an important role in the horizontal transfer of *Campylobacter* species to sewage. The untyped *Campylobacter* species isolated in the present study may well have contained *C. lari* strains.

The majority of studies that have been carried out to date investigating the prevalence of *Campylobacter* species in sewage have been limited to the isolation of *C. jejuni* and *C. coli* species. This is due to the use of sub-optimal cultural conditions for emerging *Campylobacter* species which include the use of media containing highly selective antibiotics; incubation in standard microaerophilic environments (without hydrogen) and at a higher temperature of 42°C (as opposed to 37°C); these are all factors that contribute to their poor recovery. Although, during this study a number of non-*jejuni/coli* species were isolated in raw and digested sludge, until further identification (to the species level) of these isolates is carried out, the prevalence of emerging *Campylobacter* species in sewage remains unknown. Generally, despite the majority of studies using highly selective media during cultural analysis, authors have commented on the difficulty of obtaining accurate counts or picking isolated colonies due to the presence of problematic background flora, difficulties which were also encountered during this study.

CHAPTER 7 GENERAL CONCLUSIONS AND FURTHER WORK

The prevalence of *C. jejuni* and *C. coli* species and their importance as human pathogens is well established. However, at present there remains a lack of information on the prevalence and clinical importance of non-*jejuni/coli Campylobacteraceae* species. This is largely due to the fact that isolation methods are designed to primarily detect thermo-tolerant *Campylobacter* species such as *C. jejuni* and *C. coli,* due to their perceived importance as human pathogens. These methods are thought to be sub-optimal for the isolation of these emerging *Campylobacteraceae*. Many of the more fastidious *Campylobacter* species, such as *C. concisus* and *C. upsaliensis* have been shown to be inhibited by antibiotics commonly contained within the selective isolation media, require incubation at a lower temperature (37°C as opposed to 42°C), have a hydrogen requirement or require a longer incubation time. A number of of non-*jejuni/coli* strains have been recently isolated from clinical faecal samples from paediatric patients, in numbers just as high as *C. jejuni* and *C. coli* species, when alternative culture isolation protocols and molecular techniques have been used, therefore questioning the clinical importance of these emerging species (Lastovica 2006).

The aim of this PhD investigation was to assess whether the currently used isolation and identification tools, developed primarily for the isolation of thermo-tolerant *Campylobacter* species, were suitable for the isolation of all recognised *Campylobacteraceae* species. Further to this, the aim was to develop a universal isolation medium and incubation atmosphere suitable for isolation of all *Campylobacteraceae* species, which could also be incorporated into protocols used in routine testing laboratories.

Initially during this study, a number of commonly used selective isolation media were evaluated for their ability to recover a range of *Campylobacteraceae* species. It was demonstrated that selective supplements included in Preston, Skirrow, CCDA and to a lesser extent CAT agar inhibited the growth of a number of *Campylobacteraceae* species, Further to this, a number of antimicrobial agents commonly included in selective media were evaluated for their inhibitory effects on *Campylobacteraceae*. It was demonstrated that a number of individual antibiotics, namely cefoperazone, rifampicin, polymyxin B and colistin sulphate, were inhibitory to the growth of *Campylobacteraceae* species. Vancomycin and trimethoprim were chosen to include in a newly formulated medium, since they were the two least inhibitory agents to the *Campylobacteraceae* species and, in addition, were effective against a range of background flora. Amphotericin B was also included to suppress growth of yeasts and fungi. The novel isolation medium, ABA VAT, was evaluated against the *Campylobacteraceae* reference strains and was shown to support

good growth of all strains as well as suppressing growth of most of the tested non-*Campylobacteraceae* bacteria.

Current incubation atmospheres, developed and used primarily for the isolation of thermo-tolerant *Campylobacter* species, are thought to be sub-optimal for the recovery of a number of emerging *Campylobacteraceae* species. The most widely used commercially available atmosphere generating system, CampyGen, was evaluated for growth of *Campylobacteaceae* species. Results showed that many species including *C. concisus* and the 'anaerobic' *Campylobacter* species failed to grow or grew poorly in an atmosphere provided by the CampyGen system. Surprisingly, a number of species could tolerate growth in an anerobic environment provided by Oxoid's Anaerobic Gas Generating system, even species previously shown to require strictly microaerobic conditions. Although, the presence of enzymes for anaerobic and aerobic metabolism has been demonstated in a number of *Campylobacter* species, it has been shown in *C. jejuni* that low levels of oxygen are required for the enzyme ribonucleotide reductase (RNR) to carry out DNA synthesis. It is possible that growth under this supposedly anaerobic atmosphere could have been achieved due to the use of a highly nutritious growth medium, containing the required substrates for anaerobic growth. Furthermore, the atmosphere could have contained trace levels of oxygen, at concentrations high enough for RNR to function; the likely presence of hydrogen could also have been an advantage.

It was demonstrated that hydrogen was essential for growth of a number of *Campylobacteraceae* species. Hydrogen could be a very important source of electrons for the growth of campylobacters in the gut, as many obligate anaerobes in the intestine produce hydrogen from redox reactions associated with fermentation. In *C. jejuni* metabolic pathways have been reported to exist that utilise hydrogen as an alternative terminal electron donor to oxygen for respiration and growth pathways which also may be the case for many other *Campylobacter* species (Smith *et al.* 1999; Kelly 2001)

Studies were carried out to optimise the hydrogen and oxygen concentrations in the atmosphere using the MACS workstation, with the aim to produce an atmosphere permitting the growth of all strains of *Campylobacteraceae*. An atmosphere of 3% oxygen, 10% carbon dioxide and 7% hydrogen permitted growth of all species, including the species which usually prefer anaerobic conditions (*C. curvus*, *C. gracilis*, *C. showae* and *C. hominis*) and of microaerophilic species. Three versions of this novel atmosphere were evaluated which included use of the MACS workstation and cheaper alternatives such as the gas-evacuation system (using pre-mixed gas cylinder and polythene bags) and commercially available gas sachets used in combination with anaerobic gas jars. These

alternative atmosphere generation systems were developed and evaluated so they could be applied to protocols in routine isolation laboratories. The optimal gas production system was use of the MACS workstation, although all three versions of the novel atmosphere were found suitable for growth of all *Campylobacteraceae*.

A number of novel identification tests were developed and evaluated for their ability to confirm the identity of *Campylobacteraceae* and to identify them to the species level. Initially, a number of phenotypic tests commonly used in the identification of *Campylobacter* species were assessed. It was clear that for accurate identification to the species level a large number of tests would need to be carried out to achieve the desired level of differentiation, such as the tests used in the Cape Town protocol (Lastovica 2006). However, this would have been time consuming and laborious, therefore in collaboration with Microgen Bioproducts, a miniaturised in-well biochemical test strip including 16 biochemical tests in total was evaluated. Unfortunately, reproducibility and standardisation of the test was unsuccessful, therefore further optimisation of the product is required. A novel test for differentiation of *Campylobacteraceae* from other Gram-negative bacteria was developed by Oxoid Ltd and evaluated as part of this study. The test is based on the absence of L-alanine aminopeptidase in *Campylobacteraceae.* Used in combination with the KOH string test, it was found to be a rapid and efficient screen for *Campylobacteraceae*. Novel microscopy technology in the form of episcopic differential interference contrast (EDIC) microscopy was also evaluated for its ability to allow accurate and detailed observation of cellular morphology. The technique was rapid and effective; permitting detailed three-dimensional images of *Campylobacter* cells without the requirement for complicated and lengthy staining procedures. Although EDIC microscopy was a particular help as an additional screen for *Campylobacteraceae*, there were little or no differences between the cellular morphology of *Campylobacteraceae*, which were mostly curved rod or spiral shaped. However, certain species including those preferring anaerobic conditions (*C. curvus*, *C. showae*, *C. gracilis*, *C. hominis*) predominated as straight rods.

An immunological identification test for thermophilic *Campylobacter* species based on polyclonal antibodies to *C. jejuni* that is commercially available from Microgen Bioproducts was screened against the 59 reference strains of *Campylobacteraceae*, revealing that it was unsuitable for the detection of a number of emerging species. Microgen re-developed the test to include a latex reagent suitable for detection of all *Campylobacteraceae* species and subsequent analyses showed that it was suitable. However, when this prototype latex agglutination test was used to confirm the identity of

Campylobacteraceae isolates recovered from sewage and salad vegetable samples, its level of discrimination between 'thermophilic' and non-thermophilic' species differed from that of the reference strains tested. The thermophilic reagent also cross reacted with 20% of isolates subsequently identified as *Arcobacter* species and also non-thermotolerant *Campylobacter* strains. The *Campylobacteraceae* latex reagent was able to detect 87% of the *Arcobacter* isolates (87 out of 100 isolates) and 89% (16 out of 18 isolates) of *Campylobacter* isolates. The lack of sensitivity of detection could be largely attributed to non-specific agglutination, where there was agglutination even with the control latex reagent which did not include antibody. However, the latex agglutination test was very useful at discriminating between isolates with 77% of presumptive *Campylobacteraceae* being accurately grouped either thermophilic or non-thermophilic (mostly *Arcobacter* species) groups.

A fluorescence *in situ* hydridisation technique using PNA rRNA-targetted oligonucleotide probes was evaluated. Despite numerous attempts to increase the stringency of hybridisation, the specificity of the probe was low and did not permit the differentiation of *Campylobacteraceae* from other enteric bacteria. This was unfortunate, since had the probe worked, it would have permitted the detection and enumeration of *Campylobacteraceae* species directly on samples, or used for the subsequent identification of isolates after recovery by culture methods. Originally, it was thought that this probe could have been used in combination with the thermophilic *Campylobacter* specific probe in a multipex assay which would have given information on the presence of both thermophilic and emerging species in the sample.

An attempt to accurately identify *Campylobacter* to the species level by comparative protein profiling was evaluated using a 2D-PAGE approach. Unfortunately, the number of proteins recovered on the 2D gel protein profiles was largely under-represented considering that whole-cell extracts were used. Furthermore, it was found that the technique was almost too discriminatory between species, preventing accurate comparative analysis. Rather than representing differences in protein expression, it is thought that these differences were due to either small amino acid modifications which would have had a large affect on location on the gel protein profile. Alternatively, these differences could be explained by the post-translational modification of proteins, most probably by glycosylation, which is known to be a widespread in *Campylobacter* species. Charge trains (numerous spots with the same mass but different isoelectric points) were located at similar positions on the profiles of the six *Campylobacter* species tested, subsequently identified as flagellins proteins. The glycosylation of flagellin has been

widely documented and is thought to have an important role in virulence. Furthermore, studies have shown that glycosylation is necessary for fully functional flagella, motility and gut colonisation in the host (Wassenaar *et al.* 1991).

Although, a number of non-*jejuni/coli* isolates were identified during this study (confirmed by multiplex PCR) these were not identified to the species level. It was originally planned to send live cultures to collaborators at Ghent University, Belgium, so speciation using protein profiling using SDS-PAGE could be undertaken. However, there were unforeseen problems with obtaining authorisation for sending live cultures from the University; therefore only heat killed isolates could be sent for conformational analysis using multiplex-PCR. Unfortunately, although confirmation by PCR could confirm that the isolates were *Campylobacter* species (and differentiate *C. jejuni* and *C. coli* from other *Campylobacter* species), no solid conclusions could be drawn as to the which *Campylobacter* species were recovered from sewage and vegetable samples, and whether these species were those which have been associated with disease in humans. Fortunately, the PCR method permitted the identification of *Arcobacter* to the species level. This demonstrated the high prevalence of *A. butzleri* in both raw and ready-to-eat vegetable samples. *A. butzleri* was found to be inhibited by a number of antibiotics contained in Preston and Skirrow plating media. It is thought that this, along with other factors such as inability to grow at high temperatures (42°C), and misidentification as *Campylobacter* species is likely to have contributed to the low isolation rates of this organism reported in previous studies.

Originally, it was planned to evaluate a range of samples including clinical faecal samples and poultry processing water. However due to problems encountered with ethical approval and access to poultry processing units, these samples could not be investigated.

The most prevelant species isolated during these studies was *A. butzleri*, which was recovered from the majority of both sewage sludge (raw and treated) and salad vegetable (unprocessed and processed) samples tested. Such species distribution with predominance of *A. butzleri* mirrors the prevalence of arcobacters in human infections (Vandenberg *et al.* 2004). Recent evidence suggests that *Arcobacter*, especially *A. butzleri,* may be involved in humun enteric diseases. Moreover *Arcobacter* has occasionally been linked to cases of human extraintestinal disease. Arcobacters, notably *A. butzleri*, have for some time been implicated as zoonotic pathogens and one recent study determined these bacteria to be the fourth most frequently occurring epsilobacterium in human diarrhea (Vandenberg *et al.* 2004). This is the first study to isolate *A. butzleri* from processed salad vegetables.

In conclusion, this study has confirmed the assumptions that many of the currently available isolation media and incubation atmospheres used for the recovery of *Campylobacter* species are inadequate for the recovery of a number of emerging *Campylobacteraceae* species. Furthermore, novel isolation methods, including a universal incubation atmosphere, a plating medium and confirmatory identification tests, suitable for the recovery of emerging *Campylobacteraceae* species, and which can be applied to routine testing laboratories have been developed. Application of these methods to the recovery of *Campylobacteraceae* from sewage sludge and salad vegetables showed that non-*jejuni/coli Campylobacter* species could be recovered. Although the developed protocol permitted the recovery of a number of *Campylobactereaceae* from sewage and salad vegetables, a number of problems remain with the method, mainly with selectivity (presence of background flora) and sensitivity of detection.

Possibly, the most important finding was the prevalence of *Arcobacter* species on processed salad vegetables. The methods used permitted the isolation of *A. butzleri* from processed salad vegetables in high numbers, which is a major health risk to the consumber since *A. butzleri* has recently been implicated as an important and emerging human pathogen.

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APPENDIX

Table I. Identification of Presumptive *Campylobacteraceae* isolates from Sewage sludge using Biochemical, Immunological, Morphological and Molecular Techniques. + indicates positive results, - indicates negative result. +++ indicates strong positive results, -/+ indicates weak result. NS indicates non-specific agglutination reaction. Question mark adjacent to result indicates that this result is not in agreement with final molecular identification using PCR.

Isolate no.	Sample type	Date isolated	Biochemical				Immunological (Latex test)			Morphological		Presumptive ID	Molecular ID by PCR
			KOH test	L-ALA	Oxidase	Aerobic,	Therm	Non-	Control	EDIC microscopy	Thermotolerant		
8	MAD (Digested) sludge	02/08/2005	+	-	+	+	-	-+	-/+	small curved rods	+	*Arcobacter* spp.	*A. cryaerophilus*
9	MAD (Digested) sludge	02/08/2005	+	-	+	-	+	++	-	straight rods	++	Non-therm *Campylobacter* spp.	non-*jejuni/coli Campylobacter*
10	MAD (Digested) sludge	02/08/2005	+	-	-//+	+	+++?	+	++	gull, curved, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
11	MAD (Digested) sludge	02/08/2005	+	-	+	+	+	++	+	small curved rods	-	*Arcobacter* spp.	*A. cryaerophilus*
12	MAD (Digested) sludge	02/08/2005	+	-	+	+	++?	++	-	gull, curved, spiral rods	-	*Arcobacter* spp.	*A. cryaerophilus*
18	MAD (Digested) sludge	02/08/2005	+	-	-/+	+	+	++	+	small curved rods	-	*Arcobacter* spp.	*A. butzleri*
19	MAD (Digested) sludge	02/08/2005	+	-	+	-	NS	NS	NS	gull, curved, spiral rods	-	*Campylobacter* spp.	non-*jejuni/coli Campylobacter*
20	MAD (Digested) sludge	02/08/2005	+	-	+	-	NS	NS	NS	curved rods	-	*Campylobacter* spp.	*A. butzleri*
21	MAD (Digested) sludge	02/08/2005	+	-	+	-	+	++	+	curved rods	+	*Campylobacter* spp.	non-*jejuni/coli Campylobacter*
29	Pre-digested sludge	23/11/2005	+	-	+	-	+	++	-	curved rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
30	Pre-digested sludge	23/11/2005	+	-	+	-	+	++	-	small curved rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
31	Pre-digested sludge	23/11/2005	+	-	+	-	+	++	-	stumpy, gull, curved rods	+	Non-therm *Campylobacter* spp.	*A. butzleri*
32	Pre-digested sludge	23/11/2005	+	-	+	-	-	+	-	small curved rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
33	Pre-digested sludge	23/11/2005	+	-	+	-	-	+	-	small curved rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
34	MAD (Digested) sludge	23/11/2005	+	-	+	+	+	++	-	small curved rods	-	*Arcobacter* spp.	*A. cryaerophilus*
35	MAD (Digested) sludge	23/11/2005	+	-	+	+	-	-	-	small gull shaped rods	-	*Arcobacter* spp.	*A. cryaerophilus*
36	Pre-digested sludge	23/11/2005	+	-	+	+	-	++	-	small straight rods	-	Non-therm *Campylobacter* spp.	*A. cryaerophilus*
37	Pre-digested sludge	23/11/2005	+	-	+	-	+	++	-	some curved rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
38	Pre-digested sludge	23/11/2005	+	-	+	-	-	++	-	small gull shaped rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
39	Pre-digested sludge	23/11/2005	+	-	+	-	-	++	-	stumpy, curved, spial rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
40	Pre-digested sludge	23/11/2005	+	-	+	-	-	++	-	gull, curved, spiral rods	-	Non-therm *Campylobacter* spp.	*A. butzleri*
41	Pre-digested sludge	23/11/2005	+	-	+	-	NS	NS	-	straight rods	-	Non-therm *Campylobacter* spp.	*A. cryaerophilus*
42	Pre-digested sludge	23/11/2005	+	-	+	-	-?	+	-/+	curved rods	++?	Non-therm *Campylobacter* spp.	*Campylobacter jejuni*
43	Pre-digested sludge	23/11/2005	+	-	-/+	-	-	+	-/+	curved rods	-	Non-therm *Campylobacter* spp.	non-*jejuni/coli Campylobacter*
44	Pre-digested sludge	23/11/2005	+	-	+	+	+	++	-	curved rods, some cocci	-	*Arcobacter* spp	*A. butzleri*
47	Pre-digested sludge	09/11/2005	+	-	+	+	-	-/+	-	stumpy, curved, spial rods	-	*Arcobacter* spp	*A. butzleri*
48	S2	09/11/2005	+	-	+	+	-	-/+	-	curved, gull-shape rods	-	*Arcobacter* spp.	*A. butzleri*
49	S3	09/11/2005	+	-	+	-/+	-	-/+	-	stumpy, curved, spial rods	-	*Arcobacter* spp.	*A. butzleri*
50	s4	09/11/2005	+	-	+	-/+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
51	s5	09/11/2005	+	-	+	-/+	-	+	-	stumpy, curved, spial rods	-	*Arcobacter* spp.	*A. butzleri*
52	s6	09/11/2005	+	-	+	+	+	++	+	gull, curved, spiral rods	+	*Arcobacter* spp.	*non-jejuni/coli Campylobacter*
53	s7	09/11/2005	+	-	+	+	++?	++	+	stumpy, curved rods	+	*Arcobacter* spp.	*A. butzleri*
54	s8	09/11/2005	+	-	+	-/+	+	++	+	stumpy, curved, spial rods	++	Therm *Campylobacter* spp.	*non-jejuni/coli Campylobacter*
55	s9	09/11/2005	+	-	+	+	++	++	+	stumpy, curved rods	-	*Arcobacter* spp.	*A. butzleri*

Table I. Continued.

56	s10	09/11/2005	+	-	+	+	-	++	+	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
57	s11	09/11/2005	+	-	+	+	+	-	+	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
58	s12	09/11/2005	+	-	+	+	+	++	-	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
59	s13	09/11/2005	+	-	+	+	+	++	+	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
60	s6	19/12/2005	+	-	+	+	NS	NS	NS	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
61	s7	19/12/2005	+	-	+	+	-	++	-	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
62	s8	19/12/2005	+	-	+	+	NS	NS	NS	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
63	s9	19/12/2005	+	-	+	+	-	++	-	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
64	s10	19/12/2005	+	-	+	+	NS	NS	NS	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
65	s11	19/12/2005	+	-	+	+	-	++	-	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
66	s12	19/12/2005	+	-	+	+	-	++	-	gull, curved, spiral rods, cocci	-	*Arcobacter* spp.	*A. butzleri*
67	s13	19/12/2005	+	-	+	+	-	++	-	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
68	s14	19/12/2005	+	-	+	+	NS	NS	NS	curved, straight, spiral rods	-	*Arcobacter* spp.	*A. butzleri*
69	s15	19/12/2005	+	-	+	+	-	++	-	curved stumpy rods	-	*Arcobacter* spp.	*A. butzleri*
70	S1	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
71	S2	16/01/2006	+	-	+	+	-	++	-	straight, curved rods	-	Arcobacter spp.	*A. butzleri*
72	S4	16/01/2006	+	-	+	+	-	++	-	straight, curved rods	+	Arcobacter spp.	*A. butzleri*
73	S5	16/01/2006	+	-	+	+	-	++	-	straight, curved, spiral rods,	-	Arcobacter spp.	*A. butzleri*
74	S6	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	++	Arcobacter spp.	*A. butzleri*
75	S7	16/01/2006	+	-	+	-	-	++	-	curved, straight, spiral rods	+	Non-therm *Campylobacter* spp.	*non-jejuni/coli Campylobacter*
76	s8	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
77	s9	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
78	s10	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	++?	Arcobacter spp.	*A. butzleri*
79	s11	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
80	s12	16/01/2006	+	-	+	-	-	++	-	curved, straight, spiral rods	-	Non-therm *Campylobacter* spp.	*non-jejuni/coli Campylobacter*
81	s13	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
82	s14	16/01/2006	+	-	+	-	-	++	-	curved rods	++	Non-therm *Campylobacter* spp.	*non-jejuni/coli Campylobacter*
83	s15	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
84	s16	16/01/2006	+	-	+	+	-	++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
85	s17	16/01/2006	+	-	+	+	-	-/+	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
86	s18	16/01/2006	+	-	+	+	-	-/+	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
87	s19	16/01/2006	+	-	+	+	-	-/+	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
88	s20	16/01/2006	+	-	+	+	+	+++	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
89	s21	16/01/2006	+	-	+	+	-	+	-	curved, straight, spiral rods	-	Arcobacter spp.	*A. butzleri*
90	s22	16/01/2006	+	-	+	-	-	+	-	curved rods	-	Non-therm *Campylobacter* spp	*A. butzleri*
91	s23	16/01/2006	+	-	+	+	-	++	-	curved rods	-	Arcobacter spp.	*A. butzleri*
92	s24	16/01/2006	+	-	+	+	-	++	-	curved rods	-	Arcobacter spp.	*A. butzleri*
93	s25	16/01/2006	+	-	+	+	-	++	-	curved, gull-shape rods	-	*Arcobacter* spp.	*A. butzleri*
94	s26	16/01/2006	+	-	+	+	-	++	-	curved, gull-shape rods	++?	*Arcobacter* spp.	*A. butzleri*
95	s27	16/01/2006	+	-	+	+	-	++	-	curved, gull-shape rods	-	*Arcobacter* spp.	*A. butzleri*

Table II. Identification of Presumptive *Campylobacteraceae* isolates from Salad Vegetables using Biochemical, Immunological, Morphological and Molecular techniques. + indicates positive results, - indicates negative result. +++ indicates strong positive results, -/+ indicates weak result. NS indicates non-specific agglutination reaction. Question mark adjacent to result indicates that this result is not in agreement with final molecular identification using PCR.

Isolate no.	Sample type	Date isolated	Biochemical				Immunological (Latex test)			Morphological		Presumptive ID	Molecular
			KOH test	L-ALA	Oxidase	Aerobic,	Therm	Non-	Control	EDIC microscopy	Thermophilic PNA		PCR ID
1	Pro watercress,	17/08/2005	+	-	+	+	-	-/+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
2	Pro watercress,	17/08/2005	+	-	+	+	+?	+	-	curved, gull-shaped rods	++?	*Arcobacter* spp.	*A. butzleri*
3	Pro watercress,	17/08/2005	+	-	+	+	NS	NS	NS	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
4	Pro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
5	Pro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	++?	*Arcobacter* spp.	*A. butzleri*
6	Pro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
7	Pro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
8	Pro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
9	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
10	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
11	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
12	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	+	*Arcobacter* spp.	*A. butzleri*
13	Unpro watercress,	17/08/2005	+	-	+	+	++?	++	+	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
14	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
15	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
16	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	+	*Arcobacter* spp.	*A. butzleri*
17	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods, some spiral	-	*Arcobacter* spp.	*A. butzleri*
18	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-/+	*Arcobacter* spp.	*A. butzleri*
19	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
20	Unpro watercress,	17/08/2005	+	-	+	+	-	-/+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
21	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
22	Unpro watercress,	17/08/2005	+	-	+	+	-	-/+	-	curved rods	-/+	*Arcobacter* spp.	*A. butzleri*
23	Unpro watercress,	17/08/2005	+	-	+	+	-	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
24	W31	28/09/2005	+	-	+	+	-	+	-	curved rods	+	*Arcobacter* spp.	*A. butzleri*
25	W32	28/09/2005	+	-	+	+	+?	+	-	curved rods	++?	*Arcobacter* spp.	*A. butzleri*
26	W33	28/09/2005	+	-	+	+	+	+	-	curved rods	-	*Arcobacter* spp.	*A. butzleri*
27	W34	28/09/2005	+	-	+	-	-	+	-	curved rods, some spiral	+	Non-therm *Campylobacter*	non-*jejuni/coli*
28	W35	28/09/2005	+	-	+	-	-	+	-	curved rods, some spiral	+	Non-therm *Campylobacter*	non-*jejuni/coli*
29	1 watercress	20/10/2005	+	-	+	+	-	+	-	curved, gull-shaped rods	-	*Arcobacter* spp.	*A. butzleri*
30	2 watercress	20/10/2005	+	-	+	+	-	-?	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
31	3 watercress	20/10/2005	+	-	+	+	-	-?	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
32	4 watercress	20/10/2005	-/+	-	+	+	-	-?	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*

Table II. Continued.

33	5 watercress	20/10/2005	+	-	+	+	-	-?	-	curved rods, some cocci	+	*Arcobacter* spp.	*A. butzleri*
35	unpro watercress	17/11/2005	+	-	+	-	+++	+++	-	curved rods, some spiral	++	Therm *campylobacter* spp.	*Campylobacter*
36	pro watercress	17/11/2005	+	-	+	-	+++	+++	+	curved rods, some spiral	++	Therm *campylobacter* spp.	*Campylobacter*
39	unpro watercress	17/11/2005	+	-	+	-	-	+++	-	curved rods, some spiral	+	Non-therm *Campylobacter* spp.	non-*jejuni/coli*
43		19/12/2005	+	-	+	-	++	++	-	curved rods, some spiral	+	Therm *Campylobacter* spp.	*Campylobacter*
44		19/12/2005	+	-	+	-	+	++	-	curved rods, some spiral	+	Therm *Campylobacter* spp.	*Campylobacter*
45		19/12/2005	+	-	+	-	++	++	+	curved rods, some spiral	+	Therm *Campylobacter* spp.	*Campylobacter*
46		19/12/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
47		19/12/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
48		19/12/2005	+	-	+	+	-	++	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
49		19/12/2005	+	-	+	+	+	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*
50		19/12/2005	+	-	+	+	-	+	-	curved rods, some cocci	-	*Arcobacter* spp.	*A. butzleri*

Table III. Identification of Protein Spots by Tandem Mass Spectrometry. Data generated by sequence matches to databases using Protein Lynx software.

Spot	Protein description	Mass	Accission no.	Sequence	coverage	confidence	Peptides	score	Protein ID no.
	alkyl hydroperoxide reductase *Campylobacter*	1310.7	15791702	NTPVNQGGIGQVK	28.28	100	5	149.5	1-4,6
1	hypothetical protein Pro0087	1440.8	33239539	DQAIGEIPDVKTR	10.4	100	1	34.5	5
	alkyl hydroperoxide reductase *Campylobacter*	1002.6	15791702	FPLVADLTK	21.72	100	4	131.4	1,11,25
	heat shock protein dnaK *Campylobacter*	1580.8	15792097	SLGNFNLEGIPPAPR	21.67	100	10	110.7	2,3,8-10,16,17,20,21,24
	flagellin A *C. jejuni*	1911.9	3290050	LMEELDNIANTTSFNGK	7.34	33.3	3	103.7	4,12,13,18
2	putative methyl accepting chemotaxis	1266.6	15792869	TPGNTFLFDQK	7.4	100	3	82.4	14,15,22,23
	flagellin A *Campylobacter jejuni*	1487.7	540931	NYNGIDDFKFQK	20	100	7	233.2	1-7, 14,15
	60 kDa chaperonin *Campylobacter jejuni*	1574.8	54299876	EIAQVATISANSDTR	15	100	2	56.1	9,12,13
3	flagellin B *Campylobacter jejuni*	1399.6	441236	DVDFAAESANFSK	13.91	100	5	52.6	11
	flagellin B *Campylobacter jejuni*	1399.6	15792661	DVDFAAESANFSK	22.78	100	7	161.9	2,5-13,15,17,18
	60 kDa chaperonin *Campylobacter*	1574.8	54299876	EIAQVATISANSDTR	28.33	100	4	184.6	1,3,4,10,14,16
4	flaggellin A *Campylobacter jejuni*	1487.7	14861506	NYNGIDDFKFQK	14.21	100	5	122.2	1,2,5,6
6	**flagellin A *Campylobacter jejuni***	1487.7	3290044	NYNGIDDFKFQK	6.26	100	3	142.7	1,6
7	**trigger factor peptidyl prolyl cis trans Isomerase**	1434.7	15791580	ELTQDAEQNLFK	17.12	100	8	167.3	1,2,4-7,11,13
	putative MCP type signal transduction	1487.7	15792435	SGLEDVNQSVELAK	32.17	100	9	242.9	1-3,5,7,9,14,15,17
8	malate oxireductase *Campylobacter*	1749	15792610	ALAPTVGGINLEDIAAPK	11.1	100	3	78.1	8,10,13
	malate oxireductase *Campylobacter*	1412.79	15792610	NLGVENIILVDSK	19.71	100	6	153.7	1,6
9	putative MCP type signal transduction	1487.8	15792435	SGLEDVNQSVELAK	5.83	100	2	73.31	1,2,5
	3 oxoacyl acyl carrier protein synthase	1614.9	15791809	VVTGIGMINALGLDK	13.37	100	4	121.8	1,2,4,13
	alkyl hydroperoxide reductase *Campylobacter*	1002.58	15791702	FPLVADLTK	27.27	100	5	103.6	3
10	malate oxireductase *Campylobacter*	1412.8	15792610	NLGVENIILVDSK	6.81	100	2	58.64	5, 6,14
11	**oxireductase *Campylobacter jejuni***	1531.8	15791920	IINFNANSVLGNEK	41.16	100	9	310.7	1-6,9,10,11
	oxireductase *Campylobacter jejuni*	1529.7	15791920	LGIDINEVEDDNGK	12.86	100	3	117.2	1,6,7
12	ATP synthase F1 sector beta subunit	1489.7	15791495	GIYPAVDPLDSTSR	6.24	100	2	64.98	4,8
14	**nifU protein homolog *Campylobacter***	1323.65	15791611	ITNLDVEFAMR	6.81	100	2	85.25	1,2
15	**chemotaxis protein Campylobacter**	1412.6	15791655	TGSNEMELVDFR	8.8	100	2	66.16	2,5
16	**major outer membrane protein *Campylobacter***	1840	1086940	TPLEEAIKDVDVSGVLR	54.84	100	1	66.45	1
	predicted hypothetical protein X	841.5	51460838	SIAVSIPR	0.55	100	1	72.9	1
	COG1316 transcriptional regulator	869.53	45512537	SLVVSLPR	2.37	100	1	55.43	2
17	**major outer membrane protein *Campylobacter***	1112.55	9965857	VGADFVYGGTK	8.22	8.7	2	50.35	3,4
19	**alkyl hydroperoxide reductase *Campylobacter***	1479.8	15791702	NFDVLYAEAVALR	16.67	100	3	70.53	1,2,4
	COG1994 Zn dependant proteases	855.52	46317991	ITAVLSPR	3.32	50	1	45.66	1
	hypothetical transmembrane protein	855.52	17547243	ITAVLSPR	3.32	50	1	45.66	1
	COG1316 transcriptional regulator	869.53	45512537	SLVVSLPR	2.37	100	1	39.72	2
20	chemotaxis protein *Campylobacter*	1341.71	15791653	GNVMPLIDLAQR	6.94	100	1	36.02	3
21	**inorganic pyrophosphatase *Campylobacter***	1529.9	34558091	LNAVIEIPYGSNIK	7.74	47.5	1	47.62	1,2
26	**ATP synthase F1 sector beta subunit**	1500.77	15791495	IALTGLTMAEYFR	7.96	100	3	62.3	1,3,4
28	**elongation factor TU *Campylobacter***	1667.95	15791834	ITVSLIAPVALEEGTR	26.32	100	6	142	1,2,4-6