Campylobacter species in dogs and cats and significance to public health in New Zealand

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Abstract

Campylobacter spp. are a major cause of bacterial gastroenteritis in people in the developed world, including New Zealand. Many sources and transmission routes exist, as these bacteria are common in animals and the environment. *C. jejuni* is most frequently associated with poultry whereas *C. upsaliensis* and *C. helveticus* with dogs and cats, respectively. Published data on *Campylobacter* in dogs and cats in New Zealand and on the pathogenic potential of *C. upsaliensis* and *C. helveticus* are very limited. This thesis investigated the prevalence of *Campylobacter* spp. in household dogs and cats in Manawatu region, New Zealand, and in raw meat pet food commercially available in Palmerston North, New Zealand. Five *Campylobacter* spp. were isolated and the prevalence rates were significantly influenced by the culture methods used. *C. upsaliensis* and *C. helveticus* were most frequently detected from dogs and cats, respectively and *C. jejuni* in pet food samples. An expanded panel of culture methods was used to screen working farm dogs and their home-kill raw meat diet in Manawatu. This study reported three *Campylobacter* spp. and *Helicobacter winghamensis* as being isolated from dogs for the first time. The culture methods were again shown to bias the prevalence estimates. The isolates of *C. upsaliensis* and *C. helveticus* from the household pets study and *C. hyointestinalis* from locally farmed deer were used in a study to investigate the analytical sensitivity in spiked human clinical faecal samples using the ProSpecT^{TM} *Campylobacter* Microplate Assay test that was developed for detection of *C. jejuni/coli*. The results showed the ability of the test to detect all three species and showed the influence of bacterial dose, faecal consistency and of the individual faecal samples on the test results. Further studies investigated the pathogenic potential of *C. upsaliensis* and *C. helveticus* in comparison to *C. jejuni* using an insect model of disease, *Galleria mellonella*, and whole-genome analyses, respectively. The results of the survival analysis in the *G. mellonella* study indicated that *C. upsaliensis* and *C. helveticus* have pathogenic potential, but to a lesser extent than *C. jejuni.* Additionally, several variables of experimental design were shown to significantly influence estimates of hazard rates in survival analysis. Whole genome analyses also showed indications of the pathogenic potential of *C. upsaliensis* and *C. helveticus* relative to *C. jejuni,* and how it varies between and within species in association with the core and accessory genomes, functional gene content profiles, and documented and predicted pathogenic proteins. This thesis has furthered our understanding of the epidemiology, detection, and pathogenicity of *Campylobacter* spp. in dogs, cats and humans, and confirmed raw meat animal food as a potential source of *Campylobacter* spp. for both people and animals.

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This work is dedicated to dogs and cats.

Publications

- **Zoonoses and Public Health** (*in press*) ‘Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand’ K Bojanić, AC Midwinter, JC Marshall, LE Rogers, PJ Biggs, E Acke
- **Journal of Microbiological Methods (2016 Vol. 127, p236-241)** ‘Analytical sensitivity of the ProSpecT® Campylobacter Microplate Assay for detection of *C. upsaliensis*, *C. helveticus* and *C. hyointestinalis* in spiked human clinical faecal samples’ K Bojanić, AC Midwinter, JC Marshall, LE Rogers, PJ Biggs, E Acke
- **CHRO conference 2015** (Rotorua, New Zealand) Research Abstract ‘Pathogenicity of *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus* in the invertebrate disease model *Galleria mellonella*’ K Bojanić, AC Midwinter, PJ Biggs, JC Marshall, E Acke
- **CHRO conference 2015** (Rotorua, New Zealand) Poster Abstract ‘Diagnosis of campylobacteriosis in humans from the Manawatu, New Zealand’ K Bojanić, A Nohra, LE Rogers, J Delores-Main, JC Marshall, PJ Biggs, AC Midwinter, E Acke
- **ECVIM 2015** (Lisbon, Portugal) Poster abstract ‘Pathogenicity investigation of *Campylobacter jejuni*, *C. upsaliensis* and *C. helveticus* isolated from dogs and cats using *Galleria mellonella* larvae’ K Bojanić, AC Midwinter, PJ Biggs, J Marshall, E Acke

- **ECVIM 2014** (Mainz, Germany) Poster abstract 'Whole-genome analyses of *Campylobacter upsaliensis* and *C. helveticus* isolated from dogs and cats and *in silico* investigation of pathogenic potential' K Bojanić, AC Midwinter, PJ Biggs, NP French, E Acke
- **NZVA conference 2014** (Hamilton, NZ) Invited Research lecture 'Canine diarrhoea – *Campylobacter* conundrum' K Bojanic, AC Midwinter, PJ Biggs, E Acke
- **CHRO conference 2013** (Aberdeen, UK) Research abstract 'Comparison of six culture protocols for isolation of *Campylobacter* spp. from faecal and meat samples' K Bojanic, AC Midwinter, L Rogers, PJ Biggs, E Acke
- **NZVA conference 2013** (Palmerston North, New Zealand) Research abstract '*Salmonella*, *E. coli* and *Campylobacter* spp. in Working Farm Dogs in New Zealand and their Home-Kill Diets' K Bojanic, AC Midwinter, PJ Biggs, J Benschop, N Cave, E Acke
- **WSAVA conference 2013** (Auckland, New Zealand) **Winner of WSAVA Global One Health Congress Recognition Prize** Research Abstract '*Campylobacter* spp. in dogs and cats in New Zealand' K Bojanic, AC Midwinter, E Kwan, PJ Biggs, E Acke
- **International Sheep and Beef Veterinary Congress 2013** (Rotorua, New Zealand) Research presentation '*Salmonella*, *E. coli* and *Campylobacter* spp. in Working Farm Dogs in New Zealand and their Home-Kill Diets' K Bojanic, AC Midwinter, PJ Biggs, J Benschop, N Cave, E Acke

- **Infectious Disease Research Centre Science Symposium 2012** (Palmerston North, New Zealand) Research Poster ‘ProSpecT *Campylobacter* Microplate Assay: first results but second thoughts?’ K Bojanic, AC Midwinter, J Marshall, L Rogers, PJ Biggs, E Acke
- **ECVIM 2012** (Maastricht, The Netherlands) Research Abstract ‘ProSpecT Elisa in the diagnosis of *Campylobacter* spp. infections’ K Bojanic, AC Midwinter, L Rogers, PJ Biggs, E Acke

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List of abbreviations

General

CC	Clonal Complex
COG	Cluster of Orthologous Groups
CoxPH	Cox proportional hazard
Ctrl	Control larvae
DALY	Disability-adjusted life year
DNA	Deoxyribonucleic acid
EIA	antigen test / ProSpecT® Campylobacter Microplate Assay
Fig.	Figure
HL	High bacterial inoculum load
ID	Identity number
KM	Kaplan-Meier
LL	Low bacterial inoculum load
LOD	Limit of detection
ML	Medium bacterial inoculum load
MLST	Multilocus sequence typing
NAAT	Nucleic acid-based test
PBS	Phosphate buffered saline
PBS-ctrl	Phosphate buffered saline-inoculated larvae control
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rMLST	Ribosomal multilocus sequence typing
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
spp.	Species
SSF	Semi-solid faeces
ST	Sequence type
VBNC	Viable but non culturable
WF	Watery faeces

COG-specific functional groups

A	RNA processing and modification
B	Chromatin structure and dynamics
C	Energy production and conversion
D	Cell cycle control, cell division, chromosome partitioning
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
G	Carbohydrate transport and metabolism
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
J	Translation, ribosomal structure and biogenesis
K	Transcription
L	Replication, recombination and repair
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
O	Posttranslational modification, protein turnover, chaperones
P	Inorganic ion transport and metabolism
Q	Secondary metabolites biosynthesis, transport and catabolism
R	General function prediction only
S	Function unknown
T	Signal transduction mechanisms
U	Intracellular trafficking, secretion, and vesicular transport
V	Defense mechanisms
W	Extracellular structures
X	Mobilome: prophages, transposons
Y	Nuclear structure
Z	Cytoskeleton

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CHAPTER 1

1. Introduction

1.1. General background

The importance of *Campylobacter* spp. to humans is due to it being the most common bacterial cause of acute gastroenteritis in the developed world (1-4), including New Zealand (5, 6). *Campylobacter*-associated gastroenteritis is primarily ascribed to *C. jejuni* and to a lesser extent *C. coli,* with the two species being responsible for around 80-95% and 5-15% of confirmed human infections respectively, and other species accounting for the remainder (3, 7). The disease most commonly affects children under the age of five, young adults, and immunocompromised and elderly people in the developed world (8, 9). In developing countries, clinical disease mostly affects children under two years of age and adults rarely suffer from disease but may have asymptomatic excretion which is rare in the developed world (2). Clinical signs may range from mild self-limiting enteritis to severe haemorrhagic diarrhoea with vomiting, abdominal pain and fever (10). Complicated disease may develop with bacteraemia, post-infectious sequelae such as Guillain-Barré syndrome, Reiter's syndrome, reactive arthritis, and irritable bowel syndrome (9, 11). By using the disability-adjusted life years (DALY) metric, the global health burden of *Campylobacter* was estimated at 8.4% of the total burden of diarrheal diseases, which are ranked as the fourth leading cause of DALYs worldwide (12).

Campylobacter spp. have been isolated from many domestic animals including poultry, cattle, swine, sheep, dogs, and cats, as well as wild animals of mammalian, avian and reptile species (3, 13). Infection of humans occurs most commonly through exposure by consuming contaminated food, or water, or by handling contaminated material and direct contact with animals (9). Meta-analysis of extensive epidemiological research has shown that the eating of undercooked chicken meat, unpasteurised dairy products, environmental sources (drinking water, contact with recreational waters and bird droppings), travel, direct contact with farm and pet animals, and poor food hygiene practices are significant risk factors for contracting campylobacteriosis (14).

C. jejuni was the first *Campylobacter* spp. isolated from dogs (10) and from cats (15). Since then many other *Campylobacter* spp. have been detected in dogs and cats with large variations in reported prevalence rates. The most commonly reported species include *C. jejuni*, *C. upsaliensis*, *C. helveticus* and *C. coli* (16). The clinical significance of *Campylobacter* spp. in pets is less clear compared with humans in the developed world. Clinical signs have been mostly associated with *C. jejuni* and occur mainly in young and stressed animals, or opportunistically with the presence of concurrent diseases (16). *C. upsaliensis* and *C. helveticus* are weakly associated with disease in pets but have been the most frequently detected *Campylobacter* spp. in dogs and cats, respectively and are rarely detected in other sources (13, 17). As dogs and cats are common companion animals worldwide, the exposure of humans to *C. upsaliensis* and *C. helveticus* is likely to be frequent. However, the pathogenic potential of *C. upsaliensis* and *C. helveticus* to humans remains to be further investigated, thus these species are frequently being referred to as “emerging” *Campylobacter* pathogens (13).

Apart from *C. upsaliensis* and *C. helveticus*, other species such as *C. lari*, *C. hyointestinalis*, *C. concisus* and *C. ureolyticus*, are also considered emerging *Campylobacter* pathogens (13, 18). The clinical importance of the emerging *Campylobacter* spp. started to be recognised in parallel with the research and development of new detection methods, notably microbiological culture methods. *Campylobacter* spp. are fastidious organisms that are difficult to isolate. After

Dekeyser and colleagues (19) pioneered a successful method for isolation of *Campylobacter* spp. from faecal specimens in 1969, reports of *C. jejuni* as the most frequent bacterial cause of acute gastroenteritis ensued worldwide over the next decade (9). Over the following decades a multitude of different culture methods were investigated and further developed. Initially these methods were directed at achieving the enhanced isolation of *C. jejuni*, but through this research more and more new *Campylobacter* spp. were discovered. In parallel with our increased knowledge about *Campylobacter* spp., an awareness of it's complex diversity rose. Species within the *Campylobacter* genus exhibit a vast diversity in growth requirements such as incubation temperatures, atmospheric conditions, length of incubation, nutrient requirements, and their different susceptibilities to antimicrobial agents (20). Emerging *Campylobacter* spp. are nowdays considered potentially under-recognised as a cause of disease in humans due to most methods having been optimised for detection of *C. jejuni* and *C. coli* (17, 18).

The successful methodology for isolation has opened numerous avenues for research of the epidemiology, pathobiology and ecology of *Campylobacter* spp. The epidemiology of the disease has benefited and advanced over the years of investigations, whereas the pathogenesis of the disease is still not fully understood. The pathogenic mechanisms of *Campylobacter* spp. are an active area of research. Models for investigation of pathogenicity include *in vivo* animal models and *ex vivo* eukaryotic cell cultures, complemented with molecular biology tools such as mutagenesis and recombinant DNA techniques, DNA microarray and genomic and other ~omics studies (21). Many virulence mechanisms and genes have been described such as the cytolethal distending toxins, adhesion to, and invasion of the intestinal epithelial cells, as well as the importance of flagella for motility and invasion (22, 23). Notwithstanding the advancements made, many questions remain unanswered and the research community coined the term "*Campylobacter* conundrum". It stands for the enigma of how this microaerophilic bacterium, possesing a small genome and lacking most of the well-known regulatory systems compared to other bacterial enteric pathogens, yet still constitutes a major hazard for humans (24). Why does the incidence of disease remain so high compared to other bacterial enteric pathogens (25)?

1.2. Thesis structure and format

This thesis is presented as a series of ten Chapters. Five discrete research Chapters are written in the form of manuscripts for peer-reviewed publication and are encompassed by the General Introduction, Literature Review, and General Discussion. The last two Chapters are the collated literature cited and the Appendix with the supplemental information organised by Chapters.

Chapter One

General Introduction presents a summary of general features of campylobacteriosis in humans, dogs and cats, and of *Campylobacter* bacteria. The structure and formatting of this thesis is summarised at the end of this Chapter.

Chapter Two

Literature Review describes current knowledge of *Campylobacter* bacteria and its epidemiology in humans, dogs and cats. The review of ^{m}EpiLab records is summarised at the end of this Chapter and is followed by the research aims of this thesis.

Chapter Three

Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand is *in press* in the journal *Zoonoses and Public Health*.

Chapter Four

Isolation of emerging *Campylobacter* species in working farm dogs and their frozen home-kill raw meat diets is to be submitted to the *Journal of Veterinary Diagnostic Investigations*.

Chapter Five

Variation in the limit-of-detection of ProSpecT *Campylobacter* Microplate enzyme immunoassay in stools spiked with emerging *Campylobacter* species has been published in the *Journal of Microbiological Methods* (Bojanić et al. 2016).

Chapter Six

Comparison of the pathogenic potential of emerging *Campylobacter* spp. using larvae of *Galleria mellonella* as an infection model is to be submitted to journal *Virulence*.

Chapter Seven

Whole Genome Comparison of *Campylobacter upsaliensis*, *C. helveticus* and *C. jejuni* is to be submitted to journal *Plos ONE*.

Chapter Eight

General Discussion and future research summarises the significant findings of this thesis and discusses the shortcomings and advantages of the methods used. The relevance and implications of the results with an outlook for future studies conclude this chapter.

Chapter Nine are collated cited literature according to the Journal of Clinical Microbiology style.

Chapter Ten is an Appendix of supplementary material organised by Chapters and statements of contribution to doctoral thesis containing publications of published Chapters.

CHAPTER 2

2. Literature review

2.1. *Campylobacter*

2.1.1. Historical overview

The first description of campylobacteriosis is thought to be a report of vibrio-like spiral organisms in the faeces of children with enteric disease by Theodor Escherich in 1886 (cited in 9). The bacteria were not isolated but microscopically observed and then thought not to have had an aetiological role in the disease. Similarly, in veterinary medicine, a report of epizootic abortions in ewes described frequent isolation of vibrio-like organisms from aborted foetuses (26) and in 1919 the isolation of similar organisms from bovine foetuses lead Smith to propose the name *Vibrio fetus* (27). In 1931 Jones et al. (28) described a similar organism involved in winter dysentery of calves and proposed the name *Vibrio jejuni* after which Doyle described similar organisms in swine dysentery in 1944 (29). In 1949, Stengenga documented the role of *V. fetus venerealis* in the epizootic sterility of cows and in 1959 Florent described the differentiation of two types, *V. fetus venerealis* and *V. fetus intestinalis* based on biochemical and pathogenic characteristics (cited in 9). During that time of the first half of the 20th century, infections in humans were also described as *Vibrio*-like. In 1938 in Illinois, a milk-borne outbreak of diarrhoeal illness was reported with organisms similar to *'Vibrio jejuni'* isolated from broth cultures from the blood of affected patients. Faecal cultures were unsuccessful but vibrio-like organisms were microscopically observed (30). In 1947 Vinzent reported *Vibrio fetus* isolation from

the blood of three pregnant women, two of whom aborted (cited in 9). During the 1950's working on characterisation of strains from human infections, King (31, 32) reported a 'related vibrio' to the *V. fetus* described by Vincent et al. (1947) but with different biochemical and antigenic characteristics. Through these studies, two types were proposed to be associated with enteric disease, the first *V. fetus* and the second, 'related vibrio' that was more thermophilic in nature. The name *Campylobacter* was first proposed by Sebald and Véron in 1963 to distinguish these vibrio-like organisms from *Vibrio* spp. due to differences in the guanine and cytosine (GC) content and their inability to utilise sugars (33). Reports of infections in people were still infrequent, as organisms had only been successfully isolated from blood of bacteraemic patients but not from stool specimens.

The breakthrough occurred when Dekeyser and Butzler and their team managed to isolate the 'related vibrio' from the faeces and blood of a 20-year old woman with severe diarrhoea and fever (19). The patient had no underlying diseases and no other enteric pathogen was detected. The crucial step that enabled isolation from faeces was the use of a differential filtration technique. That is, the faecal suspensions were filtered through 0.65µm filters and the filtrate inoculated onto a selective agar plate. In the following years, using this new technique the team reported an isolation rate of *C. jejuni* from 5.3% of 3800 children with diarrhoea and only 1.6% from 7200 healthy individuals (34). In the children with diarrhoea, specific complement-fixing antibodies to the *C. jejuni* isolated from stools were reported (cited in 9) and testing of strains for antibiotic sensitivity showed susceptibility of isolates to erythromycin (35). Since erythromycin has little effect on other common intestinal pathogens, the combination of resolution of clinical signs and of faecal excretion of *C. jejuni* following treatment became a therapeutic diagnostic test. Another advancement made by Skirrow and colleagues was the formulation of a compound selective agar without the need of the cumbersome filtration technique, further facilitated the isolation of *Campylobacter* species (10). With more and more reports ensuing worldwide, by the mid-1980's campylobacteriosis became known as the most frequent bacterial gastroenteritis occurring in humans. Morphological appearance of *C. jejuni* is shown in Fig. 2.1.

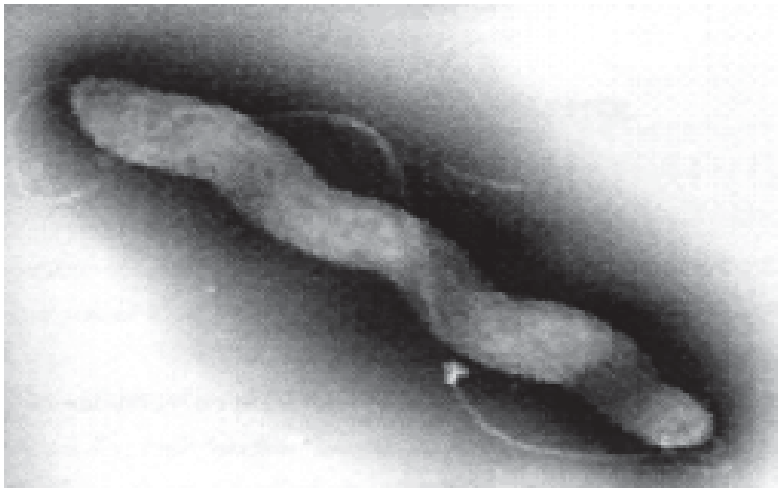

Fig. 2.1. Scanning electron micrograph of *Campylobacter jejuni*.

(Source: http://www.cdc.gov/)

2.1.2. Taxonomy and microbiology

The *Campylobacter* genus is part of the *Campylobacteraceae* family, together with the *Arcobacter, Sulfurospirilium* and *Thiovulum* genera, which with the *Helicobacteraceae* family form the Campylobacterales order within the Epsilon class of the Proteobacteria phylum (36, 37). The *Campylobacter* genus is currently comprised of 29 species, not including subspecies (bacterio.net last accessed on 15th June 2016), which are presented in Table 2.1.

Table 2.1. Identified sources of *Campylobacter* spp.[a] and association with reported diseases in animals and humans.

Taxon	Known animal source(s)	Human disease association	Animal disease association
C. avium	Chicken, turkey	None as yet	None as yet
C. canadensis	Whooping crane	None as yet	None as yet
C. coli	Pig, chicken, cattle, sheep, duck, goat, dog, turkey, ostrich, monkey	Gastroenteritis, cholecystitis, septicaemia, meningitis, abortion	Gastroenteritis, infectious hepatitis
C. concisus	Human, cat, dog	Gastroenteritis, inflammatory bowel disease, esophagitis, arthritis, brain abscess, periodontitis	None as yet
C. corcagiensis	Lion-tailed macaque	None as yet	None as yet
C. cuniculorum	Rabbit	None as yet	None as yet
C. curvus	Human, dog	Gastroenteritis, abscesses, esophagitis, periodontitis, septicaemia	None as yet
C. fetus subsp. *fetus*	Cattle, sheep, horse, turtle, kangaroo	Gastroenteritis, septicaemia, abscesses, abortion, meningitis, cellulitis, endocarditis	Abortion
C. fetus subsp. *venerealis*	Cattle, sheep	Septicaemia	Infertility
C. fetus subsp. *testudinum*	Turtles, skink, snake, human	Gastroenteritis, abscesses, bacteraemia	None as yet

Taxon	Known animal source(s)	Human disease association	Animal disease association
C. gracilis	Human, dog	Crohn's disease, ulcerative colitis, periodontitis, abscesses	None as yet
C. helveticus	Cat, dog, pig	Gastroenteritis, periodontitis	Gastroenteritis
C. hominis	Human	Crohn's disease, ulcerative colitis, bacteraemia	None as yet
C. hyointestinalis subsp. *hyointestinalis*	Pig, cattle, deer, sheep, dog, hamster	Gastroenteritis, septicaemia	Enteritis
C. hyointestinalis subsp. *lawsonii*	Pig	None as yet	None as yet
C. iguaniorum	Turtle, lizard	None as yet	None as yet
C. insulaenigrae	Seal, porpoise, sea lion	Gastroenteritis, septicaemia	None as yet
C. jejuni subsp. *doylei*	Human	Gastroenteritis, septicaemia	None as yet
C. jejuni subsp. *jejuni*	Chicken, numerous wild birds, cattle, sheep, pig, goat, dog, cat, monkey, mink, seal, insects	Gastroenteritis, septicaemia, Guillain-Barré syndrome, reactive arthritis, Miller-Fisher syndrome, cholecystitis, inflammatory bowel disease, irritable bowel syndrome, celiac disease, urinary tract infection, haemolytic uremic syndrome, meningitis	Gastroenteritis, abortion, hepatitis
C. lanienae	Cattle, sheep, pig	None as yet	None as yet
C. lari subsp. *concheus*	Shellfish	Gastroenteritis	None as yet

Taxon	Known animal source(s)	Human disease association	Animal disease association
C. lari subsp. *lari*	Wild birds, shellfish, chicken, cattle, sheep, dog, horse, seal, monkey	Gastroenteritis, septicaemia	Avian gastroenteritis
C. mucosalis	Pig, dog	Gastroenteritis	Porcine enteritis
C. peloridis	Shellfish	Gastroenteritis	None as yet
C. rectus	Human, dog	Gastroenteritis, Crohn's disease, ulcerative colitis, periodontitis, abscesses	None as yet
C. showae	Human, dog	Periodontitis, Crohn's disease, ulcerative colitis, abscess	None as yet
C. sputorum	Human, cattle, pig, sheep, dog	Gastroenteritis, abscesses	Abortion
C. subantarcticus	Albatross, penguin	None as yet	None as yet
C. troglodytis	Chimpanzee	None as yet	None as yet
C. upsaliensis	Dog, cat, cattle, birds, rodents	Gastroenteritis, septicaemia, abscesses, abortion	Gastroenteritis
C. ureolyticus	Human, cattle, poultry, horse	Gastroenteritis, abscesses, ulcers, Crohn's disease, ulcerative colitis	None as yet
C. volucris	Black-headed gull	None as yet	None as yet

[a] The data has been summarised from the cited literature in the Literature review Chapter.

Campylobacter spp. are small (1.5 to 5 µm long and 0.2 to 0.5 µm in diameter), curved to spiral gram-negative motile rods (see Fig. 2.1.) (38). Many of these species are thermophilic which denotes their optimal growth at higher incubating temperatures. For *in vitro* growth, 42 and 37°C are mostly used which reflects the temperature of their avian and mammalian hosts respectively (20). *C. fetus* is an exception with good growth reported at around 25°C (39). The atmospheric conditions for optimal growth include a decreased concentration of oxygen and increased concentration of carbon dioxide for which they are designated as 'microaerophilic' bacteria. Some species such as *C. rectus*, *C. concisus* and *C. curvus* further require the addition of hydrogen to the gas mixture (18). *Campylobacter* spp. are motile bacteria due to single or multiple flagella at one or both poles of the cell. The flagellar apparatus enables the characteristic rapid corkscrew-like, darting motility and enables the colonisation of the mucus lining of the gastrointestinal tract (40). *Campylobacter* spp. are described as fastidious due to their limited repertoire for utilising nutrients and slower growth, compared to other intestinal bacteria, requiring up to several days for the production of colonies on culture plates (18). These bacteria do not utilise carbohydrates (sugars) but derive energy from amino acids, keto acids and citric acid cycle (also known as tricarboxylic acid or Krebs cycle) metabolic intermediates (41).

The morphological characteristics of *Campylobacter* colonies differ according to the culture medium used. In general, colonies are grey in colour, flat or slightly raised, with irregular margins and tend to spread over the agar; an example presented in Fig. 2.2. Rounding of the colonies and glistening or iridescence may also be observed (38). Gram stain or wet mount phase-contrast microscopy is usually used to assess the morphology and motility, and if the characteristic appearance (curved rods with darting motility) is noted, presumption of the *Campylobacter* spp. is made. If colonies have been obtained from selective media (containing antimicrobial agents), and were grown in a microaerobic atmosphere, this characteristic morphological finding combined with an oxidase-positive biochemical result can be reliably used to report the bacteria as being *Campylobacter* spp. (38). On the other hand, with the use of a filtration technique and a non-selective agar, the presumptive isolation of

Campylobacter spp. is made by confirmation of the isolate as Gram-negative, oxidase-positive and l-alanine aminopeptidase negative (42, 43).

Fig. 2.2. *Campylobacter* colonies on mCCDA medium.

(Source: Dr. Els Acke)

It is important to note that the diversity of the *Campylobacter* genus evades the inclusion of all species by the above general phenotypic features. For instance, *C. showae* resembles straight rods (44), *C. mucosalis* has yellow coloured colonies (36), and *C. gracilis* is both non-motile and oxidase-negative (45). Phenotype refers to a combination of observable characteristics of an individual organism resulting from the interaction of its genotype (genetic make-up of an organism) with the environment thus, reflecting the nature and nurture of the organism (46). Common phenotypic tests usually consist of various biochemical tests, tolerance tests of chemical compounds; growth at different temperatures, antibiotic sensitivity profiles, serologic assays and cellular fatty acid profiles (47, 48). The most common biochemical tests employed to identify *Campylobacter* spp. are the production of oxidase, catalase activity, the hydrolysis of hippurate and indoxyl acetate, nitrate reduction and the production of H_2S (38). Several phenotypic features of common *Campylobacter* spp. are presented in Table 2.2. Although these tests were initially crucial for characterisation and identification of species, facilitated diagnostics, led to

discovery of new species, and epidemiological associations, and nowdays most have been replaced by genotypic techniques for accurate identification and characterisation (17). The main limitations of phenotypic tests include the lack of discriminatory power to differentiate closely related strains (49), the high occurrence of non-typeable strains (50), the lack of standardised tests that lead to a variation in results of the same strains between laboratories, and the lack of objectivity (17, 47). The latter limitation stems from the fact that the strain under investigation is compared to the profiles of known taxa which becomes problematic in testing rare or mutant strains of known taxa, testing of newly discovered taxa, or taxa with spurious or insufficient differential features between them.

Table 2.2. Phenotypic characteristics[a] of selected[b] *Campylobacter* species.

Taxon	Oxidase	Catalase	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Reduction of nitrate	Reduction of selenite	Resistance to cephalotin	Resistance to nalidixic acid	Requirement for hydrogen
C. jejuni subsp. *jejuni*	+	+	+	+	+	+	+	-	-
C. jejuni subsp. *doylei*	+	+	+	+	-	-	-	-	-
C. coli	+	+	-	+	+	v	+	-	-
C. upsaliensis	+	-	-	+	+	+	(-)	-	-
C. helveticus	+	-	-	+	+	-	-	+	-
C. lari	+	+	-	-	+	+	+	v	-
C. fetus	+	+	-	-	+	(+)	-	+	-
C. hyointestinalis	+	+	-	-	+	+	(-)	+	v
C. concisus	v	-	-	-	(-)	(-)	-	(+)	+
C. hominis	+	-	-	-	-	-	-	v	+
C. rectus	+	(-)	-	+	+	+	-	(+)	+
C. gracilis	-	v	-	v	(+)	-	-	v	+
C. showae	v	+	-	-	+	+	-	-	+
C. sputorum	+	v	-	-	+	+	-	(+)	-

[a] Symbols denote: +, 90 – 100% strains positive, (+), 75 – 89%, v, 26 – 74%, (–), 11 – 25%, –, 0 – 10%. [b] Adapted from Zanoni et al. (2009) (51).

Notwithstanding the limitations of phenotypic tests, it is important to note that these are still used in routine clinical laboratory settings due to their ease of use, wide availability, fast turn-around time and low cost. Additionally, routine clinical laboratories do not necessarily perform speciation of isolates but may rely on a presumptive isolation of *Campylobacter* spp. based on colony appearance and morphology on *Campylobacter* selective media in appropriate environmental conditions, as previously described. For instance, in 2005 a report on laboratory practices in the FoodNet programme reported only 124 out of 423 (31%) laboratories perform speciation of isolates (52). FoodNet is a collaborative programme in the United States conducting surveillance of foodborne pathogens diagnosed by laboratory testing of samples from patients (https://www.cdc.gov/foodnet/index.html). Similarly, in New Zealand, only 8 out of 32 (25%) of diagnostic laboratories reported speciation of isolates (53). The antibiotic sensitivity profile is perhaps the most important phenotypic characteristic from a diagnostic or, more specifically, an isolation point of view (20). That is, a crucial step toward achieving a successful isolation of *Campylobacter* spp. from faecal samples was and still is, inhibition of the common faecal flora from overgrowing the plate to allow fastidious *Campylobacter* bacteria to grow as an independent colony for a microbiologist to isolate. However, the combination of antimicrobial agents used to suppress the faecal flora may not enable all *Campylobacter* spp. to grow (54). Since the 1970's, a preponderance of methods have been developed and refined, but to this day there is no single selective culture medium that enables growth of all known *Campylobacter* spp. and, by the same token, all selective culture media may hinder the discovery of new *Campylobacter* species.

2.1.3. Sources of Campylobacter species

Most of the taxa within the *Campylobacter* genus have been associated with various diseases in human and veterinary medicine and show a considerable ecological diversity being isolated from various sources (13). Although the same species have been isolated from a multitude of sources, the *Campylobacter* genus is traditionally subdivided into groups according to the most commonly and abundantly observed

niche of isolation. As such, the three main niches of *Campylobacter* spp. are the gastrointestinal tract, the urogenital tract, and the oral cavity (13, 55). That is, intestinal *Campylobacter* spp. include *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, *C. helveticus*, *C. hominis,* and *C. ureolyticus*. Species associated with the oral cavity are *C. rectus*, *C. gracilis*, *C. concisus*, *C. showae*, and *C. curvus* and species associated with the urogenital tract are *C. fetus* and *C. sputorum*. However, most species are far less studied than *C. jejuni* and *C. coli*, the prominent gastrointestinal bacterial pathogens in humans, and *C. fetus*, the prominent urogenital pathogen of ruminants (13). Therefore, knowledge of natural reservoirs of less well known *Campylobacter* spp., and their biology in general is limited, as many species have only been reported from one host species so far (Table 2.1). The main reservoirs of *C. jejuni* are poultry, cattle, sheep and wild birds (5, 56-58) while *C. coli* is mostly associated with sheep and pigs (59, 60). *C. upsaliensis* and *C. helveticus* are predominantly detected in dogs and cats respectively (16, 17) whereas they are rarely reported and with low prevalence rates (~1 – 2%) from other sources (61-65).

Campylobacter spp. have been isolated from many environmental sources such as freshwater (5, 66, 67), seawater (68, 69), sewage (70, 71), oil fields (72), sand (73), soil (74) and feedlots (75). The occurrence of *Campylobacter* spp. in these environments is considered to arise from contamination with faecal matter harbouring *Campylobacter* spp. through direct and indirect routes because *Campylobacter* spp. do not grow and multiply outside of warm-blooded hosts (70, 76). The inability to grow and multiply below 30°C is associated with the absence of cold-shock proteins documented in *C. jejuni*, which many bacteria use for growth below optimal temperatures (77, 78). Therefore, environmental sources are not considered as reservoirs but as vehicles through which *Campylobacter* spp. spread between hosts, and the level of loading of *Campylobacter* in the environment is associated with changes in animal reservoirs (70).

Likewise, contamination of food of animal origin with *Campylobacter* spp. is considered to occur through processes and practices in production and slaughter of animals colonised by these bacteria (79). Although endogenous spread within the host to organs such as the liver and spleen may also occur it is considered an

infrequent event (56). Contamination of other food such as lettuce, spinach and other produce is due to direct or indirect faecal contamination (80). Food contaminated with *Campylobacter* spp. may serve as a source for cross-contamination of other foodstuffs, surfaces and utensils through handling, transport, preparation and storage practices employed in the food chain and in kitchens (79). Contamination with *Campylobacter* spp. can also occur through vectors such as flies (81). Therefore, the source of infection, for animals and humans, with *Campylobacter* spp. may occur through a wide variety of vehicles and transmission routes.

2.1.4. Detection and identification methods

Since *Campylobacter* spp. may be found in numerous primary and secondary sources, there is a great variety in available detection methods (17, 47). Difficulties encountered with the isolation of *Campylobacter* spp. have led to the development of other diagnostic approaches, mostly to meet the needs of diagnostic laboratories. In clinical settings, methods that have a shorter time-to-result, are less labour intensive, require less equipment and are cheaper are usually preferred (82). However, such advantages should not come at the expense of the sensitivity or specificity of the method. One of the first methods used and most readily available has been direct microscopy (wet mounts) of faecal suspensions combined with Gram staining which reportedly has a high specificity while sensitivity may be as low as 6.5% (83) to 66% (84). Other non-culture methods include immunological assays, various nucleic-acid based methods, enzyme-linked immunosorbent serologic and immunochromatographic assays (38). Each method has its own advantages and disadvantages and several studies have evaluated their performances.

Despite difficulties associated with microbiological cultures, these are still the most widely used methods of detection for routine diagnostic purposes (52, 53, 85). Unlike non-culture methods, cultures enable isolation of the bacteria that can be further subjected to various confirmatory and characterisation tests. For instance, species identification and typing procedures can serve epidemiological investigations or antibiotic sensitivity testing can be done for therapeutic purposes (17). In general, the

most important features for successful isolation of *Campylobacter* spp. are: incubating atmosphere (microaerobic or hydrogen-enriched microaerobic), incubating temperature (37 or 42°C), the selective technique (filtration or agar containing antimicrobial agents) and the method of plating (with prior enrichment or direct plating of the sample) (18, 86). The choice of culture method depends on sample type and the *Campylobacter* spp. of interest. Features of sample type that can influence the choice of methods employed include quantities of both *Campylobacter* spp. and background flora expected in the sample, the expected viability of *Campylobacter* cells, and the source of sample (faeces, food, water, environment etc.) (20, 87).

Direct plating of faecal swabs or suspensions to selective agar in a microaerobic atmosphere at 42°C for between 48 and 72 hours is the most commonly employed method for testing of human clinical samples (53, 85). This method is used because human patients usually excrete high numbers of viable *Campylobacter* cells (commonly > 10^6 CFU per gram/millilitre of faeces) (88), *C. jejuni* is the primary species of interest, and the level of background flora is high (9). If the faecal sample had a delay in transport, was used without a transport medium or kept at room temperature, the viability of the *Campylobacter* cells would have been compromised (20, 89). In such a scenario, the microbiologist should consider an enrichment procedure in a nutrient broth prior to plating onto agar to facilitate recovery of stressed and sub-lethally damaged cells due to exposure to air and room temperature (87). The enrichment step usually takes 6 to 24 hours and the incubating temperature may be reduced to 37°C. Supplementation of broth with antimicrobial agents may be delayed for a few hours (20). The enrichment procedure would also be beneficial in instances of expected low numbers of *Campylobacter* cells without the viability of cells being jeopardised. Reduced numbers of viable *Campylobacter* cells are expected in the later stages of enteral disease and in patients suffering from sequelae of *Campylobacter*-associated enteritis as sequelae usually occur several weeks to months after the initial clinical signs develop, patients may be excreting low numbers of *Campylobacter* cells (89). Enhanced detection in patients with acute diarrhoeal illness and patients suffering from sequelae may be achieved by repeated testing of stool samples (90, 91). Should *Campylobacter* spp. other than *C. jejuni*

also be of interest in human clinical samples, the use of a H_2-enriched microaerobic atmosphere combined with 37°C, filtration onto non-selective agar and incubation of five to six days is recommended (17, 18, 20, 89, 92). The incubating temperature of 42°C has a dual role; to facilitate isolation of *C. jejuni* reflecting the temperature of the avian gut (56) and to additionally suppress the background flora though it may inhibit some non-*jejuni Campylobacter* spp. (9). However, the amount of background flora may also be influenced by the particularities of a selective agar used as some have been developed for use at specific temperatures. For instance, Skirrow and semi-solid motility medium agars were developed for use at 42°C and show poorer selective properties at 37°C, whereas charcoal cefoperazone deoxycholate agar (CCDA) and charcoal-based selective media show good selective properties at 37°C (93). Additionally, several non-*jejuni Campylobacter* spp., including *C. upsaliensis* and *C. helveticus*, do not grow, or grow poorly, at 42°C and are preferentially isolated at 37°C (17, 18).

Culture methods employing a filtration technique with non-selective agar have repeatedly outperformed selective agars in their ability to isolate multiple *Campylobacter* spp. as well as the closely related *Arcobacter, Sutterella and Helicobacter* species (92-96). On the other hand, these studies showed filtration-based methods have reduced sensitivity compared to selective agars and are more labour-intensive and cumbersome to perform relative to other methods (97). The increased ability of the filtration method to isolate multiple species is attributed to the physical principles for the basis of selectivity. That is, the small pore size of filters (usually 0.65 or 0.45 µm in diameter) serve to block the larger background flora combined with the motility of *Campylobacter* cells which facilitates the passage through the pores (98). In contrast, selective agars employ antimicrobial agents to suppress the background flora but, in turn, may also suppress *Campylobacter* spp. sensitive to the agents used (20) thus, giving false negative results. However, the relationship between the agar, the antimicrobial agents and the background flora may influence the isolation of *Campylobacter* spp. in a rather complex fashion as in the example of a selective agar containing cefoperazone, amphotericin and teicoplanin (CAT) antimicrobial agents (99).

The incentive for developing such an agar were the frequent reports of catalase negative or weakly positive *Campylobacter* spp. implicated as pathogenic to humans (96, 100). This species originally isolated from dogs and subsequently named as *C. upsaliensis* (101) had less than 20% of strains able to grow on common selective agars, the majority isolated using filtration. The poor growth on selective media was attributed mainly to their sensitivity to common antimicrobial agents used in selective agars at the time such as cefoperazone, colistin, vancomycin, rifampin, tetracycline and trimethoprim (102, 103). Therefore, CAT agar was developed for the isolation of *C. upsaliensis* in addition to *C. jejuni* and *C. coli* (104) with the composition of nutrients resembling commonly used blood-free selective agar (modified CCDA, mCCDA) but varying in the composition of added antimicrobial agents. The main feature of CAT is a reduction in the concentration of cefoperazone and the use of teicoplanin. Cefoperazone was reduced from concentration of 32 mg/L to 8 mg/L, which was sufficient to suppress *Enterobacteriaceae* yet enable the growth of *C. upsaliensis* that had an average minimum inhibitory concentration of 16 mg/L. This in turn did not suppress faecal *Streptococcus* spp. for which teicoplanin was added (104). In the same study the two media were compared for the culture of 7000 human clinical samples. Out of five *C. upsaliensis* isolated, CAT agar recovered four and mCCDA only one. With regard to the detection of other *Campylobacter* spp. and level of contaminants on the plates, the results were comparable for the two methods. Subsequent studies that compared the performance of CAT agar to filtration and mCCDA in human, dog and cat faeces showed CAT to be comparable to the filtration method in overall sensitivity but with better analytical sensitivity (i.e. able to detect lower bacterial concentrations), while both methods were better than mCCDA for the isolation of *C. upsaliensis* (94). However, one study compared both methods in more detail with a description of the performance with regard to antibiotic profiles, absolute growth index, analytical sensitivity and duration of incubation for detection of *C. upsaliensis* (99). This study could not explain the basis of superiority of CAT over mCCDA by antibiotic profiles, production of colonies by absolute growth index or length of incubation and observed only a weak association with the analytical sensitivity because CAT was able to detect bacteria at concentrations of 10^3 CFU/mL and mCCDA detected 10^4 CFU/mL in spiked samples. Indeed, this is quite a small difference and the basis of the superiority remains unclear.

Researchers speculated that either the different antibiotic profiles of CAT and mCCDA alter some component or product of the faecal microflora, or exposure of *C. upsaliensis* to faeces alters its' sensitivity *in vivo* to cefoperazone, thereby making it sensitive to the higher concentrations used in mCCDA (99). These studies suggest there are complex relationships between the *milieu* of the sample and the agar as no particular component alone sufficiently explained the findings. The importance of the sample type, it's matrix or *milieu*, and the targeted organism is indirectly supported by a variety of culture methods available for different sample types (20). The composition of some common culture broths and media for isolation of *Campylobacter* spp. is presented in Table 2.3 and Table 2.4, respectively.

Table 2.3. Composition of some commonly[a] used culture broths for isolation of *Campylobacter* species.

Broth make up (g/L)	Bolton (Oxoid)	Brucella (BBL) + Oxoid SR0232E	Preston (Oxoid)	Exeter	Park-Sanders	Hunt	TECRA	m-Exeter
Meat peptone	10		10	10		10	+	10
Lactalbumin hydrolysate	5							
Yeast extract	5	2			2	6	+	
Casein hydrolysate		10			10		+	
Sodium chloride	5	5	5	5	5	5	+	5
Peptic meat digest		10			10			
Sodium sulphite					0.1			
Alpha-ketoglutaric acid	1							
Pentanedioic acid							+	
Sodium pyruvate	0.5	0.25	0.25	0.25	0.25	0.25	+	0.2
Sodium metabisulphite	0.5	0.25	0.25	0.25		0.25	+	0.2
Sodium carbonate	0.6						+	
Sodium citrate					1			
Sodium bisulphite		0.1						

Broth make up (g/L)	Bolton (Oxoid)	Brucella (BBL) + Oxoid SR0232E	Preston (Oxoid)	Exeter	Park-Sanders	Hunt	TECRA	m-Exeter
Haemin	0.01							
Ferrous sulphate		0.25	0.25	0.25		0.25		0.5
Beef extract (Lab-Lemco)			10	10		10		10
Blood (mL/L)	50		50	50	50	50		50
Dextrose		1			1			
Cefoperazone (mg/L)	20			15	32	15-30		15
Vancomycin (mg/L)	20				10	10		
Trimethoprim (mg/L)	20		10	10	10	12.5	+	10
Cyclohexamide (mg/L)	50		100		100	100		
Polymixin B (IU/L)			5,000	5,000			+	
Rifampicin (mg/L)			10	10			+	10
Amphotericin B (mg/L)				10				10

[a] Adapted from common manufacturer's product specifications available online.

Table 2.4. Composition of some commonly[a] used culture media for isolation of *Campylobacter* species.

Media make up (g/L)	mCCDA	Preston	Karmaii	CAT	Campy -Line	Campy -Cefex	Butzler	Campy-BAP	Skirrow
Meat peptone	10	10		10	10			10	
Special peptone			23				23		23
Casein hydrolysate	3			3				10	
Peptamin						20			
Yeast extract					3	2		2	
Sodium chloride	5	5	5	5	5	5	5	5	5
Starch			1				1		1
Glucose					10	1		1	
Charcoal	5		4	5					
Sodium pyruvate	0.25	0.25	0.1	0.25	0.5				
Sodium metabisulphite		0.25			0.2	0.1			
Sodium bisulphite							0.1		
Sodium desoxycholate	1			1					
Ferrous sulphate	0.25	0.25		0.25	0.5				
Haemin (mg)					10				
Alpha-kutoglutaric acid					1				
Sodium carbonate					0.6				

Media make up (g/L)	mCCDA	Preston	Karmaii	CAT	Campy -Line	Campy -Cefex	Butzler	Campy-BAP	Skirrow
Beef extract (Lab-Lemco)	10	10			5				
Agar	12	12	10	12	15	17.5	10		10
Blood (ml)		100				50	100-140	100	100
Cefoperazone (mg)	32		32	8	32	32			
Vancomycin (mg)			20		10				10
Trimethoprim (mg)		10			5			5	5
Cyclohexamide (mg)		100	100		100	200	50		
Polymixin B (IU)		5,000			3,500			2,500	
Rifampicin (mg)		10							
Amphotericin B (mg)	10		10	8				2	
Bacitracin (IU/ml)							25		
Colistin (IU/ml)							10		
Cephazolin (mg)							15		
Novobiocin (mg)							5		
Teicoplanin (mg)				4					
Cephalothin (mg)								15	
Triphenyltetrazolium chloride (g/L)					0.2				

[a] Adapted from common manufacturer's product specifications available online.

Isolation of *Campylobacter* spp. from water, food and the environment inherently differs from isolation from faeces, as in these samples bacteria are frequently exposed to air, temperatures of room, chilled or deep-freeze ambient, and chemical agents used in sanitation and hygienic procedures (20, 76, 79, 80, 86). Such environments and processes employed may lead to changes in pH, osmotic gradients, hydrostatic pressures, oxidative stress (105, 106), atmosphere composition (107) and nutrient availability affecting the survival of *Campylobacter* species (108). Therefore, in these samples lower numbers of *Campylobacter* spp. and stressed or damaged cells are expected from the outset. Due to potentially low numbers of *Campylobacter* cells and stresses exerted on cells, culture methods for water, food and environmental samples use larger volumes of samples or rinses and suspensions for samples in order to improve sensitivity (70, 86). Culture methods and protocols employing pre-enrichment steps and a 37°C incubation temperature, similar to modifications in the culture of human patient faecal samples described above, have been reported to have a higher efficacy of isolation (87, 109-111). However, certain types of samples frequently harbour high numbers of *Campylobacter* cells, such as broiler litter, and direct plating to selective agars is sufficient (112). The reduction of incubating temperature from 42 to 37°C can facilitate the detection of non-*jejuni Campylobacter* spp. (80, 113) especially if used in combination with H_2-enriched microaerobic atmosphere and filtration onto non-selective agar (61). Unlike for culture methods for human and animal faeces, international guidelines and standards for the detection and quantification of *Campylobacter* spp. from water (114), and food and animal feedstuffs have been published (115, 116).

Non-culture based methods have been developed to directly or indirectly detect the presence of the bacteria. Direct methods may include detection of bacterial antigens (antigen-based) or bacterial DNA (nucleic acid-based), while indirect methods detect changes external to bacteria but associated with their presence (*e.g.*, biomarkers and antibodies) (47). Indirect methods developed for *Campylobacter* spp. include assays for the detection of antibodies against cellular antigens and include complement-fixation assays, immunoblotting, and enzyme-linked immunosorbent assays (ELISAs) (117). In a clinical context these methods are mainly employed for the diagnosis of

Campylobacter-associated sequelae (118) as these patients may have negative results using bacterial culture methods. These serologic assays are very important for epidemiologic studies, risk assessments, and surveillance, as they can provide data on *Campylobacter* exposure at the population level thus, may address disease-to-infection ratio and immunity estimates (119), or to evaluate the public health effects of control programs (120).

Direct antigen-based methods for *Campylobacter* include ELISA, immunochromatographic assays, latex agglutination, and lateral-flow antigen and immunomagnetic separation technologies (82, 121-123). Apart from stool specimens, the tests have been developed for use in various food products (124, 125). These methods are based on mono or polyvalent antibodies produced (commonly from a rabbit) against extracted specific antigens of *C. jejuni* and *C. coli*. The advantages of these assays are their ease of use, rapid diagnosis, no requirement for sophisticated instrumentation, and relative inexpensiveness (82). Disadvantages include the lack of differentiation between *C. jejuni* and *C. coli,* potential cross-reactions with other species, and not being able to provide an isolate for further analysis such as genotyping or antibiotic sensitivity testing (47). Reports of diagnostic performance characteristics of some commonly used faecal antigen tests for the detection of *Campylobacter* spp. in humans are presented in Table 2.5. Combined approaches may be used by laboratories such as applying the antigen-based methods for initial screening purposes, followed by culture or nucleic acid-based tests of any positive samples (85); or by separation of cells using immunomagnetic technique and proceeding to DNA extraction (for nucleic acid-based tests) or culture (126).

Table 2.5. Diagnostic performance characteristics[a] of some commonly used faecal antigen tests for *Campylobacter* spp. in humans.

Antigen-based test	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predicted value % (95% CI)	References
ProSpecT™ *Campylobacter* Microplate assay (Remel)	99.2 – 99.3 87.5 – 87.6 (78 – 94) 86 – 93.2 (83 – 95) 89 – 93 96.0 – 96.1	95.8 – 98 97.5 – 97.6 (97 – 98) 97.2 – 98.2 (96 – 99) 99 99 – 100	89.4 – 95 51.1 – 56.9 (42 – 65) 76.6 – 80.4 (73 – 84) 65 – 80 NA	(121) (127) (128) (129) (130)
Premier™ CAMPY Enzyme Immunoassay (Meridian Biosciences)	75 (22 – 98) 99.2 ~ 94 86 – 97 (77 – 98)	96.5 (92 – 99) 96.1 – 98.3 > 95 97 (96.6 – 97.9)	43 (12 – 80) 90 – 95.7 ~ 81 49 – 54 (40 – 62)	(131) (121) (132) (127)
ImmunoCard STAT! CAMPY™ test (Meridian Biosciences)	98.4 – 98.5 78.8 – 79.8 (68 – 88) 80 ~ 91	94.2 – 98.2 95.9 (95.1 – 96.7) 98 > 95	92.6 – 97.8 36.6 – 41.3 (29 – 49) NA ~ 70	(121) (127) (133) (132)
RIDASCREEN™ *Campylobacter* assay (R-Biopharm)	~ 91 69 – 72	> 95 87 – 92	~ 87 36 – 48	(132) (123)
RIDA™QUICK *Campylobacter* assay (R-Biopharm)	96.8 (88.5 – 99.8)	97.2 (95.4 – 98.4)	NA	(134)

[a] Performance estimates presented as a range interval denote combined data from different analytical methods used.

Nucleic acid-based tests share the advantage of same-day results with antigen-based methods and the financial costs of their use and implementation in routine laboratories is continually decreasing thus, have become more and more used (135). Nucleic acid-based tests are based on the amplification of DNA segment using primers or probes designed for a specific target DNA sequence which is facilitated by the DNA polymerase enzyme. This method is known as the polymerase-chain reaction (PCR) (136). The choice in the design of primers and probes may be to target a specific species, a group within a particular species or genus-specific (or higher taxonomic classes) sequence (47). The method may be applied to design a presence/absence test (conventional PCR), multiple target test (multiplex PCR), quantitative test (real-time quantitative PCR) and the detection of mutations, polymorphisms and epigenetic differences in the DNA sequence (high-resolution melt analysis). In all cases, the target organism is not isolated, but subsequent procedures may be employed to isolate the bacteria as with the antigen-based methods (137, 138).

For species identification of isolates, phenotypic methods may be employed but have been shown to be limited. For instance, the use of antibiotic sensitivity testing for speciation is becoming more problematic due to the increasing frequency of resistance (38), as was reported for *C. upsaliensis* (139). Antibiotic resistance is also of concern for treatment of infections due to the increasing prevalence of ciprofloxacin resistance in *Campylobacter* species (140). Identification using biochemical tests such as positive hippurate hydrolysis has been a main feature for *C. jejuni* but hippurate-negative strains (up to 10%) have been reported. PCR test for detecting the presence of the gene responsible for hippurate hydrolysis (*hipO*) is more reliable than the biochemical hydrolysis test as the isolate could possess the gene but may not have it expressed thus giving a negative result if relying solely on the biochemical test (141). As a result, molecular methods are the preferred means for accurate species identification. Several genes have been used for species and genus identification of *Campylobacter* species. Although PCR tests are the preferred means for species identification, these tests are not without limitations (17).

One of the disadvantages of PCR tests is related to the continuing discoveries of new *Campylobacter* spp. similar to the lack of objectivity described for phenotypic schema (47). For PCR it is the development of primers and probes used that are dependent on the available information of DNA sequences at that time. As new species or subspecies are discovered, or new data become available, the primers/probes should be revalidated and reassessed in order to improve and optimise the diagnostic efficacy. This issue has been recently shown a particular problem for species of the *C. lari* group in a large, inter-laboratory study of *Campylobacter* PCR tests. The study showed that PCR tests differed markedly in diagnostic performance characteristics, sensitivity and specificity (142). This study was the largest inter-laboratory evaluation of PCR tests conducted at the time of writing this thesis and the results are presented in Table 2.6.

Table 2.6. Summary of results obtained with each of 25 *Campylobacter* strains representing 15 taxa examined with assays established in individual laboratories for various *Campylobacter* spp. The tests used (and their taxonomic range and original description, where relevant) in each laboratory is given. Sensitivity and specificity values for each test were calculated with respect to each assays taxonomic range.

Lab no.	Reference	Assay target	Taxon	Sensitivity (%)	Specificity (%)
1	Eyers *et al.* (1993)		*C. jejuni*	100	85
	Linton *et al.* (1997)		*C. jejuni*	100	100
	Van Camp *et al.* (1993)		*C. jejuni/coli/lari*	100	55
	Vandamme *et al.* (1997), multiplex PCR	Random	*C. jejuni*	66	96
			C. coli	100	100
	Eyers *et al.* (1993)	23S rRNA	*C. coli*	100	63
	Wong *et al.* (2004), multiplex PCR	lpxA	*C. jejuni*	100	100
		ceuE	*C. coli*	100	100
		23S rRNA	Thermophilic *Campylobacter*	100	100
2	Kawasaki *et al.* (2008)	gyrB	*C. jejuni*	66	100
		gyrB	*C. coli*	100	100
		gyrB	*C. lari*	78	86

Lab no.	Reference	Assay target	Taxon	Sensitivity (%)	Specificity (%)
		gyrB	*C. fetus*	100	100
		gyrB	*C. upsaliensis*	100	100
		gyrB	*C. helveticus*	0 (100[c])	100 (0[c])
		gyrB	*Campylobacter* spp. +[b]	NA	NO FPs detected
3	Wang *et al.* (2002)	hipO	*C. jejuni*	100	100
		glyA	*C. coli*	100	89
		sapB2	*C. fetus*	100	100
		glyA	*C. upsaliensis*	100	100
		glyA	*C. lari*	58	100
		23S rRNA	*Campylobacter* spp.	100	100
4	Vandamme *et al.* (1997), multiplex PCR	Random	*C. jejuni*	66	100
		Random	*C. coli*	100	100
	Linton *et al.* (1996)	16S rRNA	*C. lari*	58	100
		16S rRNA	*Campylobacter* spp.	96	100
5	Developed in-house assay (Galker)	16S rRNA	*Campylobacter* spp.	100	100
	Developed in-house assay (Galker), RT PCR	hipO	*C. jejuni*	100	100
		ORFA	*C. coli*	100	100

Lab no.	Reference	Assay target	Taxon	Sensitivity (%)	Specificity (%)
6	Developed in-house assay. Menard *et al.* (2005)	gyrA	*C. jejuni*	100	91
		gyrA	*C. coli*	100	83
7[a]	Stucki *et al.* (1995)	mapA	*C. jejuni*	100	92
	Linton *et al.* (1996)	16S rRNA	*Campylobacter* spp.	100	No FNs
8	Englen and Fedorka-Cray (2002)	hipO	*C. jejuni*	100	100
	Commercial assay (PrimerDesign), RT PCR	cadF	*C. jejuni*	100	100
9	IQ-Check (Bio-Rad)	Unknown (proprietary)	*C. jejuni, C. coli, C. lari*	100	65

[a] All results were derived from the use of DNA at a concentration of 100 ng/µL with the exception of results from lab 7 where a DNA concentration of 50 ng/µL was used throughout. [b] *Campylobacter* spp. + = *C. showae*, *C. hyointestinalis*, *C. mucosalis*, *C. curvus*, *C. concisus*, *C. sputorum*. [c] Only one set of results (out of two repeats) positive. Sourced from On *et al.* (2013) (142).

Many other *Campylobacter* spp. have increasingly been recognised as human pathogens with reports of *C. upsaliensis* (97), *C. concisus* (18) and *C. ureolyticus* (143) as being more common than *C. coli*. Although *C. jejuni/coli* have been most extensively researched there are still many questions left unanswered. Non-*jejuni/coli Campylobacter* spp. have not been extensively studied to date making it difficult to establish their public health significance (13). There is an under-appreciation of the significance of non-*jejuni/coli Campylobacter* spp. as pathogens to humans mostly because clear and unequivocal evidence of their pathogenicity is lacking, and some species can be recovered from both healthy and sick people. The potential significance of *C. upsaliensis* was first reported in 1990 (100) yet no significant research endeavour has occurred with regard to its pathogenicity and virulence.

2.1.5. Pathobiology

Campylobacter, as a human pathogen, has been described as an “accidental tourist” that has reservoirs in animals where it colonises as a commensal (23). The pathogenesis of disease in humans in still not fully understood though significant advances have been made in describing various features and mechanisms involved. Mechanims of pathogenesis identified in *C. jejuni* are presented in Fig. 2.3. Motility of *C. jejuni* has been shown as an important factor in the colonisation of the intestines providing the ability to traverse the mucous barrier and preferentially target the deep intestinal crypts (144). The flagellum is an unsheathed polymer of flagellin subunits encoded by the *flaA* and *flaB* genes with a high degree of sequence similarity (~93%) yet independent transcriptional promoters (145). Mutants in which *flaB* but not *flaA* is inactivated remain motile, unlike those with a defective *flaA* gene which renders the bacteria immotile, causing loss of their potential to adhere to, and penetrate into, human intestinal cells *in vitro* (146). Similarly, flagellar mutants were also show to have lost the ability to colonise chickens, their natural reservoir (147). Under experimental conditions that mimic the viscosity of intestinal mucus, *C. jejuni* was shown to have longer periods of straight swimming with significantly increased velocity and enhanced binding and invasion of Caco-2 cells compared to the standard growth media. These features were not observed with *Salmonella enteritidis*

(148). Apart from studies employing *in vitro* and animal *in vivo* models, the role of flagella was confirmed in an experimental infection of humans in which only motile variants were isolated from stools despite volunteers being fed mixtures of motile and non-motile phase variants (149). The flagellar apparatus of *C. jejuni* provides more than an ability to move around. The sequencing of *C. jejuni* NCTC 11168 strain reported 36 open reading frames involved with biosynthesis, export, assembly and function of the flagellar apparatus (150). Several bacterial secreted proteins were documented, most notably *ciaB*, which was required for the internalisation of *C. jejuni* into mammalian cells (151). The activity of these proteins is dependent on the functional flagellar apparatus through which secretion takes place (152). Flagellar genes were shown to be co-regulated with several virulence factors associated with flagellar glycosylation and cytolethal distending toxin production, with *flhA* as a key element involved in the coordinated regulation in *C. jejuni* (153) as well as co-regulated with genes involved in respiration and metabolism (154). Glycosylation of the flagellin is important for flagellar assembly and functioning (155) without which virulence is attenuated as shown by: (i) the decreased invasion of human epithelial cells and (ii) the reduced virulence in the ferret diarrhoeal model as only three out of 16 ferrets fed with *pseA* (the gene involved with the pseudaminic acid component of glycosylation of flagellin) mutant developed diarrhoea compared to 10 out of 16 animals fed the wild type 81–176 strain (156).

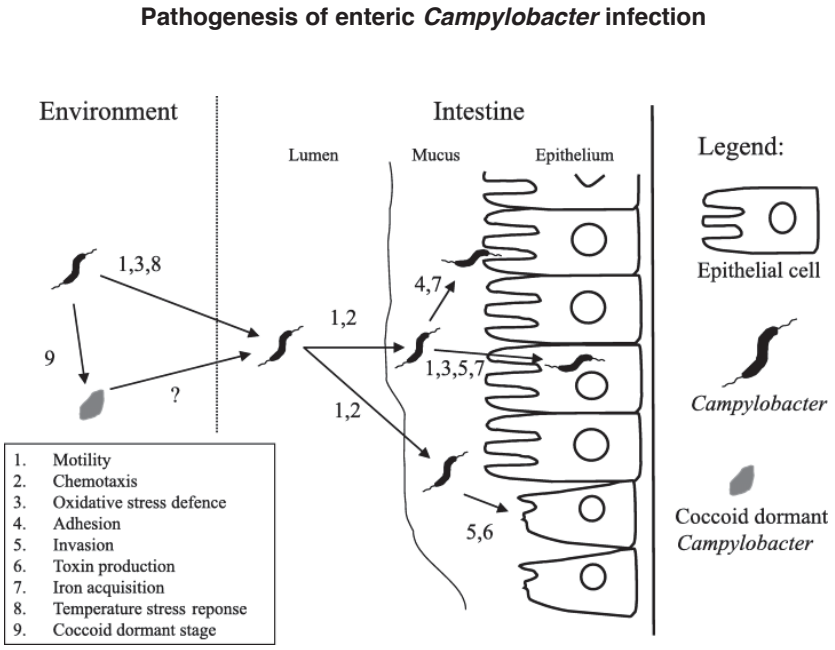

Fig. 2.3. Pathogenic mechanisms of *Campylobacter* infection.

Sourced from Van Vliet and Ketley (2001) (157).

Motile bacteria use chemotaxis as a mechanism through which they sense the environment to direct themselves toward more favourable or away from unfavourable conditions. *C. jejuni* shows a positive chemotactic response to L-aspartate, L-cysteine, L-glutamate, and L-serine amino acids and, of the carbohydrates, only to L-fucose (158). Several salts of organic acids, including pyruvate, succinate, fumarate, citrate, malate, and alpha-ketoglutarate, were also chemoattractants, as were bile (beef, chicken, and oxgall) and mucin (bovine gallbladder and hog stomach) though most constituents of bile tested individually were chemorepellents (158). Chemotaxis is regulated by a single 2-component histidine protein kinase dependent signal transduction pathway consisting of 6 chemotaxis proteins (CheA,B, R, W, Y and Z) and methyl-accepting chemotaxis proteins (MCPs also referred to as transducer-like proteins, tlp) (159). Bacteria are either attracted or repelled by chemicals sensed by

trans-membrane MCPs, and the information is transmitted to the flagellum motor via the histidine kinase CheA and the response regulator CheY. Phosphorylated CheY binds to the FliM component of the flagellar motor causing a change in rotation from counter-clockwise to clockwise which effects a change from smooth swimming forward to sideways tumbling motility and direction changes (160). *C. jejuni* mutants that lack MCP receptors (*DocB* and cj0262c), exhibit impaired colonisation of chick caeca (161) and mutants of *CheY* show delayed colonisation of mice and decreased virulence in the ferret model (162). Similarly, strains with mutations in methyl-accepting chemotaxis proteins tlp1, tlp3*,* tlp4 (*docC*) and tlp10 (*docB*), but not tlp5 displayed a 10-fold decrease in the ability to invade human epithelial and chicken embryo cells. However, these deletions did not affect the chemotactic behaviour of the mutants compared to that of the parental strain, hence demonstrating that the corresponding proteins affect host interaction (163). Further studies have shown the interaction of MCP and host differentiation along the gastrointestinal tract, as a *tlp10* mutant was reported defective in colonisation of the chicken proximal and distal gastrointestinal tract, while the *tlp6* and *tlp8* mutants showed reduced colonisation of the duodenum and jejunum (164).

In conjunction with motility and chemotaxis, adherence to host epithelial cells is also a prerequisite factor in *Campylobacter* pathogenesis. JlpA is a surface-exposed lipoprotein shown to have adhesin-like properties and a role in binding to the Hep-2 epithelial cells (165). It was demonstrated that JlpA interacts with the HEp-2 cell surface heat shock protein (Hsp) 90α and initiates signalling pathways leading to the host's pro-inflammatory immune response through the activation of NF-κB and p38 MAP kinase (166). *C. jejuni* cell-binding factor 1 was shown to contain PEB1 (periplasmic binding protein), a homologue of cluster 3 binding proteins of bacterial ABC transporters (167). In that study the researchers demonstrated that the inactivation of this operon completely abolished the expression of cell-binding factor 1 and led to 50- to 100-fold less adherence to and 15-fold less invasion of HeLa epithelial cells in culture and a significantly lower and shorter rate and duration of colonisation in the mouse infection model. Binding to fibronectin, a glycoprotein of the extracellular matrix component of epithelial cells, and subsequent internalisation of *C. jejuni* by epithelial cells was shown to be mediated through the CadF protein of *C.*

jejuni in *in vitro* studies (168). Mutants of the *cadF* gene were also shown as incapable in colonising the caeca in all 60 challenged chicks (169). The FlpA protein was also shown to mediate binding to fibronectin in human epithelial cells (170) and both FlpA and CadF in a coordinated mechanism with secreted proteins promote membrane ruffling and cell invasion (171). Investigation of some factors related to adhesion had discordant reports. A previous study showed a *capA* insertion mutant had a significantly reduced ability to adhere to and invade Caco-2 cells and completely failed to colonise and persist in chickens (172) while in contrast another study showed no effect on the ability to colonise chicken (173). Similarly, mutations in the *virB11* gene, which is part of the type IV secretion system, carried by a virulence plasmid pVir resulted in significantly reduced adherence and invasion (174) but the conjugative transfer of this plasmid did not increase the invasiveness of a recipient strain (175).

Flagella play a major role in the invasion ability of *C. jejuni* through many mechanisms. The flagella are also involved with secretion of non-flagellar proteins during host invasion as a type III secretion system (176). Several genes of this export apparatus were shown to affect the invasion ability of *C. jejuni* such as *flaA*, *flaB*, *flgB*, *flgE* and *flhA* as well as *flaC* and *cia* gene products that are delivered into the host cell's cytoplasm using this flagellar secretion system and are considered essential for colonisation and invasion (152, 177). *In vitro* binding and internalisation assays revealed that the binding of *C. jejuni ciaB* null mutants was indistinguishable from that of the parental isolate, whereas a significant reduction in internalisation was noted in INT 407 cells (151). Similarly, *ciaC* secreted proteins are required for full invasion (178) whereas the ciaI protein is important for *C. jejuni* intracellular survival in epithelial cells as it prevents the delivery of *Campylobacter*-containing vacuoles (CCV) to lysosomes (179). Invasion of epithelial cells was documented in human patients (180). CCV deviates from the canonical endocytic pathway immediately after a unique caveolae-dependent entry pathway in epithelial cells in contrast to macrophages where *C. jejuni* is delivered to lysosomes and consequently is rapidly killed (181). Studies have shown that, within cells, *C. jejuni* undergoes a significant metabolic downshift and reprograms its respiration, especially the fumarate pathway, in order to adapt and survive the low oxygen and nutrient conditions inside CCV

(182, 183). Antimicrobial peptides also have an important role in the innate immune response (184) and the *C. jejuni* homologue of the VirK family of virulence factors was shown as essential for antimicrobial peptide resistance and systemic infection in a mouse virulence model (185).

Several toxins have been identified in *C. jejuni* (186, 187) with the tripartite cytolethal distending toxin (CDT) similar to that found in other Gram negative bacteria the most commonly described (188). *CdtA* and *cdtC* gene products are responsible for toxin binding to the cell membrane and for delivery of the *cdtB* gene product, which is the active unit causing progressive fragmentation of the nucleus, and cellular distension and ultimately cell death in different cell lines (189, 190). The involvement of CDT in diarrhoea was suggested as affecting the survival or maturation of crypt cells into functional villus epithelial cells causing a temporary erosion of the villus and a subsequent loss of absorptive functions (191). Interestingly, a *C. jejuni cdtB* mutant was unaffected in it's ability to colonise the gut of adult severely immunodeficient mice, but demonstrated impaired invasiveness into blood, spleen and liver tissues (192). PCR studies have shown *cdt* to be present in many other *Campylobacter* spp., including *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus*, *C. fetus* and *C. hyointestinalis* (193), which indicates that other virulence factors must also be invlved in disease.

Carbohydrate structures are involved with many aspects of pathogenicity. Sialylation of capsular lipooligosaccharides (LOS) is associated with adhesion, invasion and immune evasion. For instance, *C. jejuni* strains expressing sialylated LOS (classes A, B, and C) invaded cells significantly more frequently than strains expressing nonsialylated LOS (classes D and E), and knockout mutagenesis of LOS sialyltransferase (*Cst*-II) significantly lowers levels of invasion compared to the wild-type strain, that can be restored by complementation of the gene (194). Sialylation of the LOS increases invasive potential and reduces immunogenicity of *C. jejuni* (195). A study revealed a correlation between genotypic diversity and the LOS locus classes of *C. jejuni* with the majority of isolates grouped by the multi-locus sequence typing (MLST) scheme to clonal complex (CC) CC-21 (correlated with LOS class C) and CC-206 (correlated with LOS class B) with statistically significantly higher levels of invasion than isolates from other CC (196). *C. jejuni* capsular polysaccharides,

mediated by the *kpsE* gene, were also shown to play an important role in adherence to, and invasion of, human embryonic epithelial cells but no significant role was shown in colonisation of the chicken gut (197). On the other hand the expression of the *kpsM*-dependent capsule undergoes phase variation at a high frequency and a *kpsM* mutant showed significantly reduced invasion of INT407 cells, reduced virulence in a ferret diarrhoeal disease model and decreased resistance to human serum (198). Similarly, the *N*-linked general protein glycosylation pathway (encoded by the multigene locus *pgl*) modifies many of the organism's proteins and *pglH* mutants had a significantly reduced ability to adhere to and invade human epithelial Caco-2 cells and to colonise chicks (199). N-linked glycosylation facilitates immune evasion, as the glycosyl moieties are immunodominant resulting in limited antibody generation against the protein fraction (160) and N-glycans were suggested to protect *C. jejuni* surface proteins against gut proteases (200).

Stress response and survival mechanisms also play important roles in *Campylobacter* biology. As an intestinal bacterium, coping with various adverse environmental conditions is important for *Campylobacter* spp. transmission between hosts, and temperature and atmospheric changes significantly influence the survival of *Campylobacter* spp. (160). Sigma factors 28, 54 and 70 encoded by the *fliA*, *rpoN* and *rpoD* genes respectively, regulate transcription of, motility, virulence, and survival genes (153, 201, 202). The regulation of iron homeostasis and oxidative stress in *C. jejuni* has been shown to be linked (203-206) and these regulators are also involved in flagellar biosynthesis (205). *C. jejuni* generates genetic diversity to improve its phenotypic fitness to survive and adapt to adverse environments encountered, as shown in a comparison of pulsed field gel electrophoresis profiles *in vitro* and *in vivo* (207). Environmental stresses encountered by *C. jejuni* in transmission between different hosts include starvation, low pH and osmotic stresses, temperature changes, desiccation, and nitrosative and oxidative/aerobic stresses (108). More than any other stress conditions, increased oxygen tension in the atmosphere will be the most viability-threatening stress that *C. jejuni* cannot avoid and consists of superoxide and peroxide stresses (208). The genes involved in the antioxidant defence system are induced and their activity increased when *Campylobacter* are exposed to reactive oxygen species that may be generated due

to an aerobic environment, aerobic respiration or produced by the host immune system against the invading pathogen (209). To date eight major detoxification enzymes/proteins have been identified and characterised within *C. jejuni*: AhpC (alkyl hydroxyperoxide reductase), SodB (superoxide dismutase), KatA (catalase), Tpx (thiol peroxidase), Bcp (thiol peroxidase), Dps (bacterioferritin), MsrA/B, and Cj1386, an ankyrin-containing protein involved in heme trafficking to catalase (210, 211).
Biofilm formation and the viable-but-non-culturable (VBNC) state are also strategies of *C. jejuni* to cope with adverse conditions. The state of bacteria in biofilms is different to those from free, planktonic living bacteria and are characterised by increased resistance to various stresses, especially oxidative, chemical and antimicrobial (212). Biofilm formation by *C. jejuni* was shown to be increased under aerobic conditions (213) and the antioxidant proteins AhpC and Tpx were shown as important mediators of oxidative damage in *C. jejuni* biofilms (214, 215).The VBNC form has been demonstrated in *Campylobacter jejuni*, representing a resting or dormant stage in which cells reduce metabolic activity and do not replicate. As a result they cannot grow in isolation media, and are characterised by increased production of degradative and substrate-capture enzymes and cell shrinkage to a coccoid form (216). Experimentally, the VBNC state can be induced by temperature, starvation, formic acid, or aerobic conditions (217). *C. jejuni* VBNC populations maintain the ability to adhere to human intestinal cells (218) and to colonise mouse intestines even after several months of dormancy (219).

As the vast majority of studies of pathogenicity has focused on *C. jejuni* and to lesser extent *C. coli*, the pathogenic potential of *C. upsaliensis* and *C. helveticus* (and other *Campylobacter* species) is poorly characterised (13). *C. upsaliensis* isolates were shown to adhere to lipids, human small-intestinal mucin and Hep-2 epithelial cells, implying bacteria can access the human host's cell membrane receptors (220). Epithelial cell lines of intestinal origin appeared to be more susceptible to invasion by *C. upsaliensis* than non-intestinal-derived cells (221). Using cytoskeletal inhibitors this study further demonstrated evidence for both microtubule- and microfilament-dependent uptake of *C. upsaliensis* by eukaryotic cells, which was also demonstrated for *C. jejuni* (222). Whole-cell preparations and lysates of *C. upsaliensis* were shown to produce a CDT-like effect on HeLa cells and human T lymphocytes, including

cytodistension, nuclear fragmentation, cell cycle arrest and apoptosis in affected host cells (223). A study of capsular LOS and polysaccharides gene clusters reported *C. upsaliensis* possessed genes homologous to the sialic acid genes implicated in the neurological disorder Guillain-Barré syndrome (224). Sequence analysis of the Fur protein, involved with ferrous iron uptake, of *C. upsaliensis* showed highly homology (87% amino acid identity) to *C. jejuni* Fur (225). Similarly, high homology of autoinducer-2 synthase, LuxS involved in chemotaxis, was shown between *C. upsaliensis* and *C. jejuni* but *luxS* was not detected in *C. helveticus* (226). However, this study showed *C. helveticus* (and *C. upsaliensis*) to have autoinducer-2 activity using reporter assays and questioned the negative result for *luxS*, suggesting it to be due to use of a *C. jejuni*-specific PCR. Both *C. upsaliensis* and *C. helveticus* strains were reported to have the ability to produce biofilms on stainless steel though inconsistently and requiring a longer incubation in comparison to *C. jejuni* (227).

No other studies of *C. upsaliensis* and *C. helveticus* relating to investigations of pathogenic potential were found in the review of the literature. However, the whole genome of *C. upsaliensis* has been published (228). In that study of whole genomes of *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, many genes involved in host colonisation, including *racR/S*, *cadF*, *cdt*, *ciaB*, and flagellin genes, were conserved across the species, but variations in LOS and polysaccharide loci appeared to be species-specific. Furthermore, *C. upsaliensis* was shown to have the greater number, length and variability of the poly G tracts that are associated with phase variation than the other three *Campylobacter* species. Another notable finding in the *C. upsaliensis* genome was the identification of a novel virulence locus, *licABCD*, with varying, but significant, similarity to genes present in *Haemophilus influenzae*, commensal *Neisseria* spp., and *Streptococcus pneumoniae*, which is possibly involved in the attachment to host cells.

2.1.6. Typing of *Campylobacter* species

Characterisation of strains serves to describe the population structure of a species and has been mostly used to research the epidemiology of disease, phylogenetics or

pathogenicity of microorganisms. Genotyping is the preferred method for both identification and subtyping of microorganisms rather than phenotyping (49). As such, early *Campylobacter* phenotyping schemes such as those based on antibiotic sensitivity profiles, serotyping, or biotyping have been superseded by schemes based on the genetic makeup of the organisms, genotyping (17, 47) hence phenotyping has been omitted in this review. Genotypic methods are divided in three main categories: (1) DNA banding pattern-based methods, which classify bacteria according to the size of fragments generated by amplification and/or enzymatic digestion of genomic DNA, (2) DNA sequencing-based methods, which study the polymorphism of DNA sequences, and (3) DNA hybridisation-based methods using nucleotide probes (49).

There is a large number of restriction enzymes that can be used to cut (digest) DNA at specific sequence. Digestion by restriction enzymes and amplification of DNA produces millions of copies of fragments available for analysis (229). Pulse-field gel electrophoresis (PFGE) is an enzymatic restriction-based method that separates large DNA molecules in a flat agarose gel by applying alternating electric fields at different angles. PFGE has a high discriminatory power and is considered a “gold standard” for typing of many bacteria (230). Restriction enzymes most commonly used with *C. jejuni* are *Sma*I, *Sal*I, *Kpn*I, *Apa*I, and *Bss*HII (229) whereas *Xho*I appears to be the most useful for *C. upsaliensis* (231). PFGE has also been successfully applied to *C. coli* (232), *C. fetus* (233) and *C. hyointestinalis* (234). The main limitation of PFGE is the time and labour consuming aspect of the method and the lack of inter-laboratory comparability due to considerable variations in the restriction enzymes and electrophoretic conditions (229).

Similar issues with inter-laboratory comparability can affect ribotyping. Ribotyping is a method based on genotyping of rRNA genes using agarose gel electrophoresis of digested genomic DNA followed by Southern blot hybridisation with a probe specific for rRNA genes (5S, 16S and 23S). Unlike PFGE, the discriminatory power of ribotyping is limited because most *Campylobacter* spp. contain only three rRNA gene copies (229). Ribotyping was successfully applied to investigate *C. upsaliensis*

outbreak in a children's daycare centre in Belgium (235) and, in combination with plasmid profiling, for comparisons of human and canine *C. upsaliensis* isolates (236).

Restriction fragment length polymorphism (RFLP) is similar to ribotyping except that hundreds of short fragments are generated. This poses difficulties in separating fragments by agarose gel electrophoresis but this can be mitigated using Southern blotting with radioactively labelled probes (237). Another way to overcome this shortcoming is to apply RFLP to a specific locus amplified by PCR, thus enzymatic digestion is applied following DNA amplification. Typing of *Campylobacter* spp. using this technique was applied to flagellar genes *flaA* and *flgE* which are a highly conserved yet variable region (238) but with variable success partly due to at least seven different procedures reported (229). It was also shown to be applicable to *C. coli*, *C. lari*, and *C. helveticus* (239). By applying a multiplex PCR to more than one locus, the PCR-RFLP method with *C. jejuni* genes *gyrA* and *pflA* reached the discriminatory power of PFGE (240).

Amplified fragment length polymorphism (AFLP) is another highly discriminatory technique using restriction enzyme digestion following amplification by PCR. The technique is based on two restriction enzymes with recognition sites of variable length that guide PCR amplification so that only those fragments flanked by both restriction sites are amplified (229). AFLP was successfully applied to characterise *C. jejuni* (241), *C. coli* (242), *C. lari* (243) and *C. upsaliensis* (244). Comparative studies in *C. jejuni* showed AFLP to have higher discriminatory power than both PFGE and PCR-RFLP (245), and was comparable to multi-locus sequence typing (MLST) and sequence analysis of clustered regularly interspaced short palindromic repeats (CRISPRs) (246). However, the disadvantages are that the AFLP technique is complex (comparable to PFGE) and requires major capital investment (an automated DNA sequencer and appropriate software) (229).

Typing methods based on fragment analysis may also be based on DNA amplification without the use of restriction enzymes. The afore mentioned CRISPR analysis is such a method based on typing of near-perfect direct repeat sequences (usually 24-48 bp) that are interspersed with (similarly sized) non-repetitive spacer

sequences (49, 246). High-resolution melting (HRM) uses PCR amplification and, through melting curve analysis, enables discrimination of DNA alleles to the level of single nucleotide polymorphisms (SNPs) (49). These methods can be combined, such as HRM analysis of hypervariable CRISPR regions of *C. jejuni* and binary gene typing (247) in order to give equivalent or better performance than the “gold standard” of PFGE (230). Another method called Rapid Amplified Polymorphic DNA (RAPD) analysis is based on the use of arbitrary short single primers to amplify genomic DNA at multiple loci and has been used to characterise *Campylobacter* spp. (49). However, while the RAPD method is inexpensive and with a fast turnaround time, the poor reproducibility of results between laboratories is a major disadvantage (248). DNA banding differences due to the influence of subjective interpretation of RAPD data were observed between strains from an outbreak of *C. jejuni* (249) and between duplicate samples (68). A slightly different approach for amplifying random genomic DNA fragments involves using primers specific for enterobacterial repetitive intergenic consensus (ERIC) sequences but this method is still limited by low reproducibility (229).

As each organism is uniquely defined by its DNA sequence, typing methods based on DNA sequencing have perhaps been the most successful due to the resolution of the data obtained and provision of the broadest range of applications (17, 49). Molecular cloning, breeding, species identification, genetic and genomic comparative studies as well as phylogenetic and evolutionary studies are available, to name a few. The major advantage of sequencing methods is the reproducibility between laboratories and the ease of sharing data that enables a ready use within research communities (49). Taken together, these are the reasons that over the last decades the sequencing technologies had the largest development of all typing methods. Of the public databases, GenBank is currently the largest online DNA sequence database (http://www.ncbi.nlm.nlh.gov/genbank).

Since the first independent development work by the Sanger (the dideoxy method), and Maxam and Gilbert (the chain-termination method) teams (for which they shared the Nobel Prize in 1980), today there are three generations of methodological design (49). The second-generation methods are characterised by various approaches that

rely on PCR amplification such as sequencing by ligation (SOLiD), by synthesis (Roche 454 Pyrosequencer, Illumina) or semiconductor-based detection of hydrogen release during DNA polymerisation (Ion Torrent). The third generation methods (*e.g.*, the Pacific Biosciences system and nanopore sequencing) are characterised by removing the reliance on PCR amplification and by signal detection during the enzymatic reaction of adding nucleotides to the complementary strand in real time.

Early DNA sequencing methods were limited to one or a few genes due to constraints on cost, time, and availability. Genes highly conserved between bacteria are useful for identification and phylogenetic analyses, for instance the 16S rRNA gene essential for bacterial survival (250, 251). The *RpoB* gene (252) that encodes the ß subunit of RNA polymerase, which is presented in Fig. 2.4, and the *gro*EL gene (253) encoding a universal 60-kDa chaperonin involved with heat-shock response, and the flagellar gene *fla* (238) have also been used for identification and/or typing of *Campylobacter* species. However, the multi-locus sequence-typing (MLST) scheme based on sequencing of multiple loci, all housekeeping genes, has been one of the most widely adopted methods (254, 255) and to date the schema exists for many different species (http://pubmlst.net/databases/default.asp). The allelic profile for each locus, a fragment of a gene in the scheme, is assigned a unique number in order of its discovery and isolates with identical sequences are assigned the same allele number. In the *C. jejuni/coli* MLST scheme, distinct allelic profiles of seven loci characterise the isolate and the sequence type (ST) is defined by the combination of alleles at each locus (256). The clonal complex (CC) groups are formed by two or more isolates that share identical allelic profiles for at least four loci and is named after the ST identified as the putative founder of the group. MLST schema exist for *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus* (257), *C. fetus* (258), *C. sputorum*, *C. hyointestinalis*, *C. curvus*, and *C. concisus* (259). The use of MLST in *Campylobacter* research has been applied to source attribution studies in New Zealand (260) and worldwide (261-264), niche adaptation (265), investigations of origin of antibiotic-resistance (266), genetic diversity (267) or distributions of a specific clone (268), and phylogenetic studies (269, 270).

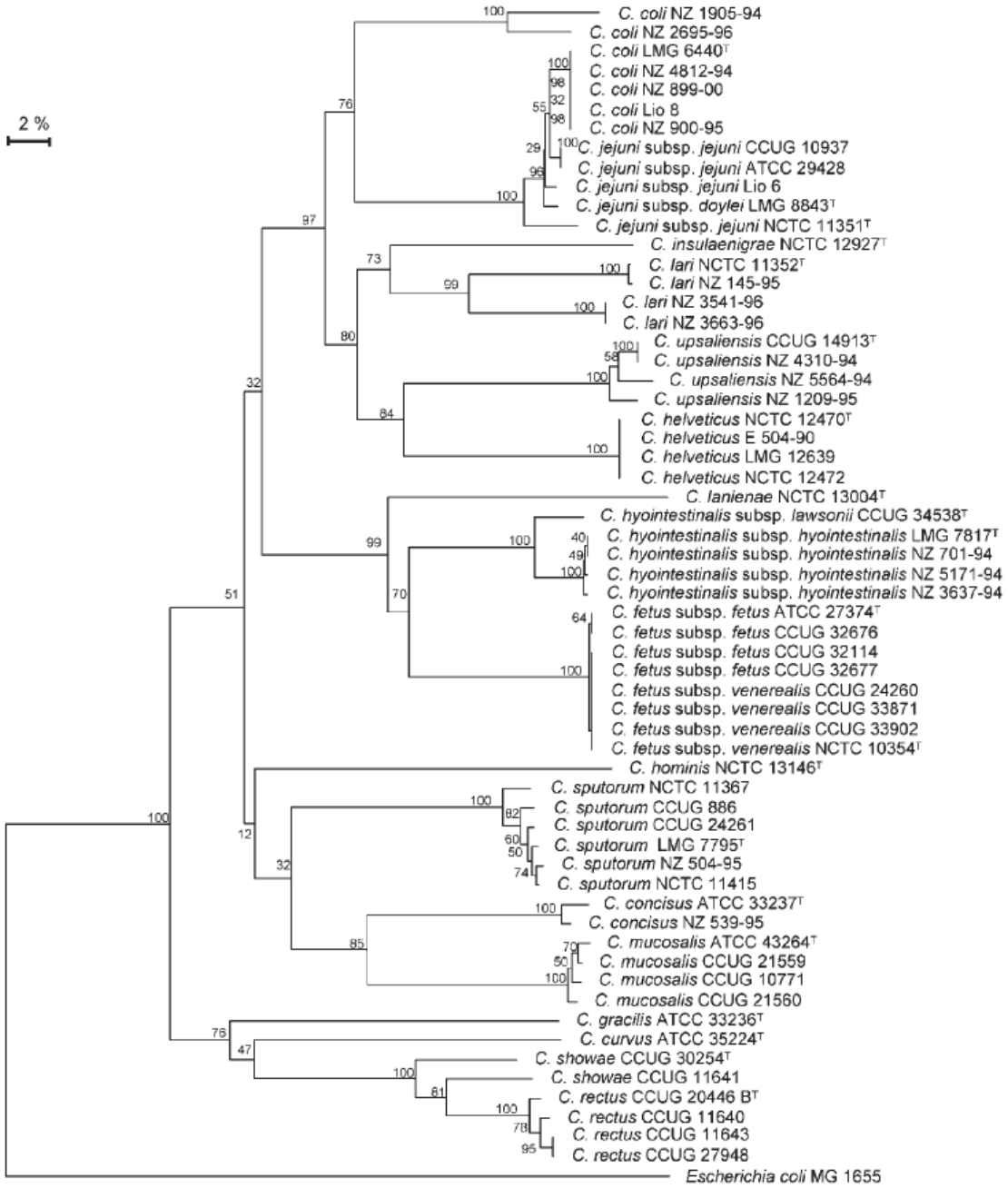

Fig. 2.4. Neighbour-joining phylogenetic tree of the genus *Campylobacter* based on partial *rpoB* gene sequences. *E. coli* was used as an outgroup. Bootstrap values of 500 simulations are indicated at major branches. Bar, 2% divergence.

Sourced from Korczak et al. (2006) (252).

As the development of sequencing methods has progressed and became more accessible and less cost-prohibitive, more complex typing schema have been developed too. For instance, unlike MLST, the whole-genome needs to be sequenced for a ribosomal MLST (rMLST). The rMLST scheme uses 53 housekeeping genes that encode the bacterial ribosome protein subunits (271). The extension of sequencing of multiple loci may be applied to whole genomes too (272, 273). Whole genome sequencing has the highest discriminatory power that can differentiate isolates down to the meroclone (variants within the colony forming unit) and clone level, whereas rMLST is suitable for differentiating from the species to strain level, and MLST can to the genus, species and the lineage or clonal complex while 16S rRNA can only discriminate to the genus level (274).

The sequencing of the *C. jejuni* genome (150) was a significant landmark and large-scale comparative genomic studies revealed extensive inter- and intra-species diversity (275) and introduced new concepts such as the core and pan-genome to species computational biology. The pan-genome of a species is a sum of all of the genes present in all strains of the respective species, whereas the core genome are those genes that are exclusively present in each and every strain of that species. The difference between these two sets of genes is a dispensable or accessory genome that represents genes present in some but not all of the species' strains (276). Therefore, the core genome is considered to represent genes involved with major genotypic (and accordingly phenotypic) traits of a species while the accessory genome contributes to the species' diversity and may confer differential features between strains such as antibiotic resistance, niche adaptation and the ability to colonise new hosts (276). Species may differ in the proportions of their core genomes in their pan-genomes (277). Species with a smaller core genome are associated with living in a highly variable environment with a sympatric lifestyle to which the large accessory pool has a greater ability to respond to. In contrast, species with a large proportion of the genome represented in the core genome are associated with a stable, or isolated environment and allopatric lifestyle (277, 278). The pathogenicity of a species has been shown to be associated with genome reduction due to gene loss and gene degradation, resulting in pseudogenes, a

pattern that was observed in comparison between facultative and obligate pathogens (279).

Whole-genome analyses in *Campylobacter* research have been mostly focused on *C. jejuni* and *C. coli*. One study showed how the pan-genome of *C. jejuni* and *C. coli* combined is around 3,000 genes but each species has a pan-genome size of around 2,600 genes. This demonstrated that the gene repertoire of the two sister species are largely overlapping (280). Another study showed evidence of a convergence of *C. jejuni* and *C. coli* species, that is, the clade 1 of the *C. coli* population was "despeciating" toward *C. jejuni* (269). However, there are debates over this phenomenon, with suggestions that interspecies genetic exchange is rare and limited, and biased by only a few housekeeping genes and the boundary between the two species is unlikely to be eroded (281). An important note is that genome association studies are in the relatively early stages, and the observed differences in results between studies can be due to sampling and analytical methods (282). Nevertheless, this is an active research area and is likely to be significantly expanded in the coming years and further combined within the genome-wide association studies framework with other "~omics" techniques such as analysis of RNA (transcriptomics), protein (proteomics), metabolites (metabolomics) and other phenotypic methods such as phenotype microarray systems (283 , 284).

2.2. Epidemiology and public health

2.2.1. Epidemiology in humans

The epidemiology of *Campylobacter* in humans is primarily dependent on the socioeconomic status that distinguishes two patterns in global incidence of the disease. Campylobacteriosis in developing countries is endemic and marked by common asymptomatic infection and seasonality (285, 286). In these regions, *Campylobacter* is associated with symptomatic infection only in the first six months of life and rarely in adults (2). Expression of illness is affected by both strain characteristics and pre-existing immunity (287) and as children age, their

Campylobacter infections become milder, they excrete fewer organisms, and *Campylobacter*-specific serum antibodies rise progressively (288). However, asymptomatic infection may also adversely affect health as campylobacteriosis was recently shown to be associated with poor early-childhood weight gain (289). In the setting of developing countries the pathogen is ubiquitous in the environment, hence, risk factors are frequently associated with environmental routes of transmission, especially drinking water (290). On the other hand, in the developed world campylobacteriosis is sporadic, except for common-source outbreaks, asymptomatic excretion is low and all age groups can be affected by clinical disease (291). The most common form of clinical disease is gastroenteritis although a range of other gastrointestinal and extraintestinal diseases are associated with *Campylobacter* species (4). This thesis is mainly focused on *Campylobacter* in the developed world and in association with gastroenteritis unless otherwise stated.

Acute gastroenteritis is one of the most common illnesses in humans in general and in New Zealand it is estimated to cause 4.6 million cases every year, of which only 0.4% are notified, and approximately 80% of cases have no identified cause of disease (292). Similarly, in Australia 68% of the reported hospital diagnoses of gastroenteritis were of unknown aetiology (293) as were 49% of foodborne cases reported in England and Wales between 1996 and 2000 (1). Campylobacteriosis is the most frequent notifiable gastroenteral disease of bacterial cause in humans in New Zealand (6) but the notification rates are thought to represent only a tip of the iceberg due to the underreporting of cases (294). Although the worldwide reports of incidence may vary due to differences in methodology and populations sampled, campylobacteriosis is considered one of the major infectious diseases in humans with a rise in global incidence in the last decade (4). The most prevalent *Campylobacter* spp. associated with human acute gastroenteritis in New Zealand are *C. jejuni* and *C. coli* (5), as is found globally, being responsible for approximately 80-85% and 10-15% of confirmed cases, respectively (3). Therefore, the majority of studies have focused on these two species unless otherwise noted.

Campylobacteriosis was reported to be more prevalent in children under five, in young adults between 15 and 24 years of age, and in the elderly in Europe (8, 295),

which is similar to reports in New Zealand (6, 296). Other reported groups with higher risks are males (296, 297), the immunocompromised (298), people with chronic conditions (14, 299) or those taking proton-pump inhibitors (300), people with a higher occupational risk due to contact with animals or meat (296, 301, 302), and overseas travellers (296, 303, 304). Overseas travel as a risk for developing campylobacteriosis was shown to be dependent on the destination (305) but domestic travel was also shown as a risk factor (306). Location of residence was also shown as an important demographic risk factor with a higher risk for people living in rural areas compared to urban dwellers (296, 301). This variable has been shown to be important for sentinel sites to be representative of the population under surveillance and in resulting epidemiological associations observed (307). People with higher socioeconomic status were reported to be at an increased risk of campylobacteriosis (308) and in turn, those socioeconomically deprived have a decreased risk (cited in 309). However, it is unknown whether the risk associated with the socioeconomic status is due to different exposure rates or reporting practices between the groups. Ethnicity has also been reported to be associated with risk of infection in the USA and the UK (297, 310) but data from New Zealand are discordant in this regard (6, 304, 311) and could be confounded by socioeconomic factors.

Campylobacteriosis is predominately a foodborne disease, hence the majority of identified risk factors for contraction of the disease are related to food and food-related practices. With regard to food consumption, commonly identified risk factors, worldwide and in New Zealand, were undercooked chicken (261, 304, 312, 313), offal (299, 314), and raw or unpasteurised dairy products (304, 315-317). Meta-analysis of sporadic *Campylobacter* infections in people also showed undercooked chicken and raw dairy products as major risk factors but not offal (14). With regard to food-related practices, the method of preparation of chicken and place of consumption was reported as a significant risk factor. Baking or roasting chicken was shown to be protective compared to barbecuing or frying (304), eating chicken at a restaurant compared to preparation at home (14, 304), and eating beef and pork were shown as protective factors yet if these meats were eaten at a restaurant they were a risk factor (14). Consumption of several food items were shown to be

protective against contraction of campylobacteriosis such as vegetables and/or fruit, eggs, fish (but raw or undercooked as a risk factor), and pasteurised dairy products (14, 299, 304).

Important limitations of epidemiological case-control studies are the unmeasured variables that are related or correlated with the variables being followed up, and the definitions and level of resolution of the latter between the studies. Consumption of certain food items such as vegetables was consistently reported as a protective factor (14) and this protective factor significantly increased with a higher amount of vegetables consumed (299) although vegetables may be a source of exposure as they have been shown to be contaminated with *C. jejuni* (318). These indicate variable and complex dietary behaviours between cases and controls that may have been unmeasured (319). On the other hand, consumption of vegetables may have provided protective effects by other unrelated mechanisms. For instance, vegetable components may improve immunity or inhibit bacterial growth (313, 320) or affect the intestinal microflora (321, 322). With regard to definitions of risk factors, contact with animals is another complex term as shown by a study in New Zealand. This study showed how any contact with animals was a protective factor, whereas contact with cattle or calves was a risk, and occupational contact with any animal carcasses was neither a risk nor protective yet contact with cattle or calf carcasses was a risk factor (304). Furthermore, unlike having any pet or any caged bird at home, having a puppy or three or more caged birds was a risk factor, as was having any pet at home with diarrhoea in the prior 10 days (304). The latter was also reported as a risk factor in several other studies (323-325). Having a dog may be a risk factor for particular groups such as children under the age of six (326). The complex interaction of sources and transmission pathways leading to exposure of humans with zoonotic agents is presented in Fig. 2.5.

Water and the environment are other well-documented common sources with many transmission routes for exposure of humans to *Campylobacter* species. They are considered to be contaminated by animals that are primary reservoirs of the bacteria (5, 80). Significant water-related risk factors for becoming ill with campylobacteriosis were shown for ingestion of untreated water from natural sources (324), recreational

water activities in natural sources (327), different water-supply systems or events such as problems with the sewage system at home during the incubation period of cases (304) but also the drinking of bottled water (328). Environmental exposure may affect the epidemiology of campylobacteriosis in complex ways. Increased environmental loading with *Campylobacter* from animals and changes in human lifestyle may also be linked with the seasonality of disease observed in humans (57, 70, 79, 108).

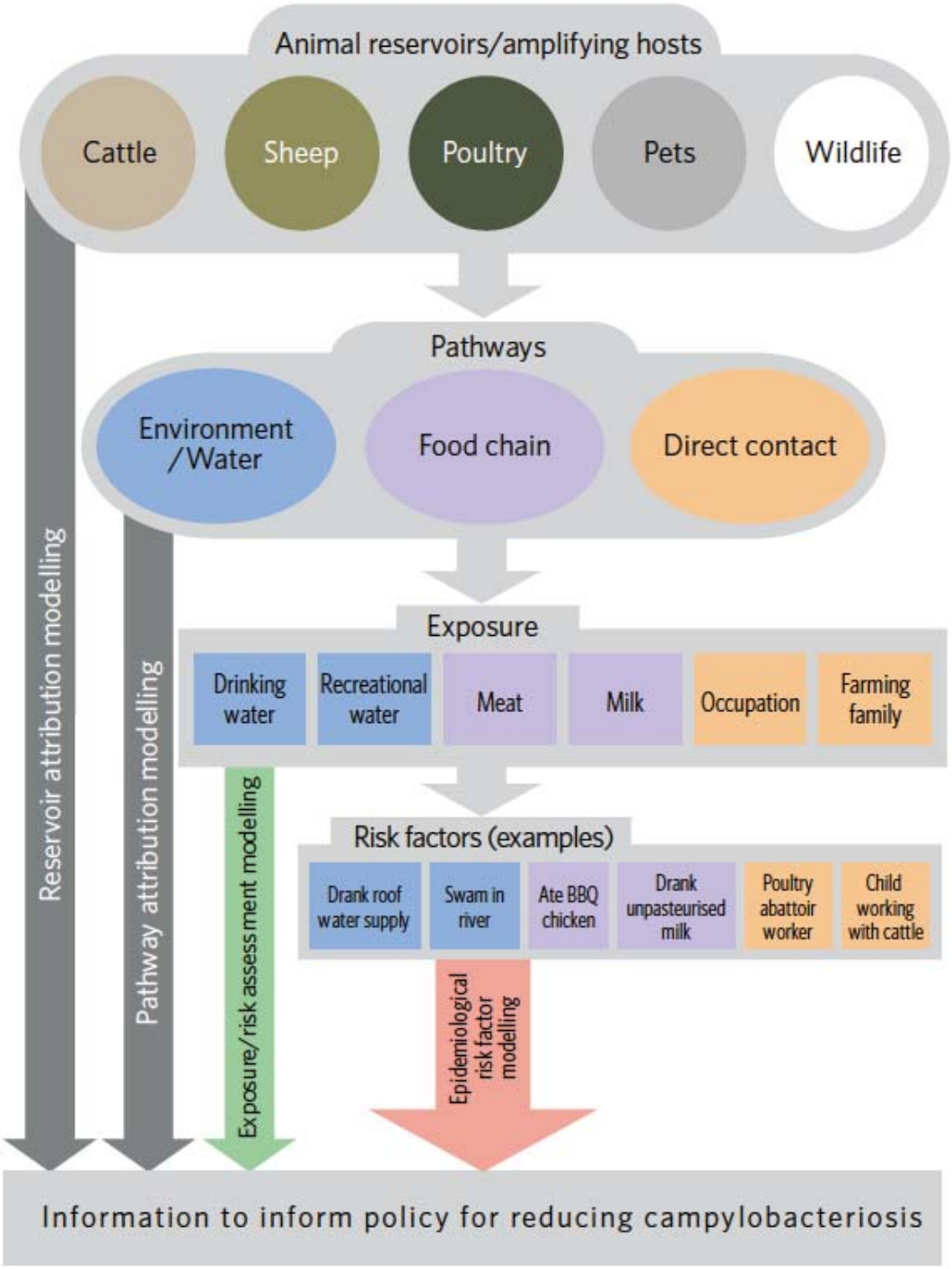

Fig. 2.5. Framework showing sources of information and modelling approaches for the transmission of zoonotic diseases, including campylobacteriosis. Note the terms reservoir, pathway, exposure and risk factor are used here for illustrative purposes, to show how various levels of data disaggregation and refinement can be incorporated into different models for informing decision making.

Adapted from WHO 2013 (329).

Although there are many sources and transmission routes for exposure to *Campylobacter*, poultry, especially chicken, is widely considered to be the major contributor to disease incidence in humans. In addition to the above epidemiological studies, case reports from surveillance data of several countries and molecular epidemiology studies support this finding. Poultry and eggs were withdrawn from sales in Belgium during May and June of 1999 due to contamination with dioxins and a coincidental drop of 40% in human cases of campylobacteriosis was observed (330). In Iceland, the sale of chilled chicken started in 1996 and the incidence of human cases of campylobacteriosis rose and peaked in 1999 at a rate of 116 per 100,000 inhabitants with 62% of broiler carcass rinses reported to be contaminated. Preventive measures were introduced relating to biosecurity and farm management practices, and public education that resulted in only 15% of broiler carcass rinses being contaminated with *Campylobacter* and the incidence in humans dropped to 33/100,000 in 2000 (331). Similar reports came from New Zealand when control measures were introduced in the poultry industry (332, 333) after molecular epidemiology studies showed significant evidence for poultry as the major source of disease in humans (260, 312, 334). Many source attribution studies worldwide have shown poultry as the major source and cattle (or with inclusion of sheep denoted as ruminants) as the second major source of *C. jejuni* in human cases, and all other sources (*e.g.*, pigs, dogs or pets, wild animals and water) as minor contributors (usually less than 5%) such as has been shown in studies in Denmark (335), Switzerland (264), Finland (336), the United Kingdom (262, 337, 338), and the Netherlands (339). The host-association of STs has been shown to transcend geographical variations (340) and temporal fluctuations (341, 342). Overall, it has been estimated that handling, preparation and consumption of broiler meat may account for 20% to 30% of human cases of campylobacteriosis, while 50% to 80% may be attributed to the chicken reservoir as a whole (309). The global incidence of *C. jejuni*- and *C. coli*-associated campylobacteriosis is presented in Fig. 2.6.

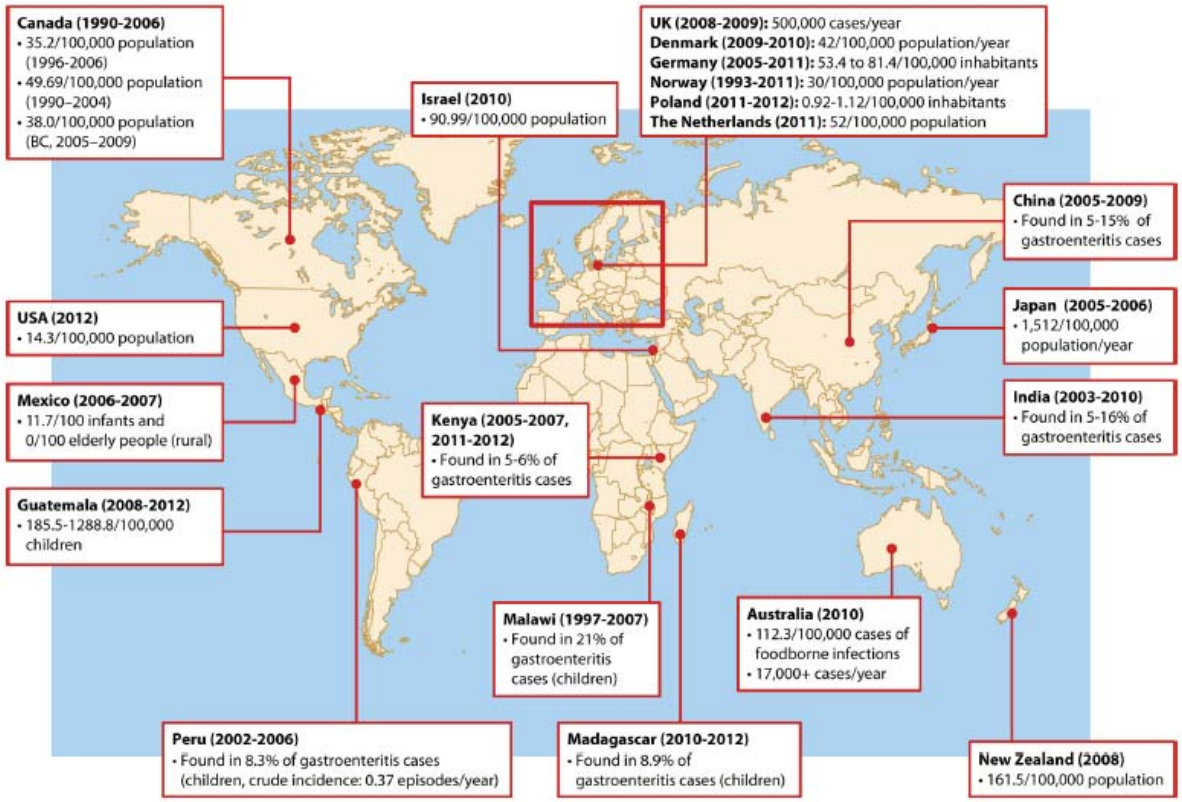

Fig. 2.6. The incidence and prevalence of campylobacteriosis caused by *C. jejuni*/*C. coli* world wide.

Sourced from Kaakoush et al. (2015) (4).

One of the limitations of source attribution studies is that the analytical framework cannot infer the transmission route. That is, being based on genetic similarity, and other parameters depending on the models used, of *C. jejuni* isolates between humans and studied sources, the method attributes the probability of a source from which the ST observed in humans has originated (312). *C. jejuni* STs that occur in many sources are called “generalists” and are difficult to assign to a particular source as shown for STs of CC-45 and CC-21 (312, 343, 344). Conversely, “specialists” are STs (or CCs) that are only, or by far predominately, observed in particular animals as shown for ST-474, ST-50, CC-257 with poultry (260, 339), ST-61 and ST-42 with cattle (345) and ST-3704 with wild birds (265). The analytical methods in source attribution studies are also limited with regard to appropriately modelling sources such as environmental water, because water is not considered a primary amplifying host but more as a vehicle contaminated by primary hosts thus, STs in water are

frequently observed in wild birds and ruminants that are likely to be the highest contributing hosts to contamination of environmental water (67, 312). Similarly, contamination of children's playgrounds is associated with STs of wild birds (346).

The epidemiology of non-*jejuni/coli Campylobacter* spp. in humans has been far less studied than that of *C. jejuni/coli*. The occurrence of these other *Campylobacter* spp. in both healthy and sick people has been used to question their significance as pathogens, for example the possible association of *C. concisus*, *C. showae*, and *C. rectus* with inflammatory bowel disease (13). There are no reports on the isolation of *C. upsaliensis* from healthy people in the developed world but only from people with diarrhoea or with extra-intestinal infections (97), which supports the association with human disease. In addition, *C. upsaliensis* has been reviewed as being one of the most frequently isolated species other than *C. jejuni* as studies showed to exceed the rate observed for *C. coli* (97) in human clinical samples. Apart from reports on the significant disease burden of *C. upsaliensis*, a few similar epidemiological features from *C. jejuni/coli* have been observed. *C. upsaliensis* was reported as more prevalent in particular groups of people; children, the elderly, the immunocompromised and those HIV positive (97). These may be more susceptible groups that would require special attention and misdiagnosis could potentially be severely harmful. On the other hand, *C. helveticus* is genetically most closely related to *C. upsaliensis* and it is difficult to differentiate between these two species. *C. helveticus* is currently not considered pathogenic to humans (13) due to weak evidence as only one study reported two out of 500 faecal samples from humans with clinical signs to be PCR positive (347). It has been reported that *C. helveticus* is associated with periodontitis in humans (17) but more studies are needed to confirm the pathogenic potential of this species.

The most recent report on diagnostic and public health management practices concerning bacterial diseases in New Zealand showed that none of the laboratories that responded to the survey used methods that could isolate non-*jejuni/coli Campylobacter* spp. (53). While it is possible some laboratories that have not responded might be using these methods, the fact that 32 out 36 did respond highlights the paucity of data on the prevalence of non-*jejuni/coli Campylobacter* spp.

Extrapolating international data to clinical medicine in New Zealand, it is highly likely there is a significant underestimation of the occurrence of non-*jejuni/coli Campylobacter* spp. in human patients. Support of this view is strengthened by a recent report in New Zealand that showed many different *Campylobacter* spp. are present in humans, with *C. upsaliensis/helveticus* (the method used could not differentiate the two species) present only in people with symptoms of diarrhoea whereas many others such as *C. concisus*, *C. gracilis*, *C. ureolyticus*, and *C. hominis* were found both in healthy volunteers and symptomatic patients (348). This was a small study with regard to the sample size but the findings are too important to be diminished by that argument. However, unspeciated reporting, as *C.upsaliensis/helveticus,* may be of a concern, as from a public health perspective it may be inappropriate to group these two species until the pathogenicity of *C. helveticus* is documented.

Due to the complex epidemiology of *Campylobacter* spp. in humans, the public health policies, guidelines and preventive measures needed are multifaceted. Control measures may be aimed at farm level, or at the processing plant within the food production chain, as well as in education of the general public of safe and hygienic practices in domestic kitchens (4, 76, 79). Identifying the most important sources of disease is important for prioritisation of food-safety interventions and setting public health goals (349). Studies have also shown the need for accessible information and guidelines with regard to the control of zoonoses from contact with pets and by pet husbandry practices (350, 351), including in sensitive groups such as immunocompromised people (352).

2.2.2. Epidemiology in dogs and cats

Campylobacter jejuni was first isolated from dogs in 1977 in the United Kingdom (10) and from cats in 1980 in the United States of America (15), whereas the first association of dogs as a risk factor for infection in humans was reported as early as 1961 (353). Since then *Campylobacter* spp. have been frequently reported in pets around the world and have been recognised as pathogens to pets in addition to the

zoonotic risk they bear to humans (354). Many *Campylobacter* spp. have been detected in the faeces of dogs and cats but the most common species have been *C. jejuni, C. coli, C. upsaliensis* and *C. helveticus* (16). The latter two species have been most commonly isolated from pets and have been almost exclusively detected in dogs and cats. However, the pathogenicity of *Campylobacter* spp. to pets has been questioned due to equivocal results obtained from epidemiological studies and its discordance with some experimental challenge studies. A summary of the reported prevalences of *Campylobacter* spp. in dogs and cats is presented in Table 2.7.

Table 2.7. Reported prevalence rates[a] of *Campylobacter* spp. in dogs and cats.

Taxon	Dogs (%)	Cats (%)	Method	Reference
***Campylobacter* species**	19	11	Rectal swab, culture[1]	(355)
	8	2	Faeces, culture[2]	(356)
	33		Faeces, culture[1]	(357)
	51		Rectal swab, culture[1]	(358)
	58 / 97		Faeces, qPCR	(359)
	44 / 50 (75 / 75)	37 (67)	Rectal swab, culture[2]	(360)
	23 / 31		Faeces, culture[NA]	(361)
	84 / 77 (100 / 100)	78 (88)	Faeces and swab, culture[2]	(362)
		3.3	Faeces, culture[1]	(363)
	6.3	4.5	Rectal swab, culture[2]	(364)
	36.4	10	Rectal swab, culture[2]	(365)
	2.2		Faecal swab, culture[2]	(139)
	17	16	Rectal swab, culture[2]	(366)
		28	Faeces, culture[1]	(367)
		0.8	Faeces, culture[1]	(368)
	7.1	7.4	Faeces, culture[1]	(369)
	4 / 9 (23 / 26)		Rectal swab, culture[2]	(370)
	4 / 9	20 / 13	Faeces, culture[1]	(371)
	20		Rectal swab, culture[2]	(372)
	44	13	Rectal swab, culture[2]	(373)
	23 / 15 (24 / 36)		Faeces, culture[1]	(374)
	10	15 / 0 (0 / 25)	Faeces, culture[1]	(375)
	6 / 8		Faeces, culture[1]	(376)
	0 / 5.5		Rectal swab, culture[2]	(377)
	- / 50 (- / 75)		Faecal microscopy	(378)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter jejuni	23	21	Rectal swab, culture[1]	(355)
	5	7	Faeces, culture[2]	(356)
	53		Rectal swab, culture[2]	(379)
	6		Faecal swab, culture[2]	(380)
	41 / 40 (33 / 56)		Faecal swab, culture[2]	(381)
	4		Faecal swab, culture[2]	(382)
	6		Faeces, culture[2]	(383)
	51		Rectal swab, culture[2]	(384)
	62 / 63		Faecal swab, PCR	(385)
	23 (10)		Rectal swab, culture[2]	(386)
	30 / 33		Rectal swab, culture[2]	(387)
	23		Rectal swab, culture[1]	(388)
	2	9	Rectal swab, culture[2]	(389)
	5 / 15		Faecal swab, culture[2]	(390)
	38	31	Faeces, culture[2]	(391)
	15 / 22		Rectal swab, culture[2]	(392)
	3		Faecal swab, culture[2]	(393)
	1		Faeces, culture & PCR	(394)
	15		Faeces, culture[2]	(395)
	7 / 46		Faeces, qPCR	(359)
	1		Faeces, culture[2]	(396)
	14 / 50	12	Rectal swab, culture[2]	(360)
	12 / 8	5 / 10	Faeces, culture[2]	(397)
		2 (28)	Rectal swab, culture[2]	(398)
	12	1	Rectal swab, culture[2]	(399)
		4	Faeces, culture[1]	(363)
	5.7	10	Rectal swab, culture[2]	(364)
	52		Rectal swab, culture[2]	(365)
	15		Faecal swab, culture[2]	(139)
	11	3	Faeces, culture[2]	(400)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter jejuni	3		Rectal swab, culture[1]	(401)
	40		Faeces, culture[2]	(402)
	- / 14	4	Rectal swab, culture[1]	(403)
	11	0	Faeces, culture[1]	(404)
	22		Rectal swab, culture[1]	(405)
	16	3	Rectal swab, culture[1]	(406)
	23		Faeces, culture[1]	(369)
	7	3 / 5	Rectal swab, culture[1]	(407)
	4 / 4 (8 / 18)		Faeces, culture[1]	(371)
	35 / 80		Rectal swab, culture[1]	(408)
	29		Rectal swab, culture[1]	(409)
	40 – 86[3]		Rectal swab, culture[1]	(410)
	58		Faeces or swab culture[1]	(411)
	11 / 11		Faecal swab, culture[1]	(412)
	3 / 22	0 / 17	Faeces, culture[1]	(413)
			Rectal swab, culture[1]	(414)
	9 (10 / 35)		Faeces, culture[1]	(415)
	4	11	Faeces, culture[1]	(416)
		10 / 16	Faeces, culture[1]	(417)
	2 / 12		Faeces or swab culture[1]	(418)
	(63 / 90)	0	Rectal swab, culture[1]	(419)
	3 / 14 (8 / 37)		Rectal swab, culture[1]	(420)
	6 – 38		Faeces, culture[1]	(421)
	10 / 17	3 / 13	Faeces, culture[1]	(374)
	10 / 16		Faeces, culture[1]	(422)
	8 / 54		Faeces, culture[1]	(423)
	11 / 17		Rectal swab, culture[1]	(424)
	1 / 0		Faeces, culture[1]	(425)
	25 / 34	14	Faeces, culture[1]	(426)
	30	3	Rectal swab, culture[1]	(427)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter jejuni	6 (36)		Faeces, culture[1]	(15)
	0 / 5 (0 / 28)	45	Faeces or swab culture[1]	(428)
	49 (39 / 38)		Faeces, culture[1]	(429)
	12 / 9 (11 / 17)		Faeces, culture[1]	(430)
Campylobacter coli	7		Rectal swab, culture[2]	(379)
	4		Faecal swab, culture[2]	(380)
	11 / 40 (13 / 22)		Faecal swab, culture[2]	(381)
	2 (2)		Rectal swab, culture[2]	(386)
	10		Rectal swab, culture[2]	(384)
	4 / 8		Rectal swab, culture[2]	(387)
	9		Rectal swab, culture[1]	(388)
	2	1	Rectal swab, culture[2]	(389)
	8 / 54		Faecal swab, culture[2]	(390)
	11	0	Faeces, culture[2]	(391)
	0 / 25		Faeces, qPCR	(359)
	0 / 0	4	Rectal swab, culture[2]	(360)
	1		Rectal swab, culture[2]	(399)
		1	Faeces, culture[1]	(363)
	1	1	Rectal swab, culture[2]	(364)
	8	0	Rectal swab, culture[2]	(365)
	1		Faecal swab, culture[2]	(139)
	2		Faeces, culture[2]	(400)
	0	1	Rectal swab, culture[1]	(401)
	2		Faeces, culture[1]	(404)
	10	0	Rectal swab, culture[1]	(406)
	5		Faeces, culture[1]	(369)
	13		Rectal swab, culture[1]	(408)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter upsaliensis	74	90	Rectal swab, culture[1]	(355)
	40		Rectal swab, culture[2]	(379)
	49 / 20 (54 / 22)		Faecal swab, culture[2]	(381)
	29		Faecal swab, culture[2]	(382)
	30 – 39		Faeces, culture[2]	(383)
	39		Rectal swab, culture[1]	(384)
	50 / 46		Faecal swab, PCR	(385)
	37		Faeces, culture[2]	(292)
	19 (12)		Rectal swab, culture[2]	(386)
	7 / 17		Rectal swab, culture[2]	(387)
	52	8	Faeces, culture[2]	(391)
	22 / 28		Rectal swab, culture[2]	(392)
	19		Faecal swab, culture[2]	(393)
	62		Faeces, culture[2]	(395)
	38		Faeces, culture & PCR	(394)
	43 / 85		Faeces, qPCR	(359)
	25		Faeces, culture & PCR	(396)
	51 / 20	36	Rectal swab, culture[2]	(360)
	31 / 12	14 / 5	Faeces, culture[2]	(397)
	1		Rectal swab, culture[2]	(399)
		19	Faeces, culture[1]	(363)
	30[3]	35[3]	Rectal swab, culture[2]	(364)
	5	30	Rectal swab, culture[2]	(365)
	59		Faecal swab, culture[2]	(139)
	2 / 3[3]		Faeces, culture[1]	(431)
	53		Faeces, culture[2]	(402)
	43		Faeces, culture[2]	(400)
	20	13	Rectal swab, culture[1]	(401)
	55 / 28)	41	Rectal swab, culture[2]	(432)
	3 / 7	0	Rectal swab, culture[2]	(403)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter upsaliensis	34	11	Faeces, culture[1]	(404)
	6	5	Rectal swab, culture[1]	(405)
	7	66	Faeces, culture[1]	(369)
	18 / 20 (13 / 26)	9 / 5	Faeces, culture[1]	(371)
	6 / 19		Faecal swab, culture[1]	(412)
	14 / 47		Faeces, culture[1]	(433)
Campylobacter helveticus	7		Rectal swab, culture[2]	(379)
	1		Faecal swab, culture[2]	(382)
	0	62	Faeces, culture[2]	(391)
	10 / 25		Faeces, qPCR	(359)
	5 / 0	8	Rectal swab, culture[2]	(360)
	2 / 1	19 / 16	Faeces, culture[2]	(397)
	0	50	Rectal swab, culture[2]	(365)
	2		Faeces, culture[2]	(400)
	0.4	46	Rectal swab, culture[2]	(432)
Campylobacter lari	2 / 0		Rectal swab, culture[2]	(387)
	0 / 9		Faeces, qPCR	(359)
	1		Faeces, culture[2]	(396)
	2 / 0	4	Rectal swab, culture[2]	(360)
	2 / 0	0	Faeces, culture[2]	(397)
	1	0	Rectal swab, culture[2]	(364)
	2		Faecal swab, culture[2]	(139)
	10		Faeces, culture[2]	(402)
	1		Faeces, culture[2]	(400)
	0.3		Rectal swab, culture[1]	(406)
	2	0	Faeces, culture[1]	(369)
Campylobacter fetus	9 / 37		Faeces, qPCR	(359)

Taxon	Dogs (%)	Cats (%)	Method	Reference
Campylobacter hyointestinalis	13 / 18		Faeces, qPCR	(359)
Campylobacter concisus	0 / 9		Faeces, qPCR	(359)
Campylobacter curvus	1 / 2		Faeces, qPCR	(359)
Campylobacter gracilis	0 / 9		Faeces, qPCR	(359)
Campylobacter mucosalis	0 / 6		Faeces, qPCR	(359)
Campylobacter rectus	1 / 3		Faeces, qPCR	(359)
Campylobacter showae	6 / 28		Faeces, qPCR	(359)
Campylobacter sputorum	1 / 18		Faeces, qPCR	(359)

[a] Prevalences are denoted, if data were available, as healthy / diarrhoeic, with brackets denoting data for juvenile and young animals, and ranges representing data from longitudinal studies. Species identification performed by [1] phenotypic, [2] PCR testing, or [NA] not available. [3] Differentiation between *C. upsaliensis* and *C. helveticus* not possible

There are a number of reports that have associated clinical signs of gastroenteritis with the faecal carriage of *Campylobacter* spp. in dogs and cats (Table 2.7). Early work with experimental infection of dogs in the late 70's and 80's showed the pathogenic potential of *C. jejuni* to dogs (412, 434-436). This association was supported by seroconversion, gross and histopathological evidence of inflammation and erosive colitis, and high numbers (>10^7 colony forming units per gram of content) of colonising bacteria in the epithelia of the colon and ileum with no mucosal invasion, although bacteria were isolated from various internal organs and blood. Organisms were also recovered from jejunal and duodenal contents but exact quantification was not performed. One of the most prominent clinical findings was that dogs, in general, did not develop clinical signs as severe as those seen in human disease, although, some human volunteers were shown to have no or mild clinical signs too (88, 149). Clinical signs included tenesmus, diarrhoea, lethargy, and inappetence. Diarrhoea mostly ranged from soft to mucoid faeces while watery diarrhoea occurred in a minority of dogs. Experimental findings were in contrast to clinical reports from naturally occurring cases where dogs showed more severe clinical signs (354). Furthermore, experimental studies showed clinical signs were not observed in all of the challenged dogs although some dogs had organisms recovered from blood and viscera. While all of the studies have been performed on a rather limited number of animals, usually between three and six, the studies suggest there may be different pathogenic properties of the strains used and/or variable susceptibility of dogs to developing the disease. There is only one experimental study involving cats in which clinical signs did not develop and *C. jejuni* was detected in faeces for only 2 to 3 days post challenge; no other findings were reported (435). Recently, a retrospective study of duodenal biopsy specimens reported *C. coli* to be associated with neutrophilic inflammatory bowel disease (437).

Most epidemiological studies investigating *Campylobacter* spp. in dogs and cats have been of a cross-sectional design. These studies have shown a vast variability in prevalence rates of intestinal carriage of *Campylobacter* spp. in dogs and cats ranging from 0 to 100% (Table 2.7). These differences could be explained by different populations sampled with regard to the age of animals or their environment (*e.g.* household pets, stray, kennelled, working dogs), sampling method (rectal swabs

vs. faeces), methods of detection (various culture protocols, PCR), animal health status (diarrhoeic vs. healthy), geographical location where the study was conducted and the time of year it was performed; all of which can result in different exposure rates and susceptibilities of the animals under investigation. These differences between the study designs are most likely the main cause of difficulties in comparing the results and deriving unambiguous inferences.

The association of diarrhoeal clinical signs and faecal shedding of *Campylobacter* spp. in dogs and cats has been variable. While some studies have reported a positive association of faecal carriage of *Campylobacter* spp. and presence of clinical signs (15, 371, 376, 392, 413, 419, 420, 422, 438), the majority of studies have not (15, 358, 363, 365, 370, 373-375, 393, 394, 397, 399, 404, 412, 419, 424-426, 429-431, 439-445), which is in contrast to the findings of experimental studies. The variable association with clinical signs has been used to question the significance of the pathogenic potential of *Campylobacter* spp. in pets. Some researchers have argued that other factors are needed for a clinical disease to develop and that in this regard *Campylobacter* spp. are not a primary but rather a secondary pathogen. Proposed factors that would predispose pets to develop disease have been co-infections with other *Campylobacter* spp. (359, 364, 402, 446), other bacterial pathogens such as *Helicobacter* spp. (367), and viruses and/or endoparasites (365, 420, 433). One important aspect when comparing the findings of epidemiological studies with experimental studies is the animal's age. Most experimental studies have been performed on puppies and kittens, either gnotobiotic or normally reared, while only one study has been performed on juvenile animals (412). In this study, only one dog out of three inoculated with *C. jejuni* developed diarrhoea and one dog out of three inoculated with *C. upsaliensis* passed loosely formed faeces making researchers question the role of *Campylobacter* spp. as primary pathogens. The majority of studies have shown an increased prevalence of *Campylobacter* spp. in younger pets compared with adults (Table 2.7) while a few studies have shown an inverse relationship in cats, *i.e.* the higher prevalence in older cats (355, 398). The decrease in prevalence as animals mature has been associated with the development of acquired immunity (354). However, in this age group diarrhoea has also been variably associated with *Campylobacter* species (Table 2.7).

Only a few longitudinal studies have been performed in pets (363, 395, 410, 446). All studies were, however, in agreement that the majority of dogs and cats were asymptomatic shedders of *Campylobacter* spp. Furthermore, the pattern of excretion has been mostly intermittent although some have found the excretion can be quite prolonged. This has been observed in dogs (395, 446) and cats (363) and it was suggested that a carrier state may exist in pets, or that *Campylobacter* spp. can be a part of the normal intestinal flora. However, in dogs it was shown there is a different pattern between excretions of *C. jejuni* and *C. upsaliensis* with the former being more commonly isolated on a single day in the study period whereas the latter was isolated during longer periods of time (446). The significance of this finding is uncertain. Lastly, longitudinal studies have shown dogs frequently appear positive on alternate days, which suggests that collection of multiple samples may aid in successful isolation of *Campylobacter* spp. (395).

Most of the studies were in agreement that the prevalence rates of *Campylobacter* spp. are higher in stray or sheltered/kennelled animals when these populations were compared to the household pets (362, 374, 404, 406, 417, 429). Some studies have found a strong disagreement in this regard. For instance, in Trinidad, which is a small country and, perhaps more importantly, an island giving it confined environmental features, a 13.8% prevalence was reported in 130 client-owned dogs (370) while no *Campylobacter* spp. were detected in 100 stray dogs (447). This finding could be explained by a relatively small sample size or the intermittent nature of *Campylobacter* spp. excretion in faeces. It has been argued that dietary factors, stress associated with living in shelters/kennels (362) and increased close contact/intensive housing (404) are responsible for the differences between shelter/kennelled and household pets. For the household dog population, studies have shown there is an increased risk of *Campylobacter* spp. shedding when more dogs are present in a household (358, 448), whereas others have not (393, 440). For stray dogs, a longitudinal study upon impoundment showed a significant increase in the rate of isolation at days 5-7 compared to day 1 at arrival (421), which could be related to all of the above factors. Different exposure rates have been also implicated as a cause of differences in *Campylobacter* prevalences for subpopulations of stray

cats. Stray cats living in a harbour area were more likely to shed *C. jejuni* than stray urban cats (398).

Years of research have significantly increased our knowledge of *Campylobacter* spp. in dogs and cats but many questions with regard to their pathobiology in pets remain unanswered and there is a need for larger and more standardised studies. Finally, the advancements of technology and molecular approaches could greatly aid in resolving some of the equivocal findings such as potential differences in the pathogenicity of species, variable prevalence rates between animal populations and the role of the host's immunity. One of the key findings of a recent meta-analysis of prevalence and concentrations of *Campylobacter* spp. in household dogs (and petting zoos) was the lack of data, the inconsistent reporting between studies, including the diarrhoeic status of animals and speciation of *Campylobacter* species (449). Additionally, the large overall variability in *Campylobacter* spp. prevalence rates between studies remained unexplained even with sub-group analyses by the region and country locations, animal species sampled, sources of populations surveyed, and diarrhoeic status of animals thus, the factors responsible for the observed variation in prevalence rates are yet to be identified.

2.3. Research aims

As discussed previously, the reported prevalences of *Campylobacter* spp. in dogs and cats has varied widely between studies worldwide. Data for New Zealand dogs and cats are limited. One study performed in the Manawatu sentinel site, screened 530 dog and 64 cat faecal samples. *C. jejuni* prevalence rates of 4.5% and 4.7% have been reported for dogs and cats, respectively (356, 450). The study design did not allow epidemiological investigations with regard to factors associated with faecal carriage due to the anonymous sampling scheme and only focused on the identification of *C. jejuni*. This knowledge gap is addressed in this thesis with two studies:

- A cross-sectional study of *Campylobacter* spp. in the urban population of dogs and cats in Manawatu.

- A cross-sectional study of *Campylobacter* spp. in the working farm dog population in Manawatu.

In order to additionally investigate the potential exposure of humans with pet food-related sources, a cross-sectional pilot survey of raw meat pet diets for the presence of *Campylobacter* spp. was also performed. Pet diets sampled included commercially available raw pet food products in Palmerston North and home-kill frozen sheep and beef meat as part of the working farm dogs' diet.

A recent survey of human diagnostic laboratory practices in New Zealand has shown that the vast majority of laboratories use standard culture methods whereas only one uses an immunoenzymatic method for detection of *Campylobacter* spp. (53). The report has shown none of the culture methods employed to be suitable for detection of non-*jejuni/coli Campylobacter* spp., which raises a suspicion for the potential underestimation of emerging *Campylobacter* spp. in New Zealand patients. With regard to the immunoenzymatic methods, the MedLab Central laboratories in

Manawatu use a faecal antigen test, the ProSpecT^{TM} *Campylobacter* Microplate Assay (Remel Inc., USA) for the detection of *Campylobacter* species. The assay has been developed for the detection of *C. jejuni* and *C. coli* but two independent studies have shown the assay has a limited capability for detection of *C. upsaliensis* though data was insufficient to explain the limitations (128, 129). The ^{m}EpiLab collaborates with MedLab Central laboratories for the purposes of source attribution studies in Manawatu. The positive samples by the antigen assay are sent to ^{m}EpiLab where a culture method is used for isolation and subsequent molecular typing of isolates. A review of ^{m}EpiLab's records has shown that between 16 and 23% (reported in 2010 and 2011, respectively) of positive samples by the above assay were culture negative in our laboratory. This discrepancy in test results could be explained by the delay in the processing of samples, which might result in the presence of dead or viable-but-not culturable (VBNC) bacteria in faecal samples. Unlike microbiological culture, non-culture methods, such as antigen and nucleic acid-based methods have the ability to detect dead and VBNC bacteria. This serves as a potential advantage over the culture methods but also questions the specificity of these methods due to the potential reaction with "free-floating" cellular components in faeces resulting in a false positive test result. Alternatively, the discrepancy in the above test results might be explained by the antigen assay having detected non-*jejuni/coli Campylobacter* spp. that have failed to be isolated by the culture method used in ^{m}EpiLab, which has been optimised for detection of *C. jejuni* and *C. coli*. This issue formed the next aim of this thesis:

- Evaluation of analytical sensitivity of ProSpecT^{TM} *Campylobacter* Microplate Assay for the detection of *C. upsaliensis*, *C. helveticus* and *C. hyointestinalis* in human spiked faecal samples.

Insects have been established as a reliable model for studying the innate immune system because, unlike the adaptive immune system, there is a high degree of structural and functional homology of the innate immune responses between insects and mammals (451). *Galleria mellonella* has been shown as a suitable model for studying several bacterial and fungal pathogens and recently also for *C. jejuni* (452).

Emerging *Campylobacter* spp. have not been studied in this manner and to ensure validity of this approach comparison with the known pathogen, *C. jejuni*, was included. The isolates of *C. upsaliensis* and *C. helveticus* obtained through cross-sectional studies were used for this next aim:

- Experimental study of pathogenic potential of *C. upsaliensis* and *C. helveticus* compared to *C. jejuni* using an insect model of disease, the larvae of the Greater Wax moth, *Galleria mellonella*.

As reviewed, there is a lack of genomic data for many *Campylobacter* spp. Other than *C. jejuni* and *C. coli*. In the early stages of this thesis project there were only two whole genome sequences of *C. upsaliensis* published and none at all for *C. helveticus*. The main interests were to explore the general features of the genomes and functional profiles and, in particular, pathogenicity markers, which formed the last aim using isolates from the cross-sectional studies:

- Whole-genome comparison of *C. upsaliensis*, *C. helveticus* and *C. jejuni*.

CHAPTER 3

3. Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand

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3.1. Impacts

- The first report of isolation of *Campylobacter* spp. from retail raw meat pet food, the overall prevalence rate 28%, with *C. jejuni* being the most common species identified.
- Four different *Campylobacter* spp. were isolated from client-owned dogs and cats with an overall *Campylobacter* spp. prevalence of 36% and 16% respectively, the most common species identified being *C. jejuni, C. upsaliensis* and *C. helveticus*.
- Prevalence rates and species distribution were highly dependent on culture methods and duration of incubation, with significantly higher isolation using CAT compared to mCCDA agar.

3.2. Summary

Campylobacter causes acute gastroenteritis in people worldwide and is frequently isolated from food, animals and the environment. The disease is predominately food-borne but many routes of transmission and sources of infection have been described, including contact with pets. The prevalence of *Campylobacter* spp. in dogs and cats varies widely and data on New Zealand pets are limited. This study aimed to investigate the prevalence of *Campylobacter* spp. in dogs, cats and retail raw meat pet food products in New Zealand and to characterise *C. jejuni* isolates using MLST. Ninety dogs and 110 cats examined at the Massey University Veterinary Teaching Hospital for elective procedures, and fifty locally purchased retail raw meat pet diets were sampled. Two culture protocols combining Bolton broth enrichment and mCCDA and CAT agars in a microaerobic atmosphere at 42°C and 37°C with species identification using PCR were performed. The prevalence of *Campylobacter*

spp., *C. jejuni*, *C. upsaliensis* and *C. helveticus* was 36%, 13%, 23%, and 1% in dogs and 16%, 5%, 5%, and 7% in cats respectively. One dog had *C. lari* confirmed, and three dogs and one cat had multiple *Campylobacter* spp. detected. Significantly more animals tested positive using CAT than mCCDA agar ($p<0.001$). Being neutered, vaccinated for *Bordetella bronchiseptica*, fed dry diets and brought in for neutering were protective factors for dogs, whereas attendance for dental treatment was a risk factor for cats. *Campylobacter* spp. were isolated from 28%, *C. jejuni* 22%, *C. lari* 6%, and *C. coli* 6% of food samples. Six isolates positive by *Campylobacter* genus PCR were identified as *Arcobacter butzleri*. Poultry meat was more likely to be positive than non-poultry meat ($p=0.006$). Of the 13 *C. jejuni* pet isolates with full MLST profiles, eight were of different sequence types (ST) and all nine food isolates were of different STs.

3.3. Introduction

Campylobacteriosis in humans is recognised as one of the most frequent infectious gastrointestinal illnesses worldwide (9) and in New Zealand it is the most commonly reported notifiable disease (6). The most prevalent *Campylobacter* spp. associated with human acute gastroenteritis in New Zealand are *C. jejuni* and *C. coli* (5), as they are worldwide, being responsible for approximately 80 – 85% and 10 – 15% of cases respectively (3). The disease is predominantly food-borne, especially through poultry, but may be acquired through many other routes as many animals carry *Campylobacter* spp., including pets (9). A case-control study in New Zealand identified consumption of raw or undercooked chicken and raw dairy products, overseas travel, direct contact with farm animals and with puppies, and where rainwater was the source of water at home, as the main risk factors for acquiring the disease (453). Similar risk factors have been also observed in a meta-analytic review of case-control studies of sporadic *Campylobacter* infection that estimated an odds ratio of 1. 96 (95% CI 1.51 – 2.54) for direct contact with pets (14).

C. jejuni was the first species isolated from dogs in 1977 in the UK (10) and from cats in 1980 in the USA (15). Since then, *Campylobacter* spp. have been frequently

reported in dogs and cats around the world. However, the reported prevalence rate of *Campylobacter* spp. in pets varies extremely between the studies, ranging from 1 – 86% in dogs (410, 425) and 0 – 66% in cats (369, 420). These differences in prevalence are partly explained by the different populations sampled with regard to the age of animals, the population investigated (*e.g.* household pets, stray, kennelled), methods of detection, animal health status (diarrhoea present vs. healthy) as well as geographical location, season and time the studies were conducted. Nevertheless, similarly to the epidemiology of *Campylobacter* in humans, undercooked/raw food (chicken, liver, tripe and unpasteurised milk), water and direct or indirect (through faecal contaminated fomites) contact with animals and the environment are considered as the main sources and modes of transmissions in pets (354). Although many *Campylobacter* spp. have been detected in the faeces of dogs and cats, the most common species are *C. jejuni, C. coli, C. upsaliensis* and *C. helveticus,* with the latter two the most common identified in dogs and cats respectively (16).

Campylobacter spp. have been detected in dogs and cats in New Zealand by veterinary laboratories but only a few studies have been performed, with *Campylobacter* isolation rates ranging from 0% (454) to 17% (356). New Zealand has among the highest notification rates of human campylobacteriosis in the developed world (311) and among the highest rate of pet ownership, as approximately half of households have cats and a third have dogs (455). The aim of the present study was to investigate the prevalence of *Campylobacter* spp. in dogs and cats in the Manawatu region of the North Island of New Zealand. The study aimed to use a combination of culture methods to facilitate detection of the different *Campylobacter* spp., and to test raw pet food products from retail stores to investigate the hypothesis that these products are one of the potential sources of exposure in households. *C. jejuni* isolates were further subjected to genotypic characterisation and compared with other sources in the ^{m}EpiLab database.

3.4. Materials and methods

3.4.1. Study design

This was a prospective cross-sectional study performed between February and July 2010 using a convenience sample of client-owned dogs and cats examined at the Veterinary Teaching Hospital at Massey University, Palmerston North, New Zealand. Only animals examined for routine vaccination, neutering, health check, and for dental treatment were included in the study. Rectal swabs were taken and information was obtained at the time of sampling regarding age (categorised as young or adult at a cut-off of six months), sex, breed, vaccination and deworming history, neuter status, diet fed (including raw meat), signs of illnesses and any recent drug treatments, the presence of other animals in contact with the case, shelter/boarding kennel history and hunting behaviour. The study was independently reviewed and approved by the Massey University Animal Ethics Committee under application number 09/70. Concurrent to sampling of pets, 50 raw pet food products were purchased at five commercial outlets located in Palmerston North. Food products were arbitrarily selected and the sellers were unaware of the intended use.

3.4.2. Bacterial culture

All rectal swabs were transported without refrigeration using Amies charcoal transport medium (Copan, Brescia, Italy) due to the close proximity of the on-site laboratory and were cultured within 2 hours of sampling using two protocols: (i) direct plating onto CAT agar (Fort Richard, Auckland, New Zealand) with incubation at 37°C in an envelope-generated (CampyGen, Oxoid Ltd., Basingstoke, UK) microaerobic atmosphere in gas-jars and (ii) 48 hours enrichment in 3mL of Bolton Broth (Lab M, Bury, UK) with subsequent plating onto mCCDA plates (Fort Richard, Auckland, New Zealand) at 42°C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) using a variable atmosphere incubator (Don Whitley Scientific, West Yorkshire, UK). CAT agar plates were checked for growth on the second and fourth

day of incubation, whilst mCCDA plates were only checked on the fourth day. All fresh raw pet food samples were cultured within two hours of purchase and frozen samples were first thawed at room temperature before culturing. Approximately 10g of the food samples were aseptically removed from the packaging and briefly mixed in a stomacher with 90mL of Bolton Broth which was split in two bottles for enrichment of 48 hours at 37°C and 42°C followed by plating onto mCCDA and CAT plates for another 48 hours; all under conditions as described above for the respective plates. Control plates were used for all incubation conditions. Colonies with morphological features indicative of *Campylobacter* spp. were checked for size and motility by dark-field microscopy. Two individual colonies (as available) each of different morphology indicative of *Campylobacter* spp. by dark-field microscopy were subcultured using Columbia horse blood agar (Fort Richard, Auckland, New Zealand). Whole plates of pure colonies were harvested for storage in 15% (weight/volume) glycerol in nutrient broth vials (Difco Laboratories Inc., Franklin Lakes, NJ, USA) at -80°C. Isolates negative by the PCR identification protocol below were regrown and tested for growth at room temperature as a phenotypic test to exclude *Campylobacter* spp. status, and if aerotolerant and non-thermophilic, were further tested by a PCR specific for *Arcobacter butzleri*.

3.4.3. Isolate identification and genotyping

Crude DNA extraction was performed by boiling fresh cultures for 10 min in an aqueous 2% (volume/volume) Chelex® solution (Bio-Rad Laboratories Inc., Hercules, CA, USA) followed by centrifugation and decantation of the supernatant into a sterile tube and storage at −20°C until each PCR was run. Each sample was initially tested by *C. jejuni* PCR and if negative, by a *Campylobacter* genus PCR. Subsequent to a positive genus PCR, samples were sequentially tested by species-specific PCRs that targeted *C. coli, C. upsaliensis, C. helveticus, C. lari, C. fetus* or *C. hyointestinalis.* In addition, *A. butzleri* PCR and 16S rRNA gene sequencing were performed for a subset of isolates negative by all species-specific PCR that exhibited growth at room temperature and room atmosphere. The detailed description of primers, amplification protocols, and references are presented in Supplemental Table

3.1. Controls were used for all PCR assays and amplified products were identified by electrophoresis in a 1% (weight/volume) agarose gel in TBE buffer (along with a 1Kb Plus ladder (Invitrogen Corp., Waltham, MA, USA)), subsequently stained with ethidium bromide and exposed to UV light using a Bio-Rad gel documentation system (Olympus Life Science Group, Richmond Hill, Ontario, Canada). Isolates confirmed as *C. jejuni* by PCR were genotyped using the *Campylobacter*-specific multilocus sequence typing (MLST) scheme of seven housekeeping genes as described previously (256). The amplifications were performed in a 25μL volume reaction using Applied Biosystems AmpliTaq Gold mastermix (Applied Biosystems, Auckland, New Zealand) and 5 pmol of each primer. Products were sequenced on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 (Applied Biosystems, Auckland, New Zealand) following the manufacturer's instructions. Sequence data were collated and alleles assigned using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/) and sequence types (ST) compared with those contained in the ^{m}EpiLab database. The ^{m}EpiLab database contained over 3,500 samples (at the time of writing) from the Manawatu *Campylobacter* sentinel surveillance site, a ten year plus project for source attribution of campylobacteriosis using concurrent sampling of sick people, animals, food and the environment (260).

3.4.4. Statistical analysis

The power analysis performed using G*Power v3.1 (456) for sampling of a total of 200 animals and 50 food samples with a two-tailed binomial test ($\alpha = 0.05$, $\beta = 0.1$) showed the effect size of 0.17 for 100 samples each from dogs and cats and for 50 samples (for raw food) the effect size of 0.23 could be detected. Isolates positive by PCR were used to report an apparent prevalence and 95% CI based on a binomial distribution using the Clopper and Pearson method (457). Univariate analyses to test the association of *Campylobacter* status with collected animal/food information were performed using Fisher's exact test of independence. Multivariate logistic regression analysis was performed using the least absolute shrinkage and selection operator (LASSO) regression. All variables with $p < 0.3$ from the univariate analysis were used for LASSO profiling by the lambda penalty parameter and cross-validation of the

model log-likelihood. The number of non-zero coefficients and 95% CIs were estimated using 10,000 bootstrapped replicates of equivalent sample sizes with replacement. Culture methods were compared using Fisher's exact test of independence and unweighted Cohen's kappa as an index of interrater agreement. The New Zealand National Dog Database (www.localcouncils.govt.nz) reports were used for the evaluation of the sampled dog population profile. Statistical and exploratory data analyses were performed using R v3.2.2 (R: A language and environment for statistical computing. R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

3.5. Results

3.5.1. Dogs and cats

The population tested consisted of 90 dogs and 110 cats with 70 (78%) dogs and 38 (35%) cats being classified as adult. The dog population consisted of 40 bitches (44%), 50 entire animals (55%), and 57 pure dog breeds (63%), a distribution that was comparable to the National Dog Database proportions of 50%, 51%, and 67% respectively. The cat population consisted of 65 queens (59%), 81 entire cats (74%) and 7 pedigree cats (6%). Of the 103 crossbred cats there were 90 domestic shorthair, six domestic medium-hair, five domestic longhair and two other crossbreeds. Eighteen dogs (20%) had clinical signs reported in the medical history of which six (7%) had gastrointestinal signs and nine dogs (9%) had received treatment, including four (4%) that received antimicrobial agents. Fourteen cats (13%) had clinical signs reported, of which eight (7%) had gastrointestinal signs and seven cats (6%) received treatment including three (3%) that received antimicrobial agents. Of the seven animals in total receiving antimicrobial treatment, two reported topical ear treatment and of the remaining five, two were within and one was over one month from the date of sampling while for two animals the dates of treatment with antimicrobials were unknown. Other treatments prescribed were dietary change (5), treatment with parasiticides (4), and systemic glucocorticoid treatment (1).

In total, 110 isolates from pets were positive by *Campylobacter* genus PCR and 107 were speciated by *Campylobacter* species-specific PCR tests; apparent prevalences are presented in Table 3.1. The three remaining, unspeciated isolates grew at room temperature and aerobically and were re-categorised as *Campylobacter* spp. negative. All three isolates were negative for *A. butzleri* by PCR and sequencing of 16S rRNA was unsuccessful. Mixed *Campylobacter* spp. were isolated in three dogs (*C. jejuni* and *C. upsaliensis*) and one cat (*C. jejuni* and *C. helveticus*).

Table 3.1. Prevalence of isolated and PCR confirmed *Campylobacter* spp. from client-owned pets and retail raw pet food diets in the Manawatu, New Zealand.

Sample	Number of samples	Prevalence[a] % (95% CI)			
		***Campylobacter* spp.[b]**	***C. jejuni***	***C. upsaliensis***	***C. helveticus***
Dogs	90	36 (26 – 46)	13 (7 – 22)	23 (15 – 33)	1 (0 – 6)
Cats	110	16 (10 – 25)	5 (1 – 10)	5 (2 – 10)	7 (3 – 14)
Poultry meat	11	73 (39 – 94)	64 (31 – 89)	-	-
Non-poultry meat	39	31 (17 – 48)	8 (2 – 21)	-	-
Overall meat	50	28 (16 – 43)	22 (12 – 36)	-	-

[a] Fourteen *Campylobacter* genus PCR positive food isolates grew at room temperature and atmosphere, thus were excluded. [b] *C. lari* was isolated from one dog, one poultry and two non-poultry meat samples and *C. coli* in three non-poultry meat samples.

Dogs not fed with dry diets were 12.3 times (95%CI 0.91 – 682.6, p = 0.03) more likely to carry *C. upsaliensis* than those dogs eating dry diets. Fifty-five animals were reported to have raw meat in their diet and 47 were fed table scraps of human food but neither of these factors were significant at α = 0.05. Intact dogs were 4.6 times (95% CI 1.6 – 14.8, p = 0.002) more likely to carry *Campylobacter* spp. and 4.6 times (95% CI 1.3 – 20.6, p = 0.01) more likely to carry *C. upsaliensis* than neutered dogs. Dogs not vaccinated for *Bordetella bronchiseptica* were 3.5 times (95% CI 1.0 – 16.1, p = 0.04) more likely to carry *Campylobacter* spp. than dogs vaccinated for *Bordetella bronchiseptica*. There were significant differences in prevalence rates between the reasons of the visit to the Veterinary Teaching Hospital for both dogs and cats. Dogs examined before neutering were 5.0 times (95% CI 1.5 – 17.8, p = 0.006) more likely to carry *Campylobacter* spp. and 6.4 times (95% CI 1.7 – 26.9, p = 0.004) more likely to carry *C. upsaliensis* (p = 0.004) than dogs examined for vaccination. Neutering as the reason of visit was not confounded by age, as five young and 16 adult dogs were examined before neutering. Cats examined for dental treatment were 23.4 times (95% CI 1.2 – 522.8, p = 0.02) more likely to carry *C. jejuni* than those examined for vaccination. The above variables and variables with p < 0.3 from univariate analysis (age, scraps in diet, kennelling/shelter history, sex, gastrointestinal clinical signs, access to outdoor, rural or urban area of residence, contact with other animals, and display of hunting behaviour) were included in separate multivariate models for dogs and cats but none remained significant, neither in dogs nor in cats, for any *Campylobacter* spp. nor for species-specific models.

3.5.2. Raw pet food products

Of the 50 food samples, six were confirmed to have leaking packaging, two samples were sold after the use-by date, and two had the same use-by date as the date on which they were purchased. There were 12 frozen food products, 19 contained tripe, and 31 were from a single animal species source (11 beef, 11 chicken, five mutton, two venison and one pork and one of horse meat origin). There were 47 isolates positive by *Campylobacter* genus PCR and 33 were speciated by *Campylobacter* species-specific PCR. The remaining 14 isolates all grew at room temperature and

aerobically, thus were re-categorised as *Campylobacter* spp. negative and adjusted apparent *Campylobacter* spp. prevalences are presented in Table 3.1. Six of these isolates were positive by *A. butzleri* PCR, of which two had the 16S rRNA gene sequenced and returned the highest similarity to *A. butzleri*. Mixed *Campylobacter* spp. were isolated from three meat samples. Univariate analyses showed poultry meat as 7.6 times (95% CI 1.5 – 46, p = 0.006) more likely to be contaminated with *Campylobacter* spp. and 14 times (95% CI 2.4 – 103, p = 0.001) more likely to be contaminated with *C. jejuni* than non-poultry meat.

3.5.3. Culture methods

There were significant differences between the two culture protocols ($p < 0.001$) with CAT 4.7 times (95% CI 2.3 – 10.3) more likely to detect an animal positive for *Campylobacter* spp. than mCCDA agar. The Cohen's kappa index of agreement between cultures was 0.23 ($p < 0.001$). Out of 12 pets positive by mCCDA, 11 carried *C. jejuni* and one *C. upsaliensis* whereas out of 46 pets positive by CAT agar, 13 carried *C. jejuni*, 26 carried *C. upsaliensis*, 9 carried *C. helveticus* and one carried *C. lari* (three had carriage of multiple species). The influence of the length of incubation on culture results could only be assessed for CAT agar. Seventeen animals negative on the second day were positive on the fourth day of incubation; nine isolates were *C. helveticus*, seven *C. upsaliensis* and two *C. jejuni* (one animal had multiple carriage). *C. helveticus* isolates were only observed on the fourth day of incubation. All isolates from food with a positive *Campylobacter* spp. PCR that exhibited growth at room temperature and room atmosphere were grown using CAT agar and were re-categorised as *Campylobacter* negative.

3.5.4. MLST of *C. jejuni* isolates

All seventeen *C. jejuni* isolates from pets were subjected to MLST typing and 13 returned full allelic profiles. Genotyping of isolates with incomplete profiles could not be performed as the isolates could not be revived from frozen cultures and other

isolates from the same sample were not available or were confirmed as different *Campylobacter* species. In dogs, three each of ST-474 and ST-45, and one each of ST-21, ST-61, ST-520, and ST-4492 belonging to four MLST clonal complexes (CC-48, CC-45, CC-21 and CC-61) were detected. In cats, two ST-696 and one ST-48 from two MLST clonal complexes (CC-1332 and CC-48) were detected. All eleven *C. jejuni* food isolates had MSLT typing attempted and nine returned full allelic profiles all of different STs (ST-137, ST-3711, ST-356, ST-45, ST-42, ST-422, ST-474, ST-48, and ST-583), belonging to six MLST clonal complexes. Occurrence of these ST across sources from the ^{m}EpiLab database is depicted in Fig. 3.1. Genotyping of the two isolates with incomplete profiles was unsuccessful as for the pet samples described above.

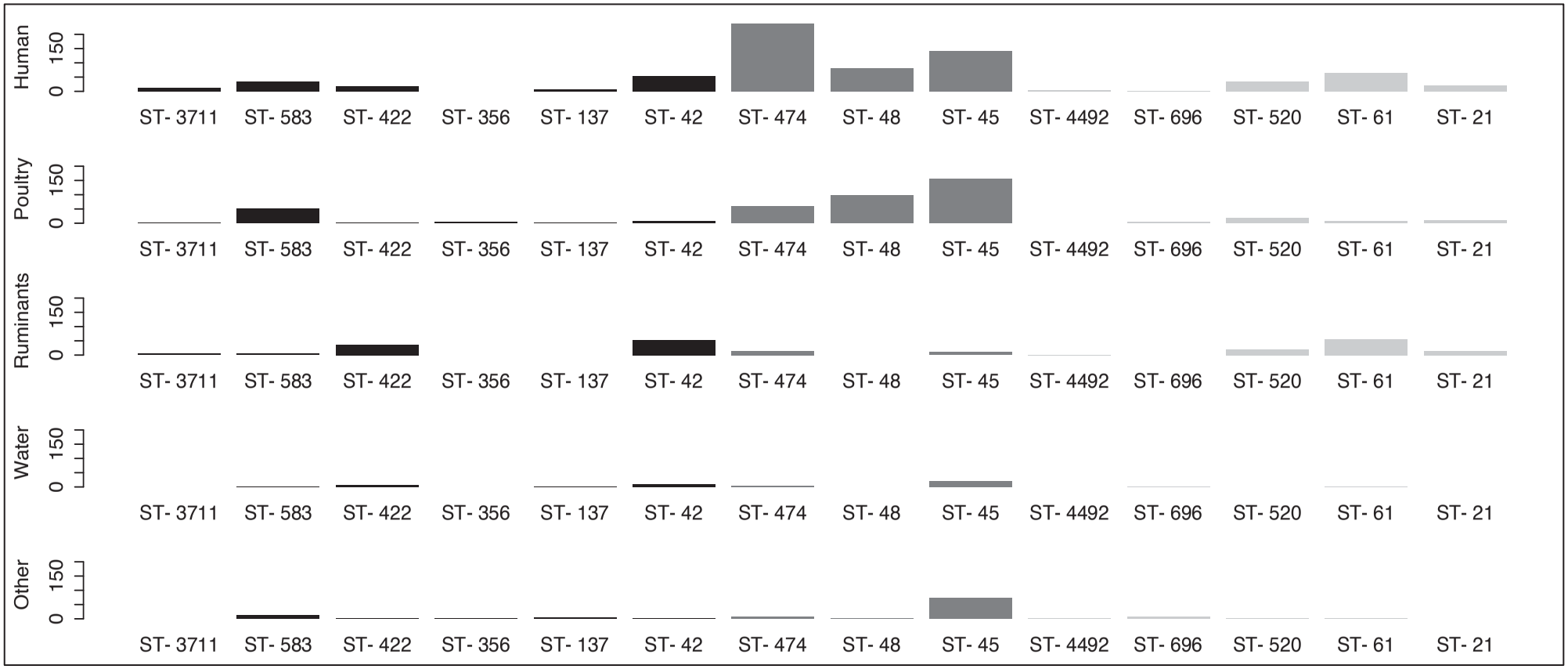

Fig. 3.1. Occurrence of *C. jejuni* MLST types observed in pets and raw meat pet food diets from Manawatu, New Zealand, across five sources from the ^{m}EpiLab database (N = 1,176). The horizontal axis shows sequence types (ST) isolated in this study from food only products (black bars), from pets only (light grey) and from both food and pets (dark grey) and their occurrence within the five sources (Other primarily denotes wild birds and to a lesser extent other wild animals and companion animals) on the vertical axiis.

3.6. Discussion

The most common species isolated from dogs was *C. upsaliensis* with the second most common being *C. jejuni*, whereas *C. helveticus* was the most common species in cats but with a minimal difference in isolation rates for the other two *Campylobacter* species. The species distribution pattern in dogs in the present study is similar to that in several other studies (360, 364, 393, 394, 401), although some investigators have reported *C. jejuni* as the predominant species in dogs (365, 403). In contrast to this study, reported prevalences of *C. upsaliensis* and/or *C. helveticus* are commonly higher than *C. jejuni* in cats, and the overall prevalence is higher than detected in this study (363-365, 445). Low prevalence figures as in this study have also been reported in cats (401, 403, 404). The relatively low prevalence in cats in this study is unlikely to be due to the skewed age distribution, as young cats (also young dogs) are generally reported to have a higher prevalence of *Campylobacter* spp. than adults (363, 364, 445). A recent study in New Zealand which focused on *C. jejuni* reported a prevalence of 5% in cats (356). That study was also conducted in the Palmerston North area with a similar sampling frame to this study as cats were sampled from the Massey University Veterinary Teaching Hospital clients, staff and cattery, thus the relatively low *C. jejuni* prevalence might truly reflect the prevalence in New Zealand cats, or at least that in the Manawatu region. In the Mohan (2015) study, environmental dog faeces were collected at Palmerston North dog-walking areas, with a reported *Campylobacter* spp. and *C. jejuni* prevalence of 13% and 5% respectively (356). The significantly lower prevalence in dogs in Mohan's study compared to the present study ($p < 0.001$ for *Campylobacter* spp. and $p = 0.007$ for *C. jejuni*) could be attributed to poor survival of *Campylobacter* spp. in the environmental samples, as they are microaerophilic and thermophilic bacteria (47). In addition, that study used only mCCDA agar (356) whereas the addition of CAT agar in the present study was shown to be very valuable, as CAT detected 29, mCCDA eight, and the CAT/mCCDA combination 32 dogs with *Campylobacter* spp. and 16, 4 and 17 cats respectively.

This is the first study in New Zealand to report epidemiological associations with the *Campylobacter* spp. status in pets. Univariate analysis showed many variables with p

< 0.3 that other studies either reported as associated with *Campylobacter* status (*e.g.* age, kennel/shelter history, being fed raw food and scraps etc.) or that may have plausible biological explanations (hunting behaviour) but none were significant by univariate analysis at $p < 0.05$, and none remained significant in the multivariate analyses. This result is most likely to be due to the relatively small sample size, for epidemiological investigations, of this study and to the conservative approach of LASSO logistic regression, which requires larger effect sizes before variables are considered statistically important compared with standard logistic regression. Nevertheless, several significant associations with *Campylobacter* status were observed. The reason of the visit to the Veterinary Teaching Hospital is a vague epidemiological variable but although the association may be a spurious one, it may have also been a proxy measure for a feature more associated with an owner rather than their animal(s). For instance, the protective association of vaccination as a reason of visit and also of having been vaccinated against *B. bronchiseptica* may suggest vigilant pet owners who care for their pet(s) through which, or by other means unmeasured in the study, renders the animal(s) less exposed to *Campylobacter* species. On the other hand, dogs presented for neutering may have been more prone to roaming behaviour resulting in higher exposure to *Campylobacter* spp. as well as influencing the owner to opt for the elective surgery and thus the animals becoming a part of the sampling frame. It is possible these associations are a bias of client-owned pets attending the veterinary practices. Similarly to this study, intact status has been reported as a risk factor for carriage of *Campylobacter* spp. in dogs attending veterinary clinics in Canada (393). As in this study, the risk factor for *Campylobacter* isolation of not feeding dry diets was reported in dogs frequenting city parks in Ontario (292). The apparent protective effect of dry diets could be due to presence of *Campylobacter* in other diets consumed, induction of gut conditions inimical to *C. upsaliensis* by dry diets, confounding due to other factors associated with the feeding of wet food, or other unexamined covariates. The association of *C. jejuni* and examination for dental treatment in cats is unclear. Potentially diet-associated factors may be associated with both parameters. A range of non-*jejuni Campylobacter* spp. have been detected in humans with periodontal disease (458) and in cats and dogs with oral/dental

disease (459) and further studies are needed to elucidate the validity of all these associations.

The sampled dog population was considered representative of the New Zealand pet population, as the demographic data compared closely to the National Dog Database. Unfortunately, similar data for cats do not exist, and in this study, age was skewed towards young cats, a demographic that may limit interpretation of results to the whole cat population of New Zealand. A small number of pets had clinical signs reported in the history, although these signs were not their reason for the visit to the Veterinary Teaching Hospital and these animals did not undergo a diagnostic investigation because these signs were not considered significant, being very mild. Also, the frequency of mild clinical signs reported in animals in this study might not have been any different from the general pet population; a study in UK reported that out of 772 dogs that did not present for veterinary examination, approximately 15% had diarrhoea and 19% had vomiting in the two-week study period (460). Only two animals were confirmed to have received systemic antimicrobial treatment in the last month prior to sampling. Notwithstanding the potential limitations of the study, five different *Campylobacter* spp. were isolated from dogs, cats, and pet food.

This is the first study to report the presence of *Campylobacter* spp. in retail raw pet food diets, as no studies with similar attempts identified in a review of the literature were successful (461-463). Although culture protocols differed to a certain extent between all these studies, the success of this study might be explained by the use of a larger food sample (approximately 10g) compared to less than 1g of food or a swab of homogenized food in the above studies. The larger sample might have facilitated isolation, as food samples are generally contaminated with lower concentrations of bacteria than are observed in the faeces of animals (464-466). As a common food pathogen in meat for human consumption, the presence of *Campylobacter* in raw meat diets for pets could be expected; the results of this study are similar to observations in retail meat for human consumption. The higher prevalence of *Campylobacter* spp. in poultry meat compared to other meat types for human consumption has been reported in New Zealand (312, 467) and elsewhere in the world (468-470). *C. upsaliensis* and *C. helveticus* have each been isolated from 3%

of beef meat samples but not detected in chicken and pork meat for human consumption with a filtration technique combining pre-enrichment and plating using non-selective media (61). Therefore, this study may have had insufficient sample size to detect rarely occurring *Campylobacter* spp. in meat or no detection was due to use of a selective enrichment broth rather than CAT agar which supported growth of *C. upsaliensis* and *C. helveticus* in the pet rectal swabs.

The superiority of CAT to mCCDA agar in this study was statistically significant and primarily attributable to the isolation of *Campylobacter* spp. other than *C. jejuni,* although CAT also isolated more *C. jejuni* than mCCDA did. The two agars were initially developed for use in the detection of human *Campylobacter* isolates and only differ in the composition and concentrations of antimicrobial agents, and while mCCDA was developed primarily for *C. jejuni* (471), CAT was intended for a wider range of thermophilic *Campylobacter* spp., including *C. upsaliensis* (104). The isolation of a wider range of *Campylobacter* spp. is difficult due to the vast diversity in growth requirements between each species including, but not limited to, temperature, atmospheric conditions and incubation period (17, 18), as well as the differing antimicrobial sensitivities of the diverse species (104). When the two agars were used simultaneously in pets, a higher isolation rate for both *C. jejuni* and *C. upsaliensis* with CAT than mCCDA has been reported (54), but also comparable (402), and lower rates (405) have been reported. The first two studies used both methods at 37°C for 4 – 6 days in a microaerobic atmosphere and the last study used CAT at 37°C and mCCDA at 42°C for 2 days in a hydrogen-enriched microaerobic atmosphere; all of which could partly explain their discordant results. In humans, isolation of *C. jejuni* was reported to be better at 37°C than 42°C for mCCDA (96), and at 37°C mCCDA and CAT were comparable (92), but studies of *C. upsaliensis* reporting the superiority of CAT over mCCDA are more likely to be attributable to a better isolation at lower bacterial concentrations (99) and the support of growth for a larger strain diversity (472). The hypothesis of strain variation influencing successful isolation could also be supported by the large genotypic variations reported in *C. upsaliensis* from three continents (473). Notwithstanding the many potential reasons for discrepant results between the different isolation

methods, at least for the present study it is clear that detected prevalences would be skewed if only mCCDA was used.

CAT agar has also been reported as more optimal than mCCDA for *Arcobacter* spp. isolation (472). The cross-reaction of *A. butzleri* with the *Campylobacter* genus PCR seen in this study has been previously reported (474) and is of concern to studies employing this method of taxonomical identification, and should therefore be addressed. *Arcobacter* spp. are emerging human pathogens with many sources in the environment, animals, and meat samples (475). To the best of our knowledge this study is first to report isolation of *Arcobacter* spp. in raw meat pet diets. A study in Australia reported 25 out of 30 beef carcasses at a pet food abattoir contaminated with *Arcobacter* spp. (476) and the lower prevalence in this study could be attributed to the use of retail pet food and a suboptimal culture method.

It is important to appreciate the direction of transmission cannot be ascertained, neither by this nor other cross-sectional studies. Pets may be a source of infection for people and *vice versa* but both can have a common exposure, for instance, from food or water. The inconclusive direction of transmission is supported by studies using MLST showing the majority of genotypes observed in pets frequently occur in humans but also in poultry, particularly, ST-45 which is the most common genotype reported in pets around the world and is poultry associated also (300, 386, 477). Source attribution studies in New Zealand have shown poultry as a major source of human infection (260, 312). In this study, ST-45 and ST-474 were the most common genotypes in dogs, and together with ST-48 from cats, are the STs most commonly detected in both chicken and humans (Fig. 3.1) plus all three have been isolated from raw pet food in the present study (Fig. 3.1). Out of the 341 STs in the ^{m}EpiLab database these three STs accounted for 33% (462/1401) and 35% (312/890) of human and chicken isolates respectively (data not shown). A study in Finland reported infection of people with ST-45 was significantly associated with contact with both dogs and cats but not with eating chicken and was negatively associated with pork and fish consumption (336). In contrast to other countries, ST-45 was rare in humans (264) and significantly more frequent in dogs with diarrhoea than dogs without diarrhoea in Switzerland (386). In this study, ST-696 and ST-4492 from cats

and a dog respectively are too rare across all sources (Fig.3.1) to allow confident associations but do suggest that in this, as in all the above studies, that pets show a large diversity in sequence types. This diversity of *C. jejuni* STs could be attributed to the pets' lifestyle that exposes them to many sources of *C. jejuni* and is of importance to molecular epidemiology studies. The diversity of *C. jejuni* STs isolated from the raw pet foods may be inferable to the assorted different species found in pet food and the possibilities for cross-contamination in the manufacturing process. A longitudinal study employing a pulse-field gel electrophoresis genotyping method reported that all genotypes of *C. jejuni* except one were observed in only single dogs and only occasionally more than once (139). In contrast, the majority of dogs shed the same or closely related strains of *C. upsaliensis* for several months (up to 21 months or longer), which may imply dogs act as reservoirs of the species. Furthermore, *C. upsaliensis* is highly prevalent in dogs and cats and very rarely observed in other sources; hence pets may be a source of infection that humans are frequently exposed to.

C. upsaliensis is among the leading emerging *Campylobacter* pathogens and, with suitable culture methods, isolation rates higher than those of *C. coli* were reported in patients with gastroenteritis in Europe (102, 478), Africa (18) and North America (479). In New Zealand, a recent survey of laboratory practices showed none of the public health laboratories used culture methods suitable for detection of non-*jejuni/coli Campylobacter* spp. (53) whereas in a relatively small sample, many *Campylobacter* spp., including *C. upsaliensis/C. helveticus* were detected in humans with diarrhoea using molecular methods (348). Given that most commonly used culture methods are optimised for *C. jejuni/coli*, a potential for misdiagnosis and underestimation of disease associated with the emerging *Campylobacter* spp. is now widely recognised in humans (13, 17).

Detection of many potential human pathogens in both pets and pet food highlights the implications for public health of this study. Studies have shown there is a need to raise awareness of the risks involved for both pet owners and non-owners because contact with, or ownership of pets is a feature covering many potential routes of disease transmission (350, 351, 480). Transmissions may occur through direct

contact (*e.g.* petting or playing with pets) or indirectly through a contaminated shared environment. This study highlights raw pet food as a potential source of infection in households, both from direct contact or indirect contamination during transport, storage, preparation, and consumption. The observation of leaking packaging is of particular concern as it could lead to unapparent contamination of the environment and/or other (human) foodstuffs.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Krunoslav Bojanić

Name/Title of Principal Supervisor: Dr Els Acke

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CHAPTER 4

4. Isolation of emerging *Campylobacter* species in working farm dogs and their frozen home-kill raw meat diets

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4.1. Highlights

- *C. rectus*, *C. lari* subsp. *concheus*, *C. volucris* and *H. winghamensis* isolated in dog
- Higher isolation observed with 37ºC, a microaerobic atmosphere with H_2 and CAT agar
- *Campylobacter* spp. in 31 dogs overall with methods varying from two to 25 dogs
- Cultures varied by total contaminant overgrowth and selectivity for target colonies
- Cross-reaction of PCR for *Campylobacter* spp. with *Arcobacter* and *Helicobacter* spp.

4.2. Abstract

Campylobacter spp. are fastidious organisms, difficult to isolate but frequently detected in animals, food, and the environment. Most culture methods are optimised for isolation of *C. jejuni* and *C. coli*, which are considered major pathogens of the genus. The aim of this study was the isolation of a wide range of *Campylobacter* spp. from working farm dogs and their home-kill raw meat diet in Manawatu, New Zealand. Seven culture methods were used on 50 dog faecal and six on 50 meat samples. Cultures combined filtration, enrichment broths and agars at 37 and 42°C in conventional and hydrogen-enriched microaerobic atmospheres with PCR used for the speciation of isolates. Overall 356 isolates of *Campylobacter* spp. were recovered from 31 dogs with successful isolation by individual methods from two to 25 dogs, resulting in multiple significant differences in pairwise comparisons ($p<0.05$). The most common species were *C. upsaliensis* and *C. jejuni* and less common were *C.*

coli and *C. lari*. Species reported and/or isolated for the first time in dogs included *C. rectus*, *C. lari* subsp. *concheus*, *C. volucris* and *Helicobacter winghamensis.* Six isolates from dogs positive by *Campylobacter* genus PCR were confirmed as *Arcobacter cryaerophilus* (1), and *A. butzleri* (5). Overall, there were 20 isolates from three meat samples positive by *Campylobacter* genus PCR; one meat sample positive for *C. jejuni*, one for *C. rectus* and one subsequently identified as *A. butzleri*. The performance of methods for target species, growth of contaminants and *C. jejuni* multi-locus sequence type results are described.

Keywords: microbiological culture, *Campylobacter*, *Arcobacter, Helicobacter*, canine, farm, zoonosis, raw meat

4.3. Introduction

Campylobacter-associated enteritis is one of the most common bacterial gastrointestinal diseases in humans and the organisms are frequently detected in many animals, food and in the environment (9). Campylobacteriosis is predominately foodborne, especially from poultry meat but is also significantly associated with travel, contact with environmental water and animals including pets (14). *C. jejuni* and *C. coli* are the most common species associated with disease but many other species have been implicated as pathogens. These species are referred to as “emerging” pathogens and are generally considered underrepresented (3, 13). The underestimation of emerging *Campylobacter* spp. is mostly attributed to the bias of culture methods that are optimised for the recovery of *C. jejuni/coli* (18). The narrow selection of culture methods is related to the fastidious nature of these taxa and the vast diversity of growth requirements amongst them such as incubating temperatures, atmospheric conditions, length of incubation, nutrient requirements, and their different susceptibilities to antimicrobial agents (20). Difficulties with the isolation of *Campylobacter* spp. are not only associated with the constant refinement and development of culture protocols but also the cause for adoption of different methodologies for detection. Studies employing ELISA and PCR have shown enhanced sensitivity for detection of *Campylobacter* spp. compared with culture

methods (132, 481, 482) and also their ability to detect a wider range of species, many of which are challenging to isolate (483, 484).

Dogs were first associated with campylobacteriosis in humans in 1960 (353) and the first species isolated from dogs was *C. jejuni* in 1977 (10). Since then, many studies worldwide have reported the frequent isolation of *C. jejuni* from sick and healthy dogs with pathogenic involvement more likely to occur in young animals or be precipitated by contributing factors such as stress, crowding and concurrent diseases (16). Similar to humans, the distribution of species observed is significantly dependant on the methods of detection, but of all the species isolated from dog's faeces, *C. upsaliensis* is by far the most common, with *C. jejuni* and *C. helveticus* being recovered relatively frequently, while *C. coli*, *C. lari*, *C. hyointestinalis*, and *C. concisus* are rare (54, 139, 397, 400, 402, 485, 486). The distribution of species isolated is in sharp contrast to those detected by PCR methods. One molecular study reported the presence of 14 different *Campylobacter* spp. in dogs (359). For seven of these species there are no reports of isolation from dogs in the literature (*C. rectus, C. mucosalis, C. showae, C. gracilis, C. fetus, C. sputorum, C. curvus)* beyond identification of *C. rectus*/*showae* in saliva and/or dental plaques by molecular methods (459, 487).

Recently, a culture method was described for the recovery of *Campylobacter* spp. from meat for human consumption that enabled the isolation of 17 *Campylobacter* spp. (43), including all of the above species not previously recovered by culture in dogs. The aim of the current study was to apply this culture method and a combination of conventional culture methods to screen working farm dogs and their raw meat home-kill food with the primary aim of isolating emerging *Campylobacter* species. In the region of this study, an increased risk for campylobacteriosis in humans has been reported with factors associated with farming and the rural environment (307, 488). In the same region, a study in predominately urban dogs environmental deposited faeces reported a 5% prevalence of *C. jejuni* (356). For the present study, multiple culture methods were used to examine the hypothesis that a wide range of *Campylobacter* spp. may be cultured from dogs and meat, and the difference in results and experience with the protocols were evaluated. Working farm

dogs and their home-kill meat diet were chosen, as prevalences in these dog and meat types have not been evaluated and both could pose an infection risk to farmers. *C. jejuni* isolates were subjected to multi-locus sequence typing (MLST) for addition to the ^{m}EpiLab surveillance database.

4.4. Materials and methods

4.4.1. Study design

This was a prospective cross-sectional study using convenience sampling. Participants were recruited from previous studies where farmers agreed to be contacted for future investigations and by telephone survey using data available on the New Zealand electoral roll where the registrants' occupation was recorded as "farmer". The eligibility criteria for sampled premises were location within the Manawatu region, having a minimum of three working farm dogs, and feeding dogs raw meat home-kill food at least once fortnightly. Sampling was performed over July – August 2012 and March – May 2013. Fifty farms were visited in the morning and the dogs observed for defecation to allow sampling the freshly voided faeces; if any dog did not defecate faeces was obtained by rectal digital recovery. Raw home-kill meat for feeding of working dogs was sampled either frozen from the freezer or from meat put to thaw that morning being prepared for feeding the dogs that afternoon. Dogs and meat were arbitrarily selected for sampling and one sample from each was taken per farm. All samples were refrigerated without transport medium and cultured within 4 hours from sampling. The study was independently reviewed and approved by the Massey University Animal Ethics Committee under protocol number MUAEC 12/23.

4.4.2. *Campylobacter* isolation

Culture methods consisted of in-house prepared anaerobe basal agar (AB) (Oxoid, UK) with 5% lysed horse blood, commercially available CAT and mCCDA agar (Fort Richard, New Zealand), filtration using 0.6µm pore size mixed ester membranes (Whatman, UK) performed in bio-hazard cabinets, no or prior enrichment in Bolton broth (BB) (Lab M, UK) or Campylobacter Enrichment Broth (CB) (Lab M, UK), H_2-enriched (82% N_2, 10% CO_2, 5% H_2, 3% O_2) and a conventional microaerobic atmosphere (MA; 85% N_2, 10% CO_2, 5% O_2) in gas cabinets (Don Whitley Scientific, UK), and gas-jars using envelope-generated (CampyGen, Oxoid Ltd., UK) MA in a temperature-controlled room. Plates were checked daily for growth from day 2 (direct plating) or day 3 (if enriched) to day 6 of incubation. Control plates were used in all culture protocols. Colonies exhibiting morphological features indicative of *Campylobacter* spp. were checked for size and motility by dark-field microscopy and Gram-reaction using potassium hydroxide (489). For suspect *Campylobacter* spp., up to two individual colonies (as available) each of different morphology were subcultured using Columbia horse blood agar (Fort Richard, New Zealand). Additional colonies of the same morphological features were also subcultured if the newly grown colony had a minimal difference of two days from the previous colony of same morphology. Whole plates of pure colonies were harvested for storage in 15% (weight/volume) glycerol in nutrient broth (Difco Laboratories Inc., NJ) at -80°C. Plates were considered unreadable if over three quarters of the streaked area was overgrown by non-target organisms. The selectivity of each method was expressed as the proportion of presumptive *Campylobacter* isolates from culture plates that subsequently tested positive by *Campylobacter* genus PCR.

Culture of faecal samples from dogs. Seven culture methods were performed. Cotton swabs were applied to fresh faecal samples and cultured as follows: (A) four swabs placed in CB for 48 hours followed by passive filtration of 0.2mL for 20 minutes to AB with the inoculum distributed over agar surface using sterile disposable hockey-stick spreaders in H2-MA at 37°C (CB_H2_AB), (B) same as (A) but following enrichment a swab onto CAT (CB_H2_CAT), (C) a direct swab onto CAT at 37°C in envelope-generated MA (CAT_MA), (D) a swab placed in BB for 48

hours followed by a swab onto mCCDA at 37°C in H2-MA (BB_ H2_mCCDA), (E) a swab placed in BB for 48 hours followed by a swab onto mCCDA at 42°C in MA (BB_MA_mCCDA), (F) a direct swab onto CAT at 37°C in H2-MA (CAT_H2), and (G) a swab suspended in 10mL of phosphate-buffered saline pH 7.3 (Difco Laboratories Inc., USA) directly followed by passive filtration as in (A) onto AB agar at 37°C in H_2-MA (AB_H2). CB_H2_CAT was performed on 38 and AB_H2 on 21 samples.

Culture of meat samples. Six culture methods were performed. Twenty-five grams of meat was initially "stomached" for 30 seconds with 225mL of CB using a Colworth Stomacher 400 (Seward, Worthing, UK) in a Seward Classic 400 bag, and then divided into 5 aliquots of approximately 45mL of meat suspensions in screw-top bottles. Three meat suspensions had BB selective supplement added (SR0183, Oxoid Ltd, UK). All meat suspensions were cultured with an initial enrichment of 48 hours in the respective environmental conditions as follows: (i) 0.2mL of CB suspension filtered (procedure performed as for faeces) onto AB at 37°C in H_2-MA (mCB_H2_AB), (ii) a swab of CB suspension onto CAT at 37°C in H2-MA (mCB_H2_CAT), (iii) a swab of CB suspension onto CAT at 37°C in MA (mCB_MA_CAT), (iv) a swab of BB suspension onto mCCDA at 37°C in H2-MA (mBB_H2_mCCDA), (v) a swab of BB suspension onto mCCDA at 42°C in H2-MA (mBB_MA_mCCDA), and (vi) a swab of BB suspension onto CAT at 37°C in MA (mBB_MA_CAT). As a check for faecal contamination, meat samples were also cultured for *Escherichia coli* by placing 25g of meat in buffered peptone water with aerobic cultivation overnight at 37°C from which three 100-fold dilutions in 0.9% phosphate-buffered saline were spiral-plated (Don Whitley Scientific, UK) onto MacConkey agar (Fort Richard, New Zealand) with incubation at 37°C for an additional 24 hours. Lactose fermenting colonies, up to four as available, were subcultured onto blood agar for a spot indole test and if positive, reported as *E. coli*.

4.4.3. *Campylobacter* identification and typing

Crude DNA extraction was performed by boiling a small loopful of fresh cultures for 10 min in a 2% (weight/volume) Chelex solution (Bio-Rad Laboratories Inc., CA) followed by centrifugation and decantation of the supernatant into a sterile tube with storage at −20°C until PCR was performed. Each faecal isolate was initially tested by *C. upsaliensis/helveticus* PCR (490) and each food isolate by *C. jejuni* (491) and *C. coli* (492) PCRs; if negative followed by *Campylobacter* genus PCR (490). Subsequent to a positive genus PCR, faecal and food isolates were sequentially tested by species-specific PCR for *C. lari, C. fetus*, *C. hyointestinalis* (490), and *Arcobacter butzleri* (493). A subset of isolates negative by the species-specific PCRs was selected for 16S rRNA gene amplification and sequencing (494). Controls were used in all PCR assays and amplified products and 1Kb Plus ladder (Invitrogen Corp., USA) identified by electrophoresis in a 1% (weight/volume) agarose gel in Tris-Borate-EDTA buffer, subsequently stained with ethidium bromide and exposed to UV light using a Bio-Rad gel documentation system (Life Science Group, Canada). The dog isolates confirmed as *C. jejuni* by PCR were genotyped using the multilocus sequence typing scheme (MLST) of seven housekeeping genes (256). The amplifications were performed in a 25μL volume reaction using Applied Biosystems AmpliTaq Gold mastermix (Applied Biosystems, Auckland New Zealand) and 5 pmoles of each primer. Products were sequenced on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 (Applied Biosystems) following the manufacturer's instructions. Sequence data were collated and alleles and sequence types (ST) assigned using the *Campylobacter* PubMLST database (URL http://pubmlst.org/campylobacter/) and occurrence compared with other sources in ^{m}EpiLab database. The ^{m}EpiLab database contains over 3,500 samples (at the time of writing) from the Manawatu *Campylobacter* sentinel site, a ten plus year project for source attribution of campylobacteriosis using concurrent sampling of human cases, animals, food and the environment (312).

4.4.4. Statistical analysis

Results of culture methods were compared using Fisher's exact test of independence. Statistical and exploratory data analyses were performed using R v3.2.2 (R: A language and environment for statistical computing. R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

4.5. Results

From a total of 50 dog faecal samples, there were 408 presumptive *Campylobacter* isolates with 356 testing positive by *Campylobacter* genus PCR, of which species-specific PCRs returned 232 *C. upsaliensis*, 81 *C. jejuni*, 14 *C. coli*, and one *C. lari*. Five isolates were PCR positive for *A. butzleri*. Fifteen isolates with negative species-specific PCRs from various dogs and from every culture method, as available, returned five *Helicobacter winghamensis*, four *C. upsaliensis*, two each of *C. rectus* and *C. volucris*, one *C. lari* subsp. *concheus* and one *A. cryaerophilus* as the most similar species by 16S rRNA sequencing. The remaining eight isolates could not be identified. A relative comparison in performance between culture methods is presented in Table 4.1. Overall, two dogs (4%) were positive for three *Campylobacter* spp., five (10%) for two and 24 dogs (48%) for one species only. Three dogs (6%) were positive for *Campylobacter* spp. by one method only, six (12%) by two, nine (18%) by three, ten (20%) by four, and three (6%) dogs by five methods. Combining all the methods, 24 (48%) dogs were *Campylobacter* spp. positive on day two, four (8%) on day three, two (4%) on day four and one (2%) on day five (*C. lari*) of incubation.

Table 4.1. Number of positive working farm dogs[1] from Manawatu, New Zealand (N = 50) using seven culture methods and isolates identified by PCR.

Method[2]	*Campylobacter* spp.	*C. upsaliensis*	*C. jejuni*	*C. coli*	Other
CB_H2_AB	2 [a]	- [a]	- [a]	1	1
CB_H2_CAT	8 [b]	7 [b, c]	- [a]	-	2
CAT_MA	25 [c]	21 [d]	5 [a, b]	-	2
CAT_ H2	24 [c]	18 [d]	5 [a, b]	1	2
BB_ H2_mCCDA	21 [c]	8 [b]	8 [b]	3	5
BB_MA_mCCDA	6 [a, b]	3 [a, b]	2 [a, b]	1	-
AB_H2	11 [c]	8 [c, d]	3 [b]	-	-
Overall	31	21	9	3	8

[1]Shared superscript letters within each column denote no significance by Fisher's exact test ($\alpha < 0.05$). [2]CB (non-selective enrichment broth); BB (selective Bolton broth); AB (non-selective anaerobe basal agar); CAT and mCCDA denote respective agars; MA (microaerobic); H2 (H_2-enriched MA). All methods performed at 37°C except BB_MA_mCCDA at 42°C. CB_H2_CAT used on 38 and AB_H2 on 21 dogs.

Species isolated for the first time in dogs were as follows: *C. volucris* by the methods CB_H2_CAT and CAT_MA (on fourth and second day of incubation respectively), *C. lari* subsp. *concheus* by the method BB_ H2_mCCDA (on fourth day of incubation), *C. rectus* by the method CB_H2_AB (on fourth day of incubation) and *H. winghamensis* by the methods CB_H2_CAT, BB_ H2_mCCDA, and CAT_ H2 (on third and fourth day of incubation). The proportion of readable plates was 26%

(13/50), 79% (30/38), 98% (49/50), 96% (48/50), 100% (50/50), 98% (49/50), and 100% (21/21) with the methods CB_H2_AB, CB_H2_CAT, CAT_MA, BB_H2_mCCDA, BB_MA_mCCDA, CAT_ H2, and AB_H2 respectively, and selectivity was 55% (6/11), 80% (16/20), 93% (97/104), 94% (72/77), 100% (20/20), 93% (104/112), and 63% (41/65) respectively. Forty *C. jejuni* isolates from eight dogs (two to nine isolates per dog) were subjected to MLST typing and 33 returned full allelic profiles. Eight different STs belonging to five different clonal complexes were observed and two dogs carried STs of different clonal complexes. The occurrence of these ST in other sources from the ^{m}EpiLab database is depicted in Supplemental Fig. 4.1.

From 50 home-kill meat samples, there were 52 presumptive *Campylobacter* isolates with 17 testing positive by *Campylobacter* genus PCR from three samples (6%). Of these 17, four isolates were positive by *C. jejuni* PCR and all were grown using mBB_MA_mCCDA from one meat sample (2%) while one other sample (2%) grew 11 isolates using mCB_H2_CAT, mCB_MA_CAT, mBB_H2_mCCDA, and mBB_MA_CAT, which were all positive for *A. butzleri* by PCR. Sequencing of the 16S rRNA gene for the two remaining isolates returned *C. rectus* as the most similar species isolated using mCB_MA_CAT in the third meat sample (2%). The proportion of readable plates was 38% (19/50), 89% (34/38), 94% (47/50), 100% (50/50), 100% (50/50), and 98% (49/50) with the methods mCB_H2_AB, mCB_H2_CAT, mCB_MA_CAT, mBB_H2_mCCDA, mBB_MA_mCCDA, and mBB_MA_CAT respectively. The presence of *E. coli* was detected in 48% (24/50) of the meat samples.

4.6. Discussion

The main findings of the study are the significant differences in isolation of *Campylobacter* spp. between the culture methods and the isolation of four species previously either not reported or not isolated from dogs. These results are of potential public health importance as all species identified in dogs and their food are implicated as pathogens for people (13). Of the emerging species, *C. volucris* was

described in black-headed gulls (495) and since then reported in an immunocompromised human patient with bacteraemia (496). *C. lari* subsp. *concheus* was initially isolated from shellfish and subsequently in humans, seagulls and river water (66, 497). *C. rectus* was detected previously using molecular methods in dog faeces (359) and oral swabs (487) but the clinical significance is uncertain in dogs. In people, *C. rectus* is associated with periodontitis/gingivitis, various gastrointestinal diseases and extra-intestinal infections and, apart from dogs, no other potential sources have been identified (13). *H. winghamensis* was described as a novel species upon isolation from people with clinical signs of gastroenteritis (498) and so far only one study using molecular methods has reported its occurrence in animals and rodents (499). All of the above species have been rarely reported, thus it is not clear if the animals are the true reservoirs for the organism or if they are just transient carriers. With regard to the faecal carriage of *C. jejuni* and *C. upsaliensis*, a longitudinal study in dogs reported carriage of *C. jejuni* of short duration and with genotypically diverse isolates using pulse-field gel electrophoresis whereas the carriage of *C. upsaliensis* was of long duration of clonal strains (139). Four out of eight *C. jejuni* STs isolated in this study are very rarely observed in the ^{m}EpiLab database and the other four STs are common in several sources which supports the heterogeneous exposure of farm dogs (Supplemental Fig. 4.1). Other studies using MLST also reported a high diversity of STs in dogs including strains frequently seen in humans and food (300, 477). In contrast to this study, *Campylobacter* spp. were isolated from 13% and *C. jejuni* from only 5% of 498 dog faecal samples in the Palmerston North area (mostly dog walking areas) (356). In that study, a culture method similar to the BB_MA_mCCDA used in the current study. Lower prevalences using the BB_MA_mCCDA method alone compared to other methods and overall results in this study (Table 4.1) suggest the different results between the two studies are due to the culture methods used. However, the two studies also had different dog populations, faecal sample handling and sample sizes that make results less directly comparable.

The benefit of applying multiple culture protocols in this study is evident from the significant differences observed in pairwise comparison of methods, both in the overall isolation rate and for *C. upsaliensis* and *C. jejuni* in particular (Table 4.1). For

the less common species *A. cryaerophilus*, *C. lari* subsp. *concheus*, *H. winghamensis* and *C. rectus*, the common denominator was isolation in H_2-MA, while for *C. volucris* the use of CAT agar and for *Arcobacter* spp. use of BB_H2_mCCDA appeared to be the most suitable. The requirement of hydrogen for isolation of many emerging *Campylobacter* spp. as well as the enhanced recovery of *C. jejuni* has been previously recognised (17, 18). Interestingly, *C. rectus* isolated from meat samples in this study grew in pure MA although it is considered to have a requirement for hydrogen to grow (36). In this study, too few of the emerging species were isolated for statistical comparison, but with regard to *C. jejuni* and *C. upsaliensis*, CAT_MA and CAT_H2 only differed in the presence of hydrogen and no significant differences were observed (Table 4.1). Although not statistically significant ($p = 0.06$), a surprising finding was the difference in isolation rate of *C. jejuni* between BB_H2_mCCDA and BB_MA_mCCDA (Table 4.1). The two methods differ by temperature and presence of hydrogen, and the enhanced isolation of *C. jejuni* at 37°C rather than at 42°C with mCCDA has been reported (96). *C. upsaliensis* had the largest difference in isolation rate between methods in this study (Table 4.1).

Comparison of CAT and mCCDA for isolation of *C. upsaliensis* in veterinary studies are conflicting as a higher isolation rate with CAT compared to mCCDA has been reported (54, 94) but also equivalent rates (402), and a lower rate with CAT (405) for both *C. upsaliensis,* and *C. jejuni*. Similarly to the current study, in human studies mCCDA was outperformed by both CAT (104) and by the filtration method (96) for the isolation of *C. upsaliensis.* In contrast, filtration was reported as superior to CAT (95) but in the present study the two were comparable (Table 4.1). CAT was originally developed according to the antimicrobial resistance profiles of several thermophilic *Campylobacter* spp. (104) but was also shown to result in better growth and isolation of a greater diversity of *C. upsaliensis* strains than mCCDA (472) and enhanced detection of lower bacterial concentrations compared to mCCDA (99). However, the latter study could not explain the difference in sensitivity between the two agars, neither by the absolute growth index for any length of incubation time nor the antimicrobial composition of the media, leading investigators to speculate that the growth of *C. upsaliensis* is indirectly affected by the interaction of faecal microflora and culture agars. In this study, mCCDA was always used in conjunction with BB and

a comparable rate of isolation of *C. upsaliensis* in CB_H2_CAT with both BB/mCCDA methods was observed, while direct plating on CAT outperformed all three of them (Table 4.1). Improved recovery and motility of *C. upsaliensis* in CB compared to BB has been reported (43). These observations could explain the poorer performance of methods using BB in this study but not that of CB. The poor performance of CB_H2_AB is likely to be due to overgrowth of contaminants, as the modification to CB_H2_CAT both improved the readability of plates and the isolation of *Campylobacter* although the isolation success was still significantly less than that of other methods (Table 4.1). This suggests that contaminating organisms may inhibit *Campylobacter* cells in CB. In this study only one dog was identified as positive after four days of incubation but the relatively low number of samples is likely to preclude weighting of this observation and incubation up to six days has been advised for higher isolation success (18).

The main objective for this study was to isolate a diverse set of *Campylobacter* spp. using the novel culture method, CB_H2_AB, which, with the exception of *C. rectus* being isolated from one dog, failed in both faecal and meat samples. The failure was primarily ascribable to frequent overgrowth by contaminants (mostly *Proteus* spp. and less frequently *Pseudomonas* species). The CB_H2_AB was developed on fresh beef samples only, with no report of overgrowth by contaminants (43). Perhaps the home-kill meat (48% of which showed faecal contamination) and the dogs' faeces used in the current study contained too many non-target bacteria, making the antimicrobial-free method unsuitable. Overgrowth by contaminants on plates using the filtration method (without enrichment) and using selective plates was reported, but usually in less than 10% of plates (94, 99) which is similar to methods using antimicrobial agents (faeces and meat) used in this study. CB_H2_AB with an enrichment duration reduced to 24 hours was also successfully applied previously in many types of fresh meat products (61) and porcine samples including caecal contents (62). As the overgrowth of contaminants in this study could not be explained by the procedures in production, storage and usage, the modified (m)CB_H2_CAT methods were added during the study. The readability of the plates rose to 79% and 89% with faeces and meat respectively. In addition, increasing the agar content of AB to 4% to limit the swarming growth of *Proteus* spp. was attempted

in 12 samples, but was unsuccessful. To investigate the overgrowth of contaminants associated with the CB enrichment rather than the filtration technique in CB_H2_AB vs. swab to CAT in CB_H2_CAT, the AB_H2 method was added during the study. Although AB_H2 was only applied to 21 faecal samples the readability rose to 100% which suggests CB caused overgrowth of contaminants to a level too high for the filtration method and partially too high for the CAT to cope with.

Another modification that could be useful in optimising CB_H2_AB and other methods in this study used to isolate *Campylobacter* from food samples is the inclusion of a pre-enrichment step (usually up to 4 hours) with either delayed addition of antimicrobials to broth, a lower incubating temperature or both. This step is suggested when low number or injured *Campylobacter* cells are expected, such as in frozen products (20, 110). The absence of pre-enrichment step in the current study could explain the low prevalence of *Campylobacter* spp. observed in frozen home-kill meat by all methods used. There were 44 sheep and 6 beef samples in this study and the prevalence of *C. jejuni* in retail fresh meat or liver for human consumption in New Zealand is 25% and 8% in sheep and beef meat respectively (312). It could be expected that home-kill meat has a greater contamination level than commercially available meat due to a lack of hygienic measures in farming environments and considering the intended use of the two meat types with the regard to preparation, handling, and storage practices. However, the sample size of this study is low and limits the confidence for comparison of the studies.

The selectivity estimates were used to describe the workload with the different culture methods given the protocol for identification of species used. The rationale was, that suspect *Campylobacter* colonies that were negative by *Campylobacter* genus PCR were subcultured, stored and re-tested for no benefit for the time and resources invested. The variation in colony morphology of *Campylobacter* spp. between agar plates was reported (38) but is not related to selectivity expressed herein which denotes the ratio of PCR-confirmed over presumptive colonies. Low selectivity suggests the isolates should not be considered *Campylobacter* spp. and should be confirmed by further identification tests. Lower estimates were observed with filtration methods and CB than with the use of antimicrobial agents. It is possible

the antimicrobials have supressed a wider range of species than the filtration method, thus providing a lesser diversity of bacteria on the agar, of which even fewer were *Campylobacter*-like. In contrast, a greater diversity of bacteria passed through the filters, many of which grew on a non-selective agar and more of which were *Campylobacter*-like. However, the aim of the study was to isolate a variety of *Campylobacter* spp. hence screening of isolates that otherwise may not be included was expected. *C. showae* resembles straight rods (44), *C. mucosalis* has yellow coloured colonies (36), and *C. gracilis* is non-motile (45); all are examples of isolates that would not be included if the focus was strictly on the phenotypic characteristics of the common species. Additional biochemical or phenotypic tests (47) could have been applied for presumptive isolates in this study, which could change the selectivity estimates by reducing the number of isolates passed to PCR testing. However, the addition of more screening tests for isolates increases the workload and cost. The cross-reaction of the PCR for *Campylobacter* spp. with *Arcobacter* spp. has been reported (474) but the cross-reaction with *H. winghamensis* is newly observed. These are closely related genera within *Campylobacteraceae* that can be isolated using similar culture methods (92). The taxa have relatively high similarity both phenotypically and genotypically (500) which makes their cross-reaction less surprising.

Conflict of interest statement

The study was funded by the Centre for Working and Service Dogs, Massey University, New Zealand and authors declare no conflict of interests.

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CHAPTER 5

5. Variation in the limit-of-detection of the ProSpecT *Campylobacter* Microplate enzyme immunoassay in stools spiked with emerging *Campylobacter* species

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5.1. Highlights

- EIA can detect *Campylobacter hyointestinalis*, *C. upsaliensis* and *C. helveticus*
- Analytical sensitivity of the EIA varies between and within *Campylobacter* species
- Faecal consistency and the individual faecal sample tested influence EIA results

5.2. Abstract

Campylobacter enteritis in humans is primarily associated with *C. jejuni*/*coli* infection. The impact of other *Campylobacter* spp. is likely to be underestimated due to the bias of culture methods towards *C. jejuni/coli* diagnosis. Stool antigen tests are becoming increasingly popular and appear generally less species-specific. A review of independent studies of the ProSpecT® *Campylobacter* Microplate enzyme immunoassay (EIA) developed for *C. jejuni/coli* showed comparable diagnostic results to culture methods but the examination of non-*jejuni/coli Campylobacter* spp. was limited and the limit-of-detection (LOD), where reported, varied between studies. This study investigated LOD of EIA for *C. upsaliensis, C. hyointestinalis* and *C. helveticus* spiked in human stools. Multiple stools and *Campylobacter* isolates were used in three different concentrations (10^4-10^9 CFU/ml) to reflect sample heterogeneity.

All *Campylobacter* species evaluated were detectable by EIA. Multivariate analysis showed LOD varied between *Campylobacter* spp. and faecal consistency as fixed effects and individual faecal samples as random effects. EIA showed excellent performance in replicate testing for both within and between batches of reagents, in

agreement between visual and spectrophotometric reading of results, and returned no discordance between the bacterial concentrations within independent dilution test runs (positive results with lower but not higher concentrations). This study shows how limitations in experimental procedures lead to an overestimation of consistency and uniformity of LOD for EIA that may not hold under routine use in diagnostic laboratories. Benefits and limitations for clinical practice and the influence on estimates of performance characteristics from detection of multiple *Campylobacter* spp. by EIA are discussed.

Keywords: Analytical sensitivity, *Campylobacter*, Concordance, ELISA, Faecal consistency, Heterogeneous sample, Performance characteristics

5.3. Introduction

Campylobacteriosis is one of the most common bacterial gastrointestinal illnesses in the developed world (501) with *C. jejuni* and *C. coli* being responsible for approximately 80 - 85% and 10 - 15% of cases respectively (3). Other *Campylobacter* spp., generally referred to as “emerging pathogen” species, have been isolated from diarrhoeic stools and implicated as human pathogens with the more commonly reported species being *C. upsaliensis*, *C. hyointestinalis*, *C. ureolyticus*, *C. concisus*, *C. lari*, and *C. fetus* (18, 478, 479, 483, 502-504). The main difficulty for isolation and detection of a wider range of *Campylobacter* spp. is the lack of an all-encompassing method to suit the vast diversity in growth requirements between species including, but not limited to, temperature, atmospheric conditions and incubation period (17, 18). Since the most commonly used culture methods are optimised for *C. jejuni/coli*, a potential for misdiagnosis and underestimation of disease associated with the emerging *Campylobacter* species is now widely recognised (13, 17).

Diagnostic laboratories have to vary widely in the methods and protocols used, due to differences in regulations (or by adherence to best-practice guidelines), clinical relevance, and available resources. Although our knowledge about

campylobacteriosis comes from decades of research primarily based on culture methods, the development of new methods is changing their routine use in clinical diagnosis as well as in research studies. Nucleic-acid amplification tests (NAAT) are used increasingly in clinical practice (481, 505, 506) although stool antigen tests are more widely accepted because these tests are fast to undertake, easy to use, require minimal equipment and are cost competitive. A recent survey of laboratory practices for the diagnosis of campylobacteriosis by the Foodborne Diseases Active Surveillance Network in the USA reported the proportion of diagnostic laboratories using non-culture methods increased from less than 3% in 2004 to 15% in 2014 and almost all of the non-culture methods were commercial stool antigen tests (52, 507). The performance of methods and protocols are very important because of their impacts on both patient care and public health surveillance and planned interventions (508, 509). Currently there is a lack of formal guidance and best practice recommendations regarding the use of non-culture methods for the detection of *Campylobacter* infection in stool specimens (52).

The ProSpecT® *Campylobacter* Microplate enzyme immunoassay (Remel, Lenexa, KS, USA) (EIA) is a stool antigen test developed specifically for the detection of *C. jejuni* and *C. coli* in the diagnosis of *Campylobacter* enteritis in humans, with several independent studies reporting both comparable results to culture (121, 128-130, 510) and possible detection of non-*jejuni/coli Campylobacter* species (128, 129). The manufacturer reported the analytical sensitivity of EIA to be $5x10^5$ CFU/ml and a review of the literature revealed estimates for limit-of-detection (LOD) ranging from $3x10^4$ – 10^5 CFU/ml in 0.9% NaCl solution to $3x10^6$ CFU/ml in faecal suspensions for *C. jejuni* (510), and for *C. upsaliensis* at $3x10^7$ CFU/ml (129). This variation in LOD of EIA with regard to the bacterial species and the testing matrix is interesting considering that the common procedure for estimating LOD is methodologically very constrained. In contrast, the inferences drawn are applied to faecal samples - perhaps the most heterogeneous group of clinical specimens the diagnostic laboratories deal with. The aim of this study was to evaluate the ability of EIA to detect non-target *Campylobacter* spp. and to determine if estimates of LOD are influenced by the *Campylobacter* species isolate used, and the faecal specimen characteristics. *C. hyointestinalis, C. upsaliensis* and *C. helveticus* were selected as

they are emerging *Campylobacter* spp. and had not been included in the validation of EIA by the manufacturer (technical information sheet, Remel Inc.). In addition, apparent discordance of results on repeat test runs and between visual assessment and spectrophotometry was assessed.

5.4. Materials and Methods

5.4.1. Isolates

All isolates were recovered from faeces of healthy animals; *C. upsaliensis* and *C. helveticus* from local household dogs (n = 5) and cats (n = 8) presenting to the Massey University Veterinary Teaching Hospital for elective procedures, and *C. hyointestinalis* from locally farmed red deer (n = 8). Sources were selected for their potential to expose the local human population to *Campylobacter*. Cultures were performed using mCCDA and C.A.T. *Campylobacter* selective agars (Fort Richard, Auckland, New Zealand) in a microaerobic atmosphere at 37 and 42°C for 4 days. Genus and species identification was confirmed by PCR as described by Linton et al. (490). Overall, eight isolates each of *C. upsaliensis* and *C. hyointestinalis* subsp. *hyointestinalis,* and five *C. helveticus* isolates were used in the study.

5.4.2. Patient samples

Anonymous stool specimens submitted for screening for enteric pathogens to the regional laboratory between February and May 2012 were used. Samples were submitted from patients whose referring clinician deemed that their symptoms (including abdominal pain and/or diarrhoea) were consistent with gastroenteritis. On the day that stool specimens were collected, they were stored at 4°C and were sent directly to our laboratory the following morning, after the screening tests were performed. This screening included testing for *Campylobacter* spp. (by EIA), *Salmonella* spp., *Shigella* spp., *Yersinia* spp., and, if the patient was less than five

years of age, for *E. coli* O157 and rotavirus. In brief, for *Salmonella* and *Shigella*, samples were cultured onto XLD agar and selective selenite enrichment broth for 24 hours at 37°C in normal atmosphere then sub-cultured onto XLD for a further 24 hours. For *Yersinia*, samples were cultured onto CIN agar for 48 hours at 30°C in normal atmosphere and plates were read at 24 and 48 hours. For *E. coli* 0157 samples were cultured onto sorbitol MacConkey agar for 48 hours at 37°C in normal atmosphere and plates were read at 24 and 48 hours. All media was supplied by Fort Richard, Auckland, New Zealand. Rotavirus was tested using the Rida®Quick immunochromatographic kit (R-Biopharm AG, Darmstadt, Germany). Only specimens that tested negative for all the above pathogens were sent to our laboratory. Faecal aliquots were made and stored immediately at -20°C in accordance with the instructions of EIA's manufacturer. In addition, faeces were cultured as above as further evidence of the stool's *Campylobacter* spp.-free status before spiking experiments were performed. All specimens were used and EIA tested within one month from the initial testing. The Central Regional Ethics Committee, Ministry of Health determined the study did not require full ethical review (CEN/11/EXP/088).

5.4.3. EIA testing

Inocula of *Campylobacter* spp. were prepared using whole-plate growth of pure cultures suspended in phosphate buffered saline pH 7.3 (Difco Laboratories Inc., Detroit, MI, USA). Three 100-fold dilutions were thoroughly mixed with watery faeces (WF) in a ratio of 1 part suspension to 9 parts of faeces (v/v). Semi-solid faeces (SSF) were emulsified according to EIA's manufacturer instructions using the Bacterial Diluent provided. Bacterial suspensions were added to the emulsified SSF, as for WF, to ensure better homogenisation of the bacterial inocula and SSF. Spiked faecal samples were tested by EIA and results determined according to the manufacturer's instructions both spectrophotometrically using a VersaMax ELISA Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA), and visually by two trained laboratory personnel (KB, LR) who were not blinded. Each isolate was tested in all three bacterial loads in aliquots of the same faecal specimen, except for

two *C. hyointestinalis* isolates for which the low load in WF was omitted in one of the test runs due to unavailability of assays. Controls were included in all assays. Before spiking, the faecal samples were re-tested by EIA because different batches of reagents were used by the submitting and study laboratories.

5.4.4. Quantification of bacterial loads

In order to quantify the bacterial loads in spiked faeces, 100μl of at least two dilutions of each bacterial suspension were spread using a spiral plater (Don Whitley Scientific, West Yorkshire, UK) and sterile hockey-stick spreader on Columbia horse blood agar plates (Fort Richard, Auckland, New Zealand) and cultured as above. Bacterial loads were also estimated visually by comparison to a 2.0 McFarland turbidity standard and by a turbidometer (Biolog, Hayward, CA, USA) as occasionally the *C. helveticus* isolates exhibited swarm-like growth preventing colony counting on the plates. Bacterial colony counts were performed manually and/or using an aCOLyte plate reader (Synbiosis, Cambridge, UK), depending on the amount of bacterial growth. For each of the species tested, the three 100-fold dilutions used gave bacterial concentrations of 1.1 - 3.0 x 10^4 - 10^5, 10^6 - 10^7 and 10^8 - 10^9 CFU/ml in faeces (WF) or faecal suspensions (SSF) and were categorised into three levels: low (LL), medium (ML) and high (HL) respectively.

5.4.5. Statistical analysis

Statistical and exploratory data analyses were performed using R (R: A language and environment for statistical computing. R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). For univariate analyses, Fisher's exact test was used to evaluate the association of faecal consistency, individual isolate and faecal sample identity number (faecal ID) with bacterial loads observed as LOD for each of the species tested. Multivariable logistic regression analysis was performed using generalised linear mixed effects models where the dependent variable was EIA result and independent variables were

species, bacterial load and faecal consistency as fixed effects, whereas faecal ID number was specified as a random effect. Bacterial load as an ordinal category and actual concentrations as a continuous variable were used to evaluate linearity and variation in predicted probabilities of obtaining a positive result by EIA. Model building was performed using a forward step-wise elimination procedure and model selection was based on parametric bootstrapping, ANOVA tests and information criteria, while goodness of fit was assessed by likelihood ratio tests. The significance of regression coefficients and their pair-wise differences were additionally assessed by a Wald χ^2 test.

5.5. Results

A total of nine faecal samples were used, and a total of 196 test procedures were performed in the study. All nine faeces remained EIA negative when re-tested with different batches of reagents showing a 100% concordance (95% CI 66 – 100) between batches of reagents. Results by visual assessment compared to spectrophotometry had a 100% concordance (196/196, 95% CI 98 – 100). The first 76 tests were performed in duplicate and also demonstrated a 100% concordance between replicates (95% CI 95 – 100). The remainder of the tests were performed only once and 9% (4/44) of these tests had indeterminate reading on SSF (two isolates of *C. hyointestinalis* in HL and two of *C. upsaliensis* in ML) by both spectrophotometry and visual assessment and were reported as positive but repeat testing was not performed. No discordant results (*e.g.*, a positive result in LL but not ML or HL) between bacterial loads were observed in any of the test runs. A detailed summary of LOD by faecal consistency, isolate and faecal ID for each species is presented in Table 5.1.

Table 5.1. Limit-of-detection of ProspecT™ *Campylobacter* Microplate Assay in human stools spiked with three *Campylobacter* species.

Factor Factor levels	Number of times the bacterial load[a] was observed as the limit-of-detection			
Campylobacter hyointestinalis	Low load	Medium load	High load	No detection
Faecal consistency				
Semi-solid			4	4
Watery/Liquid	3		1	4
Isolate				
vp11a				2
vp12b	1			1
vp14a	1		1	
vp24b			2	
vp26a				2
vp28a			1	1
vp30b			1	1
vp35b	1			1
Faecal sample				2
A				4
B			2	2

C	3		1	
D			1	
E			1	
F				

Campylobacter upsaliensis	Low load	Medium load	High load	No detection
Faecal consistency				
Semi-solid		4	4	
Watery/Liquid	2	6		
Isolate				
acp5b	1	1		
acp18a		1	1	
acp64a	1	1		
acp72b		1	1	
acp135a		2		
acp136b		1	1	
acp170b		2		
acp179b		1	1	
Faecal sample		5		
B		2	1	
C			1	
G	2	1		

H		2	2	
F			1	
I				

Campylobacter helveticus	Low load	Medium load	High load	No detection
Faecal consistency				
Semi-solid			5	
Watery/Liquid	3			
Isolate				
acp102b	1		1	
acp105a			1	
acp108a	1		1	
acp114b	1		1	
acp141a			1	
Faecal sample				
F			1	
G			3	
H	3		1	
I			1	

[a]Low, medium, and high load correspond to 10^4 – 10^5, 10^6 – 10^7, and 10^8 – 10^9 CFU/ml of faeces, respectively.

After removing duplicate tests, 120 observations were available for statistical analysis. For all *Campylobacter* spp. evaluated, the univariate analysis using Fisher's exact test showed the bacterial loads observed as LOD to be significantly associated with the types of faecal consistency and faecal ID used ($p < 0.05$) except for faecal consistency with *C. hyointestinalis* ($p = 0.13$); isolates were not significantly associated with LOD for any of the *Campylobacter* species ($p > 0.05$). In the multivariable logistic regression analysis, species, faecal consistency and bacterial loads were statistically significant fixed effects and faecal ID a significant random effect. Holding other covariates constant, a Wald χ^2 test on regression coefficients showed all three species in pair-wise comparisons as significantly different between each other ($\chi^2 > 4.3$, d.f. = 1, $p < 0.04$). Holding other covariates constant, the results by bacterial load showed a significant increasing log-linear relationship ($\chi^2 = 15.9$, d.f. = 1, $p < 0.001$). The linear relationship was supported by no significant difference between linear and polynomial regressions with neither second-order ($p = 0.8$) nor third-order polynomials ($p = 0.4$) when bacterial concentrations were used as a continuous variable. Additionally, with bacterial load used as an ordinal factor, the linearity was supported by the significance of the linear term ($z = 4.3$, $p < 0.001$) and not the quadratic term ($z = 0.943$, $p = 0.3$). Holding other covariates constant, WF was significantly more likely to test positive than SSF ($\chi^2 = 18.7$, d.f. = 1, $p < 0.001$). The relative importance of the fixed effects, in descending order, was bacterial load, faecal consistency and bacterial species. The model-predicted probabilities of obtaining a positive EIA result across the range of bacterial concentrations accounting for variation in fixed and random effects (with 95% CI) are depicted in Fig.5.1. The final model had a marginal R^2 of 0.67 and a conditional R^2 of 0.89.

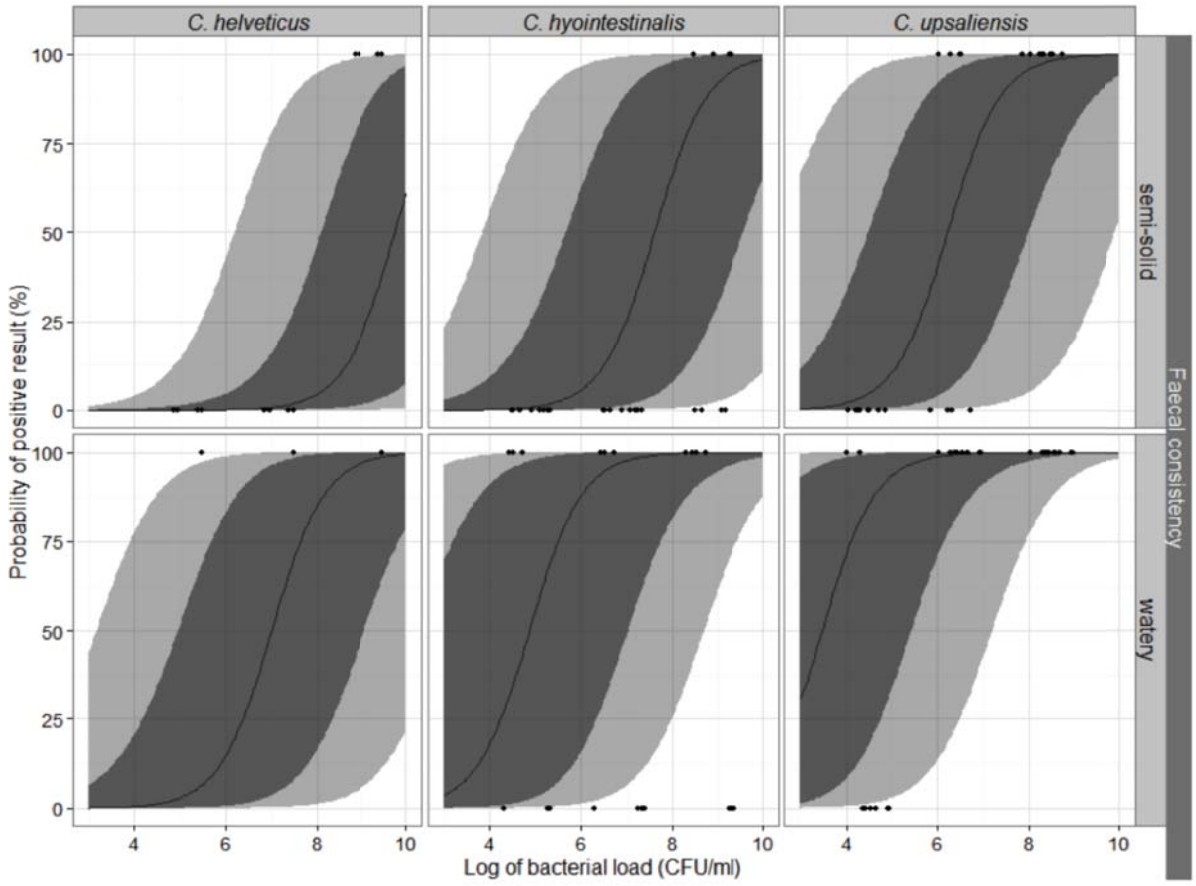

Fig. 5.1. Model predicted probabilities of obtaining a positive result using ProSpecT^{TM} *Campylobacter* Microplate Assay in human clinical stools spiked with *Campylobacter* species. The line represents point estimates of predicted probabilities, the dark shaded areas 95% CI based on variation of the fixed effects (species, faecal consistency and bacterial concentrations) and the light shaded areas 95% CI with the addition of the random effect (faecal sample ID). Points at 0 and 100% represent negative and positive results of the raw data respectively.

5.6. Discussion

The ability of EIA to detect *Campylobacter* spp. in addition to the target species (*C. jejuni/coli*) is both an asset and a liability in the diagnostic laboratory. The *Campylobacter* genus includes well-established and 'emerging' pathogens as well as opportunistic and commensal species, and the distribution of species between

asymptomatic and symptomatic people varies between developed and developing countries (13, 17, 511). Of the species evaluated in this study, *C. upsaliensis* and *C. hyointestinalis* are recognised as enteric pathogens; *C. upsaliensis* is among the leading emerging *Campylobacter* pathogens and, with suitable culture methods, higher isolation rates compared to *C. coli* were reported in patients with gastroenteritis in Europe (102, 478), Africa (18) and North America (479). However there is currently very little evidence for *C. helveticus* being a pathogen (13, 97, 347). Hence, the non-target species coverage of EIA may lead to both 'false positive' and 'false negative' results in the laboratory diagnosis of campylobacteriosis if case definitions are not optimally suited to the clinical setting where the test is applied.

It is interesting that in the independent studies investigating EIA (128, 129, 285), none reported cross-reactions of EIA with non-*Campylobacter* spp., but only, (if attempted), with non-*jejuni/coli Campylobacter* species. Several studies have made assumptions about the possibility of non-*jejuni/coli Campylobacter* spp. detection by stool antigen tests (121, 131, 132) and several have confirmed this occurrence (128, 133, 285). In a recent large, multi-centre, prospective study employing four stool antigen tests (EIA included), 206/2767 culture negative samples were positive by at least one antigen test (111th Annual Meeting of the American Society for Microbiology, abstract 0518, 2011). The study design considers these as 'false positive' results but non-*jejuni/coli Campylobacter* spp. are not taken into account and these could have well explained at least a portion of positive results. The study concluded that stool antigen tests should not be used as the sole diagnostic tests but results should be verified by culture methods. However, in order to maximise the diagnostic success rate and provide benefits for the patients, routine diagnostic protocols and practices should consider campylobacteriosis as more than "jejunosis". Approximately 70% of gastroenteritis cases do not have an established diagnosis and emerging pathogens may well explain a certain portion of the undiagnosed cases (17). That is, if culture methods do not enable detection of non-*jejuni/coli Campylobacter* spp., a negative culture with a positive stool antigen test should signal the possibility of an 'emerging' *Campylobacter* species. Otherwise, stool antigen tests will always be deemed false positive (as culture was negative) and

potential patients with non-*jejuni/coli* campylobacteriosis will consequently be missed (131).

Recent studies employing NAAT have showed the importance of species information due to the extent and variety of *Campylobacter* spp. present in faecal specimens from clinical cases in the developed world (138, 347, 506) and cases and controls in the developing world (285). More importantly, the study using quantative NAAT suggested differences in the level of bacterial burden between *Campylobacter* spp. and severity of gastrointestinal symptoms in the developed world (138), whereas the association of *Campylobacter* spp. with the burden-level between asymptomatic and symptomatic people varied between developing countries (285). In addition, the latter study reported NAAT outperformed EIA for detection of low burden-level *Campylobacter* spp. infections (285) and this is in line with this study which shows a decline in probability of positive EIA result as the burden-level decreases. These two studies highlighted the importance of quantification of the *Campylobacter* burden-level, which puts the test LOD as a performance characteristic metric under the spotlight.

The second finding of this study is that LOD of EIA fluctuates between the variables that are frequently constrained by the experimental procedures. LOD of stool antigen tests are commonly determined by testing at least two replicates of serial dilutions of faecal specimens spiked with a known concentration of the targeted bacterial agent/antigen, usually mixed in a ratio of 1:9 to preserve the characteristics of the background faecal matrix. Prior to spiking, the stool specimens are tested by the assay under evaluation and preferably by another diagnostic method to support the absence of the target agent/antigen. Therefore, the methodological design is most commonly limited to the use of one or a few representative organisms (usually the type strain and/or a widely used isolate of particular interest) and by a limited number of faecal specimens (descriptions and numbers are usually not reported). Although it is possible for methodological design constrained in such a way to affect the estimates of LOD, these influences are rarely evaluated. A consequence is that the estimate of LOD appears as a consistent feature across the population the assay is applied to, and also as a suitable performance characteristic for comparison of

different methods. In the above independent studies, the reporting of LOD of EIA for several *Campylobacter* species has a limited description of methodology (faeces of different consistency and the number of faecal samples were either not used or not reported) and reported a point estimate for LOD of EIA (129, 510). In contrast to a uniform estimate of LOD the present study shows, as depicted in Fig. 5.1, the variation expected when mimicking routine conditions of use. Routine conditions hereby denote the variability of faecal specimen characteristics and pathogen genotypes occurring in samples submitted to the diagnostic laboratories. Similarly to this study, the probability of successful detection in different bacterial loads were reported in studies using culture methods for *C. jejuni/coli* and *C. upsaliensis* in association with *Campylobacter* species' isolates (94, 99) and faecal samples used (104). These observations suggest that the experimental estimates of LOD may vary considerably and should be cautiously applied for comparison of diagnostic tests in samples with large heterogeneity in the absence of a quantitative method or procedure and species information provided.

Surprising findings of this study were the significant differences in LOD between SSF and WF detected and the random effect of the individual faecal sample. The random effect of faecal sample may be indicative of differences between faeces with regard to severity of gut inflammation, or presence of substances that may cause inhibition of enzymatic reactions in antigen tests similarly to inhibition of nucleic acid polymerases with NAAT. The difference in LOD of EIA between 0.9% NaCl solution and faecal suspension has been reported previously (510), but to the best of our knowledge this is the first report on the influence of the individual faecal sample and of faecal consistency *per se*. It should be emphasised that this is the influence on LOD and not the association of positivity rates with faecal consistency that has been reported in clinical diagnostic studies of enteric viruses (512), and also for *Salmonella* spp. but not *C. jejuni* (513). The higher content of particulate matter in SSF might be responsible for the inaccessibility of the antigen epitopes to EIA reagents. The influence of faecal consistency might be important for diagnosing campylobacteriosis in the later stages of acute enteral disease or in the (post)convalescent period when formed stools are expected to occur more frequently. This may be of importance in Guillain-Barré syndrome, a recognised, albeit rare, sequela of *C. jejuni* or *C.*

upsaliensis infection (514). It is presumed that counts of bacteria are lower in faeces of these patients due to the onset of neurological symptoms up to several weeks after the acute gastroenteritis (89) when patients may not have diarrhoea. *C. jejuni* infections have also been associated with post-infectious irritable bowel syndrome (515) and these patients may also pass more formed stools than patients with acute gastroenteritis.

It is important to appreciate the way that the choices of species accepted as "true pathogens" and the ability (or application) of the method to provide species information will influence the estimates of diagnostic performance characteristics. These two features can vary markedly between different diagnostic methods and protocols, thereby influencing the conclusions one draws from their use or comparison. Stool antigen tests commonly provide a dichotomous, unspeciated result unlike culture and NAAT for which in-line procedures (*e.g.*, phenotypic tests, primer selection) can provide species information. Therefore, studies opting for culture (121, 128-130, 510), or NAAT methods (121), or a combination of methods (132)(111^{th} Annual Meeting of the American Society for Microbiology, abstract 0518, 2011) as a "gold standard" or case definition lead to different determinations of performance characteristics. By using NAAT as verification method, stool antigen tests (EIA included) outperformed cultures (121) whereas, with a combination of NAAT and antigen tests as a "gold standard", the stool antigen tests were reported as both superior (132) and inferior (111^{th} Annual Meeting of the American Society for Microbiology, abstract 0518, 2011) to cultures. These findings also suggest that stool antigen tests could vary markedly between each other and grouping of tests by method of action may be problematic.

5.7. Conclusions

This study showed EIA has variable ability to detect several non-*jejuni/coli* *Campylobacter* spp. within the range of 10^4 – 10^9 CFU/ml of faeces. The *Campylobacter* genus is diverse and with the stool antigen tests being used frequently in diagnostic laboratories, the need for thorough investigation of cross-

reactions with species closely related to the target organisms is critical. Any potential diagnostic benefits for patients from such cross-reactions should be accounted for in developing best practice guidelines. The LOD of EIA varied between *Campylobacter* spp. and was influenced by the faecal consistency and by the individual faecal sample used. These factors limit the use of LOD of EIA as a comparative performance characteristic metric and may be important contributor to the interpretation of results as true or false positive and negative between EIA and other diagnostic tests.

Compliance with Ethical Standards: This study been deemed by the Central Regional Ethics Committee, Ministry of Health as not requiring the full ethical review (CEN/11/EXP/088).

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Conflict of Interest: Previous and on-going studies by the authors have used the EIA as a diagnostic tool.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Krunoslav Bojanić

Name/Title of Principal Supervisor: Dr Els Acke

Name of Published Research Output and full reference:

K Bojanić, AC Midwinter, JC Marshall, LE Rogers, PJ Biggs, E Acke. Analytical sensitivity of the ProSpecT® Campylobacter Microplate Assay for detection of C. upsaliensis, C. helveticus and C. hyointestinalis in spiked human clinical faecal samples. Journal of Microbiological Methods (2016 Vol. 127, p236-241). doi: 10.1016/j.mimet.2016.06.016

In which Chapter is the Published Work: Chapter 5

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: >80%

  and / or
- Describe the contribution that the candidate has made to the Published Work:

Krunoslav Bojanic — Digitally signed by Krunoslav Bojanic Date: 2016.10.09 18:04:20 +02'00'

Candidate's Signature

9/10/2016

Date

Els Acke — Digitally signed by Els Acke

Principal Supervisor's signature

9/10/2016

Date

GRS Version 3– 16 September 2011

CHAPTER 6

6. Comparison of the pathogenic potential of emerging *Campylobacter* spp. using larvae of *Galleria mellonella* as an infection model

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6.1. Abstract

Campylobacter-enteritis in humans is primarily associated with *C. jejuni*/*coli* infection. Other species occur relatively infrequently; while this could be attributed to the bias of diagnostic methods, the pathogenicity of non-*jejuni/coli Campylobacter* spp. is questionable and they are denoted as 'emerging pathogens'. *C. upsaliensis* and *C. helveticus* are emerging pathogens commonly isolated from dogs and cats. *Galleria mellonella* larvae were shown as suitable models of mammalian innate immune systems due to high functional and structural homologies and have been applied to research of *C. jejuni*. This study compared pathogenicity of 34 *C. jejuni* isolates, 22 *C. upsaliensis*, and 13 *C. helveticus* with saline-inoculated and undisturbed larvae control groups in a total of 5,878 larvae. *C. upsaliensis* and *C. helveticus* showed significantly higher survival of larvae compared to *C. jejuni*. Histopathological changes were indistinguishable between *Campylobacter* species. *C. jejuni* could be isolated from haemocytes and haemolymph up to eight days post-inoculation whereas *C. upsaliensis* and *C. helveticus* could only be isolated from haemolymph in the first two days. Dose, temperature- and atmosphere-dependent survival of larvae was confirmed with all *Campylobacter* spp. but infection dynamics varied between the species. Mixed effects Cox proportional hazard regression modelling showed a significant variation in the hazard rate between batches of larvae, strains, and biological, but not technical, replicates as random effects, and species and bacterial dose as fixed effects. Inoculation of larvae with heat- and cold-inactivated whole cells and cellular components induced varying degrees of sickness and death of larvae between these assays and *Campylobacter* species.

Keywords: *Galleria mellonella*, pathogenicity, virulence, *Campylobacter*, dose response, emerging pathogens, zoonosis, survival

6.2. Introduction

Campylobacteriosis is one the most common bacterial gastrointestinal diseases in people worldwide and is mostly associated with *C. jejuni* and *C. coli*. (9) The disease is predominantly food-borne, especially through poultry, but as *Campylobacter* spp. are commonly isolated from the intestinal tract of many animals, direct contact with animals and contact with contaminated water and environments are also important transmission routes of infection. (9) Diagnostic methods are commonly optimised for detection of *C. jejuni/coli,* hence many other *Campylobacter* spp. are considered underreported although implicated as human pathogens, often referred to as 'emerging' pathogens. (13, 18) *C. upsaliensis* and *C. helveticus* are the most common *Campylobacter* spp. reported in dogs and cats, and are frequently detected with a high prevalence rate (13, 16) whereas they are rarely reported and with low prevalence rates (~1 – 2%) in other sources. (61-65) *C. upsaliensis* is one of the main emerging *Campylobacter* pathogens, as several studies have reported higher isolation rates than those of *C. coli* when suitable culture methods were performed. (97) On the other hand, no or few isolations have also been reported with suitable methods employed. (92, 104, 129) *C. helveticus* is the species most closely resembling *C. upsaliensis* (516) but has only been reported once in humans. (347) Considering the two species are common in pet animals but infrequent and disparate in humans, the pathogenic potential of these taxa remains uncertain. Discordant reports in humans could also suggest that different (and possibly unidentified) sources of infection exist, resulting in varying exposures of humans to these taxa, or that strain variation and host factors may play important roles in the development of disease.

Mechanisms of pathogenesis of *Campylobacter* spp. can be investigated by various approaches such as animal models of disease (*e.g.* primates, rodents, chickens etc.), eukaryotic cell cultures, and molecular biology tools (*e.g.* genetic and genomic studies). (21) Invertebrates may also be used as an infection model for microbes due to a high degree of functional and structural homology with the mammalian innate immune system. (451) The larvae of the greater wax moth, *Galleria mellonella*, have been described as a model for many fungal and bacterial pathogens, including

species of *Acinetobacter, Escherichia, Enterococcus, Serratia, Pseudomonas.* (517, 518) Recently, the wax moth larvae were also described as an infection model for *C. jejuni* for the evaluation of the role of selected genes in the mortality of larvae. (519) That study showed that mutant strains of *C. jejuni* lacking defined virulence factors showed an attenuated ability to kill the larvae compared to the respective wild types. Subsequently, histological changes and survival of *C. jejuni* in the larvae were described and the model was further applied to evaluate differences in virulence between genotypes of *C. jejuni* according to the multi-locus sequence-typing (MLST) scheme. (452) That study showed extensive histological changes in larvae upon inoculation of *C. jejuni*, intracellular survival of *C. jejuni* within larval haemocytes, and that the sequence type (ST) ST-257 was more virulent than ST-21 in the larvae model.

The aim of this study was to use *Galleria mellonella* wax moth larvae as an infection model for comparison of the pathogenic potential between *Campylobacter* species. *C. upsaliensis* and *C. helveticus* were selected as the emerging pathogens to which humans are likely to be exposed from contact with pets and were compared to the established pathogen *C. jejuni*. Survival of larvae inoculated with viable bacteria and heat- and cold-inactivated cells and cellular components, histopathological changes, cultures of larval haemolymph and haemocytes, and dose-, temperature-, and atmosphere-dependent survival of larvae were used as comparative features of larval infection between *Campylobacter* species.

6.3. Results

In total, 5,878 larvae obtained from seven different batches were used for inoculation with *C. jejuni* (2,137 larvae), *C. upsaliensis* (1,751), *C. helveticus* (1,272), phosphate-buffered saline (PBS) (338), and Mueller-Hinton broth (10) while 385 served as undisturbed controls. The main assay was the inoculation of larvae with viable bacteria using three 100-fold dilutions followed by incubation in the optimal environment and consisted of 1,831 larvae (1,073 events) for *C. jejuni*, 1,460 (489 events) for *C. upsaliensis*, and 982 (338 events) for *C. helveticus.* The Kaplan-Meier

(KM) survival curves for *Campylobacter* spp. with the three dilutions used in the main assay are presented in Fig. 6.1. Overall, the survival at 24 hours post-inoculation with the doses of ~10^4, 10^6 and 10^8 *C. jejuni* CFU was 99% (80 – 100% between strains), 95% (65 – 95% between strains) and 47% (5 – 95% between strains) respectively (Fig. 6.1, strain data not shown). The KM survival curves in Fig. 6.1 show a clear dose-dependent survival for all of the *Campylobacter* species. With the low bacterial load (225 *C. jejuni*, 205 *C. upsaliensis* and 180 *C. helveticus* larvae), the log-rank test showed no significant differences in survival of larvae inoculated with different *Campylobacter* species. In the medium load (785 *C. jejuni*, 625 *C. upsaliensis* and 392 *C. helveticus* larvae) survival of larvae inoculated with *C. jejuni* was significantly lower than that of larvae inoculated with either *C. upsaliensis* or *C. helveticus* ($p < 0.001$), the latter two not being significantly different to each other. In contrast, in the high load (821 *C. jejuni*, 630 *C. upsaliensis* and 410 *C. helveticus* larvae) survival of larvae was significantly different between all *Campylobacter* spp. ($p < 0.001$) with *C. jejuni* having the lowest survival followed by *C. helveticus* and *C. upsaliensis* with the highest.

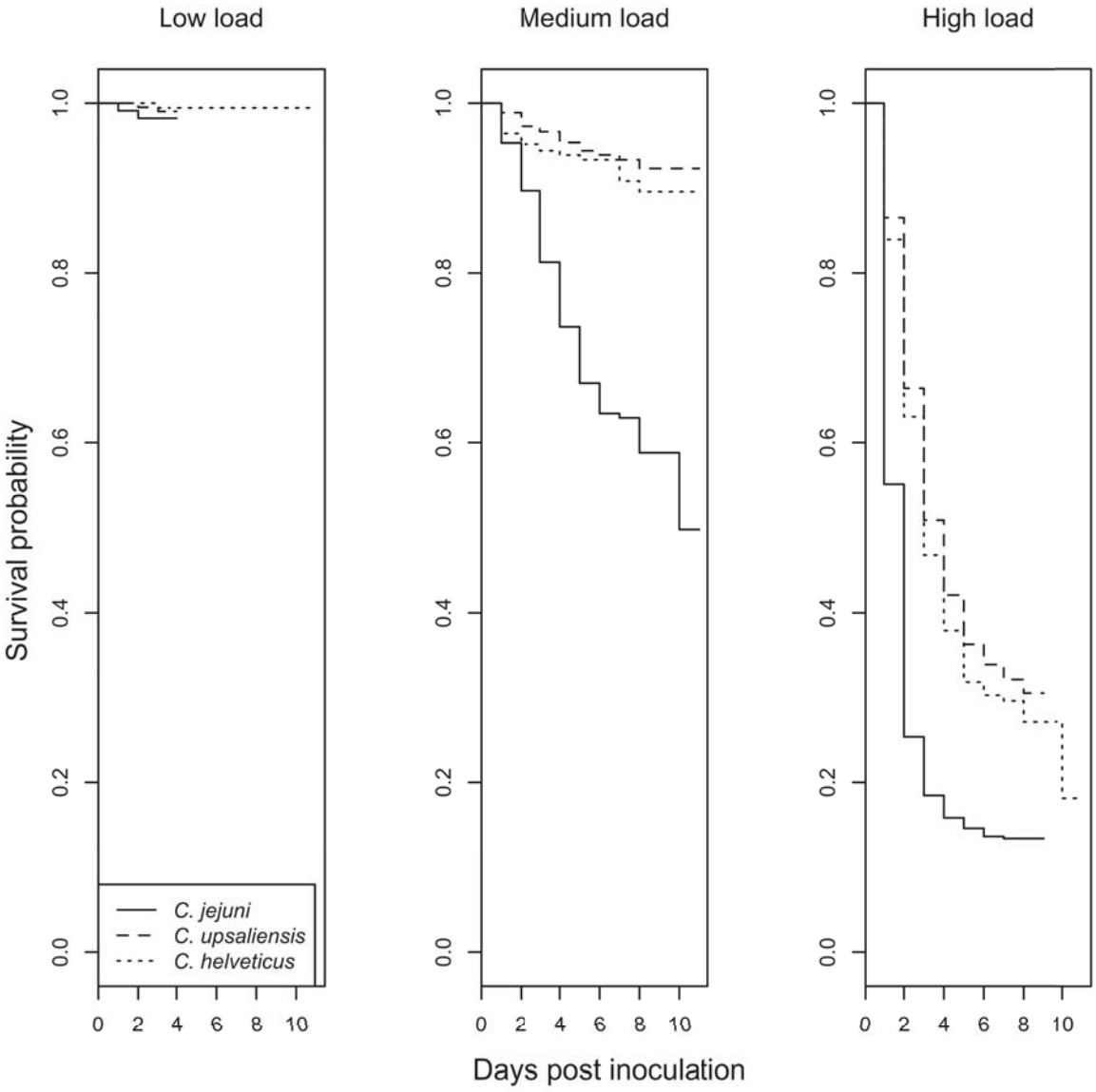

Fig. 6.1. Kaplan-Meier survival curves of larvae (n = 4,273) inoculated with three 100-fold dilutions of *Campylobacter* species.

There were 301 PBS-inoculated (27 events) and 355 (27 events) undisturbed larvae used in the optimal environment as controls in the main assay and their KM survival curves are presented in the “Optimal” subplot in Fig. 6.2. The comparison of the dose response shown in Fig. 6.1 with survival of control larvae in Fig. 6.2 showed no significant difference between any *Campylobacter* spp.-infected larvae with low bacterial loads and the control groups. The uninfected larvae and larvae infected with low *Campylobacter* doses never exhibited macroscopic melanisation, and reduced

responses - righting reflex and response to physical stimuli - were only observed in the latter. Therefore, the low dose study was discontinued in the remainder of the study. Signs of morbidity in infected larvae with medium and high doses were always present. With the medium bacterial load, only survival of *C. jejuni*-inoculated larvae was significantly different from survival of the control groups ($p < 0.001$), whereas with the high bacterial load all *Campylobacter* spp.-inoculated larvae had significantly different survival from the control groups ($p < 0.001$). The survival of PBS-inoculated and undisturbed larvae was not significantly different from each other (Fig. 6.2).

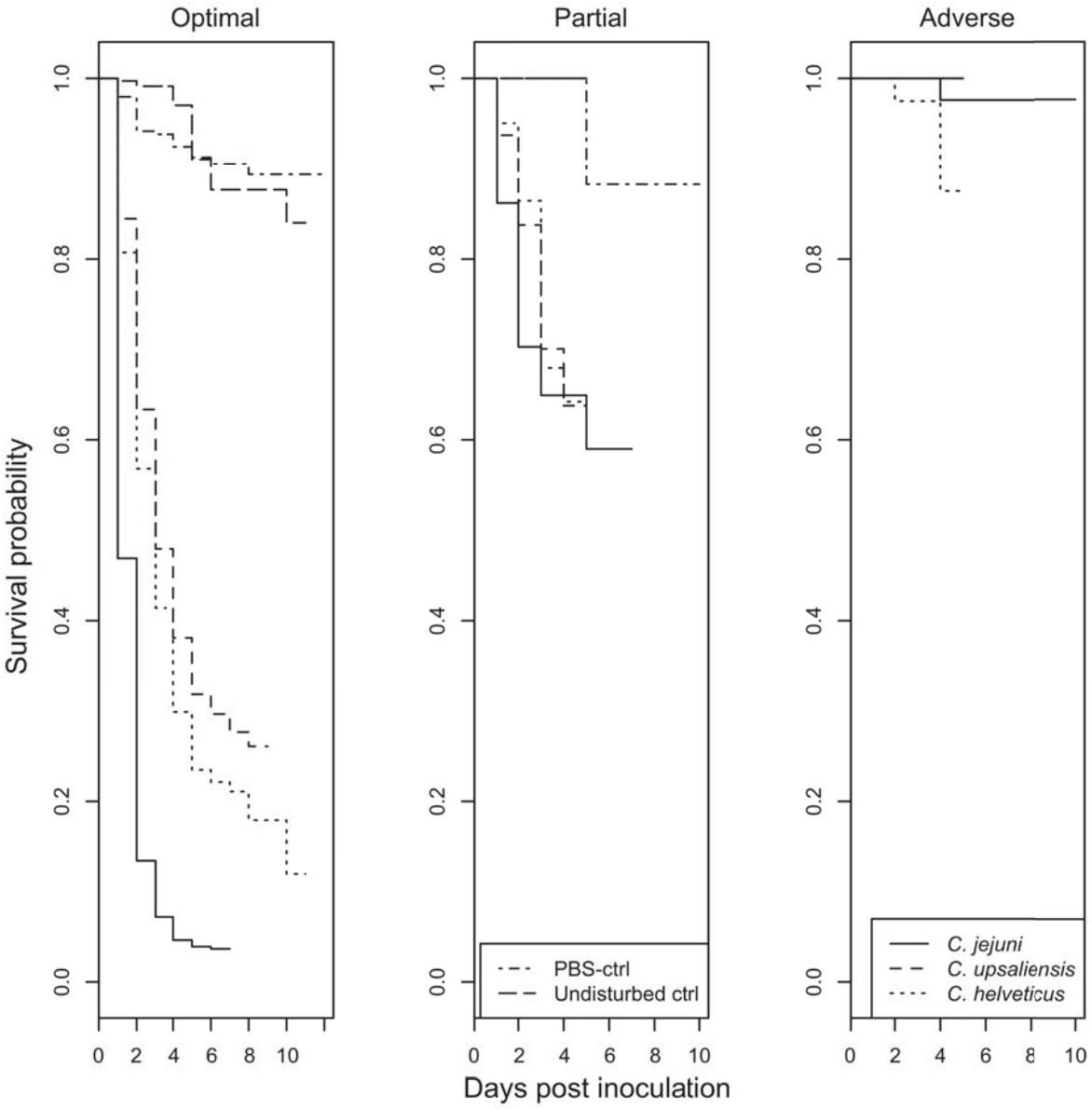

Fig. 6.2. Kaplan-Meier survival curves of larvae inoculated with *Campylobacter* spp. in different environmental conditions according to *in vitro* growth requirements.

The bacterial doses used had counts and estimates of viable bacteria CFU in the inocula obtained from spread plating. The linear functional form of bacterial dose was satisfied by evaluations of the Cox proportional hazard (CoxPH) model assumptions. The covariates exhibited mild violations of the assumption of proportionality of hazard ratios over time, mostly as a decline in low and medium loads and an increase in high loads after the fourth to fifth day post-inoculation, which corresponds to variation of the magnitude of effect between species and plateauing of survival curves in the medium and high loads (Fig. 6.1). The final CoxPH mixed effects regression model for the main assay included the species and dose as independent fixed effects. The larvae batch shipment and a nested structure of strain, bacterial load, and biological and technical replicates were significant random effects in the final model. The model showed significant variation between species with a hazard rate (the rate of death) for *C. upsaliensis* and *C. helveticus* of 21% (95% CI 14 – 30%, $p < 0.001$) and 34% (95% CI 22 – 52%, $p < 0.001$) respectively, of the hazard rate of *C. jejuni*. The hazard rate of *C. upsaliensis* was 61% (95% CI 39 – 95%, $p = 0.03$) of the hazard rate of *C. helveticus*. The increase of one log unit in the bacterial dose increased the hazard rate by 4.6 times (95% CI 4.1 – 5.2, $p < 0.001$) as an independent fixed effect. Of note, a CoxPH model with only fixed effects was significantly better with inclusion of the interaction term between species and dose, but with the addition of random effects the interaction term did not significantly improve the model ($p < 0.001$). With regard to the random effects, the final model showed the variation in hazard rate of larval survival for 95% of batch shipments in the range of 61 – 165% of the average. For the nested structure of strains and replicates, the variation in hazard rate for 95% of bacterial strains was in the range of 81 – 141% of the average, for biological replicates 77 – 135% that extended to 41 – 300% of the average between bacterial loads used, and for technical replicates 95 – 107% of the average. The technical replicates did not significantly contribute to the final model but were kept in order to quantify the effect of a commonly employed experimental procedure. The mixed effects CoxPH model for control larvae groups showed the hazard rate for survival of undisturbed larvae at 83% of the PBS-inoculated larvae hazard rate but with no significant difference between the two. The random effect of the batch shipments on the survival of control larvae groups ranged from 25 – 319% of the average, and for technical replicates from 76 – 146% of the average.

For the subsets of data, the CoxPH mixed model showed that STs of the CC-21 complex do not have a significantly different hazard rate from CC-61, CC-42, CC-403, CC-3961, and CC-2381, but do have a significantly different hazard rate from CC-45 (p = 0.041), CC-48 (p = 0.036) and CC-1332 (p = 0.018) estimated at 187% (95% CI 103 – 344%), 184% (95% CI 104 – 326%) and 452% (95% CI 129 – 1,583%) of the CC-21 hazard rate, respectively. At the ST level, the sign (direction of) of the effect and significant difference of ST-21 hazard rate was maintained in relation to ST-45 and ST-696 (the only member of CC-1332 tested) isolates and was significantly different to ST-42 with an inverse effect showing a hazard rate of 40% (95% CI 18 – 88%) of the hazard rate of ST-21; all other STs of the respective CCs were not significantly different to ST-21. All STs of CC-48 maintained the sign of effect but lost any significant difference in hazard rate that seems to have been mostly driven by ST-474 (p = 0.066). In addition, the hazard rate of animal isolates of *C. jejuni* was 179% (95% CI 107 – 300%) of the hazard rate of human isolates of *C. jejuni,* while the dose effect with animal isolates was at 76% (95% CI 58 – 98%) of the hazard rate of the dose with human isolates. No significant differences were observed in the hazard rates of *C. jejuni* isolates when STs were categorised as "generalists" vs. "specialists" and in "frequently" vs. "STs rarely or never observed in human clinical cases". There were no significant differences between the hazard rates of human, pet and zoo animal sources of *C. upsaliensis*. There were insufficient different sources or other features for *C. helveticus* isolate analysis.

Survival of larvae in different environmental conditions was evaluated with 302 larvae in partial conditions (94 *C. jejuni*, 80 *C. upsaliensis*, 81 *C. helveticus*, 27 PBS-inoculated and 20 undisturbed larvae) and 142 (42 *C. jejuni*, 40 each of *C. upsaliensis* and *C. helveticus*, and 10 each of PBS-ctrl and undisturbed larvae) larvae in adverse environmental conditions. There were 301 PBS-ctrl and 355 undisturbed control larvae used in the optimal environmental conditions, and as only the high doses of *Campylobacter* spp. were used in the partial and adverse environments, a subset of the main assay matching the high dose was included for comparison of the KM survival curves presented in Fig. 6.2. The reduced survival of infected larvae in optimal vs. partial, and partial vs. adverse environments was

significant for all *Campylobacter* spp. ($p < 0.001$). Survivals of larvae in the uninfected control groups was not significantly different between the different environmental conditions. In the partial environment the survival of all infected larvae was significantly lower than that of the uninfected control groups ($p < 0.01$) but the differences between *Campylobacter* spp.-infected larvae were not significant. In the partial environment a significantly lower survival rate was associated with the maintenance of the 37°C incubation temperature rather than with the maintenance of the H_2-enriched microaerobic (MA) atmosphere for infected larvae (data not shown, $p < 0.001$). This was not seen for the uninfected control larvae. In the adverse environment a significant difference was observed between survival of *C. upsaliensis*- and *C. helveticus*-infected larvae ($p = 0.02$), but the survival of the uninfected control groups was not significantly different from the survival of any *Campylobacter* spp.-infected larvae. However, the infected larvae exhibited melanisation of their cuticle and reduced responses to stimuli, unlike the uninfected control groups. Melanisation of infected larvae was noted from day one and throughout the observation period in optimal and partial conditions with all *Campylobacter* spp., but in adverse conditions larvae inoculated with *C. jejuni* reverted to normal colour on day two of observation. Of note, progression of larvae to the next biological cycle, prepupa, was occasionally observed only in uninfected control groups in normal atmosphere irrespective of the incubation temperature.

There were six assays with 590 larvae inoculated using whole-cell lysates of inactivated bacteria, the cellular soluble and insoluble lysate components, and their secretory products. The KM survival curves for *Campylobacter* spp. in these assays are presented in Fig. 6.3. Inoculation of larvae with cold-inactivated whole-cell lysates showed significant differences only between the survival of larvae inoculated with *C. helveticus* and those of both *C. jejuni* and *C. upsaliensis* lysate inoculated larvae ($p < 0.01$). The removal of cold-inactivated insoluble material from the whole-cell lysate significantly improved the survival of larvae inoculated with *C. jejuni* ($p <0.001$) and *C. helveticus* ($p < 0.001$) but not that of larvae inoculated with *C. upsaliensis* lysates. In addition, for the cold-inactivated soluble fraction assay, the survival of larvae inoculated with *C. jejuni* lysates did not differ from the survival of control groups which was seen for both *C. upsaliensis* ($p < 0.01$) and *C. helveticus* ($p < 0.01$)

lysates. There were no significant differences between the survival of larvae inoculated with *Campylobacter* spp. lysates for all three heat-inactivated lysate assays and the secretory products assays. The removal of heat-inactivated insoluble material from the whole-cell lysate significantly improved larvae survival for each of the *Campylobacter* spp. ($p < 0.001$) and thus, the difference in survival from that of the uninfected control groups was not significantly different anymore. Also, survival of larvae inoculated with *Campylobacter* spp.-secreted products was not different from survival of the Mueller-Hinton broth-inoculated and undisturbed larvae. All inoculated larvae exhibited melanisation from day one of observation but it was observed that the intensity of melanisation declined over time. The larvae inoculated with either cold- or heat-inactivated soluble cellular material also showed signs of morbidity with low mortality, similar to the observations in the adverse environment assays above, whereas the larvae inoculated with bacterial secretory products showed no signs of morbidity at all.

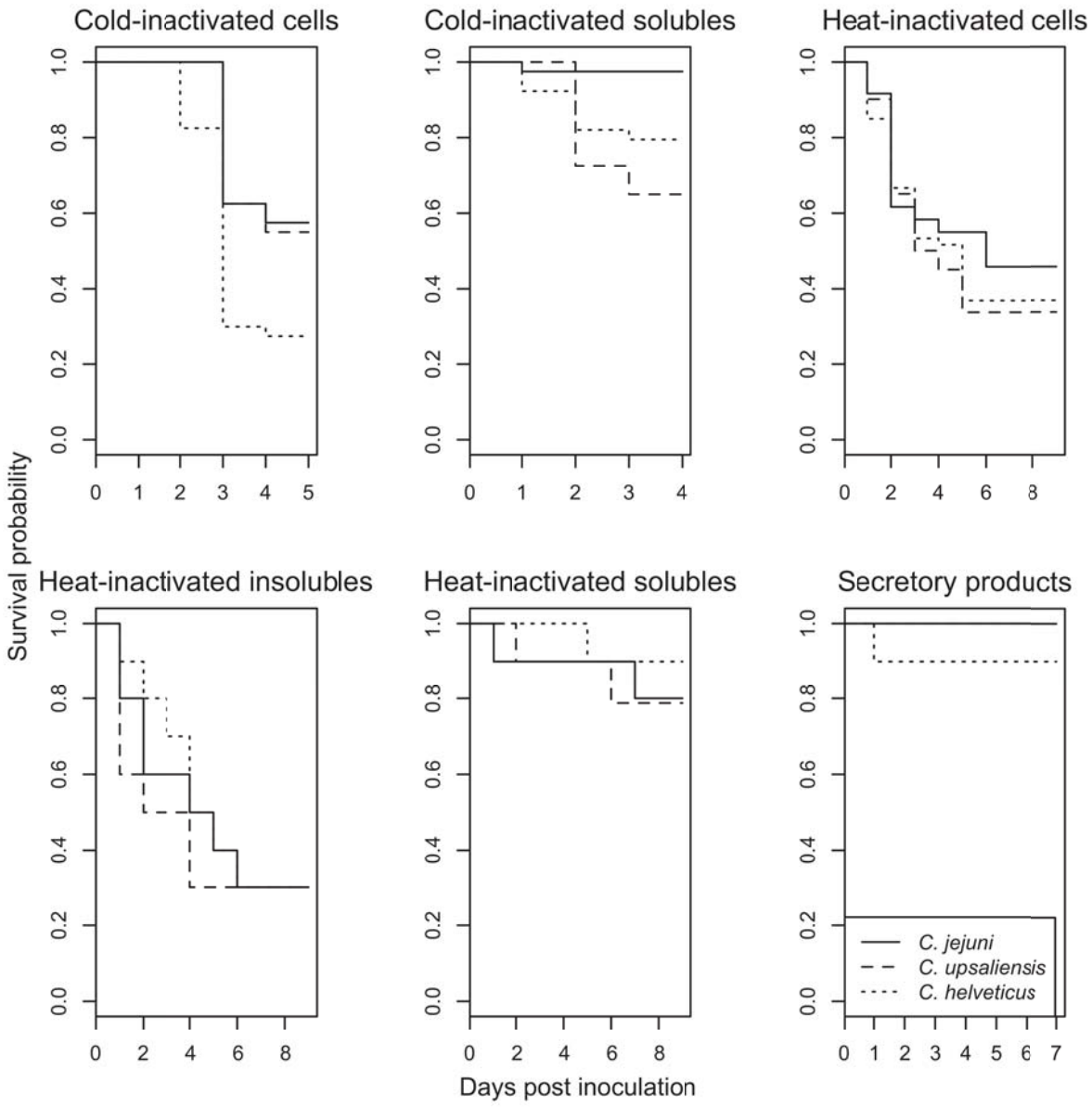

Fig. 6.3. Kaplan-Meier survival curves of larvae inoculated with temperature-inactivated whole-cells and cellular components, and secreted products of *Campylobacter* species.

Microscopic evaluation was performed on two to four larvae pooled from the same experimental run from 11 experimental runs of *C. jejuni*, 10 of *C. upsaliensis*, 12 of *C. helveticus*, three of PBS-inoculated and three undisturbed control larvae. There was severe loss of tissue architecture in most longitudinal sections and so only transverse sections were scored and evaluated. There were on average 4.4 sections (2.0

standard deviation) per experimental run; all were evaluated and scored. The gut tissues in both infected and uninfected larvae could not be evaluated due to the heavy autolysis. The summary of histopathological scores in infected and uninfected larvae is presented in Table 6.1.

Table 6.1. Distribution of histopathology scores* in larvae infected with *Campylobacter* spp. and the uninfected control larvae groups.

Feature	Score	*C. jejuni* (n = 11)	*C. upsaliensis* (n = 10)	*C. helveticus* (n = 12)	PBS-inoculated (n = 3)	Undisturbed larvae (n = 3)
Fat body	1	4	0	1	0	0
	2	3	7	3	0	1
	3	4	3	8	3	2
Haemolymph	1	3	5	8	1	0
	2	7	5	4	2	3
	3	1	0	0	0	0
Haemocytes	1	2	5	3	0	0
	2	6	3	7	3	3
	3	3	2	2	0	0
Nodules	0	1	3	3	3	3
	1	6	3	5	0	0
	2	4	4	4	0	0
Pigment	0	2	2	3	3	3
	1	5	2	0	0	0
	2	3	2	5	0	0
	3	1	4	4	0	0
Bacteria	0	2	3	2	2	1
	1	6	6	9	1	2
	2	2	1	1	0	0

* All evaluations were conducted blinded to treatment group. Haemocytes were scored as 1 (low number of individual haemocytes), 2 (low to moderate number of clusters) and 3 (numerous clusters or sheets of haemocytes), and haemolymph and fat body as 1 (fat body taking ≤ 25% of cross sections), 2 (~ 50% of sections) and 3 (≥ 75% of sections). Pigment was scored as 0 (no pigmentation), 1 (faint pigmentation at 4x magnification), 2 (moderate pigmentation visible at 1.25x magnification) and 3 (obvious dark pigmentation at 1.25x magnification). Nodules as aggregations of pigmented haemocytes (520) were scored as 0 (none observed), 1 (few per section) and 2 (over 10 per section). Bacteria were scored as 0 (none observed), 1 (filling of ~ 25% of gut lumen) and 2 (filling over 25% of gut lumen) in gut tissue and as presence/absence in haemolymph.

Overall, the scores of fat body and haemolymph between infected and uninfected larvae were similar but the two features showed large variations between larvae from the same experimental runs, thus were not statistically analysed. The scores of haemocytes were not significantly different between infected and uninfected larvae ($p = 0.7$) and between *Campylobacter* spp. within infected larvae ($p = 0.4$). However, only the haemocytes of infected larvae exhibited pigmentation of the cytoplasm. Apart from the cytoplasm of haemocytes of infected larvae, pigment deposition was also observed in tissues (fat body, muscle, epithelial cells, haemolymph and gut lumen) and nodules. The overall pigment scores (Table 6.1) were significantly higher in infected than uninfected larvae ($p = 0.002$) but there was no difference between *Campylobacter* spp. within the infected group ($p = 0.4$). The pigment scores in haemocytes, nodules and tissues between *Campylobacter* spp. within infected larvae were not significantly different (all three pigment locations with $p > 0.3$). Nodules were only observed in infected larvae, which was significantly different from uninfected larvae ($p = 0.001$). Nodules were mostly observed beneath the cuticle and around the gut and there was no difference in scores between *Campylobacter* spp. within infected larvae ($p = 0.8$). Coccoid bacteria were almost always observed in infected larvae but filamentous rods and cocci were also noted, though infrequently, in both infected (two each of *C. jejuni* and *C. upsaliensis*) and uninfected (one PBS control and two undisturbed controls) larvae. Scores of abundance of bacteria were significantly higher in infected than in uninfected larvae ($p < 0.001$) but not between *Campylobacter* spp. within the infected larvae ($p = 0.7$). Within infected larvae, bacterial scores had a significant ($p < 0.01$) positive association with nodule scores (Spearman's rank correlation *rho* of 0.45) and with pigment scores (Spearman's rank correlation *rho* of 0.41, $p = 0.01$). Similarly, bacterial scores had a significant ($p = 0.04$) positive association with bacterial loads used in the inocula of infected larvae with the Spearman's rank correlation *rho* of 0.36.

The isolation of *Campylobacter* spp. from the haemolymph and haemocytes of larvae was attempted with 40 each of *C. jejuni* and *C. upsaliensis* and 30 *C. helveticus* inoculated larvae on days one, two, three, five and eight post-inoculation. The isolation was successful with all of the *Campylobacter* spp. from the haemolymph and from the haemocytes only with *C. jejuni*. The isolation of *C. upsaliensis* and *C.*

helveticus from the haemolymph of larvae was only successful in the first two days post-inoculation while *C. jejuni* was successfully isolated, including from the haemocytes, up to 8 days post-inoculation. *Campylobacter*-like colonies were not observed in cultures of haemolymph from uninfected control groups (10 PBS-ctrl and 10 undisturbed larvae) on day one, two, three and five of observation.

6.4. Discussion

The results show a clear distinction between the survival of larvae infected with the established pathogen *C. jejuni* and the emerging pathogens *C. upsaliensis* and *C. helveticus*. In addition, several limiting features in the use of larvae for comparison of survival rates were observed and suggest some possible explanations of the observed differences. *C. upsaliensis* and *C. helveticus* showed a significantly reduced ability to kill larvae compared to *C. jejuni,* but histopathological findings could not distinguish between the larvae infected with different *Campylobacter* species. The histopathological findings of both infected and uninfected control groups correlate well with results of the study investigating *C. jejuni* infection of *Galleria mellonella* larvae. (452) In both studies loss of integrity of the gut wall, activated and apoptotic haemocytes, pigment and nodule formations (observed macroscopically as melanisation) throughout the tissues and haemocoel were recorded in the infected larvae, as in the present study for all *Campylobacter* spp., but not in the control larvae groups. The consistent histological changes in larvae infected with *C. jejuni* and clear differentiation from the changes in uninfected controls between the two studies, indicate the applicability of larvae as an infection model for *C. upsaliensis* and *C. helveticus* as well. The coccoid bacteria observed microscopically were presumed to be *Campylobacter* cells. In support of this presumption are the successful isolation of *Campylobacter* spp. from larval tissues and the significant positive correlation of the bacterial scores with pigment and nodules scores and the bacterial loads in the inocula used for infection. The poorer success in evaluation of longitudinal sections compared to transverse sections in this study was also reported with use of *Candida albicans* in *Galleria* larvae (521). That study reported the injection of larvae with formalin as the preferred method of fixation to preserve tissue

architecture, which could explain the poor success in the evaluation of gut lesions in the present study with the submersion of larvae in formalin.

Inactivated *Campylobacter* cells and cellular materials in this study were also shown to induce signs of morbidity and could cause death of larvae with all three *Campylobacter* spp. with little difference between them (Fig. 6.3). The melanisation of larvae was evident, and is a sign of the activation of the immune system; this may also cause substantial damage to the host and lead to death as well. (522) These assays employed the same high doses and optimal environmental conditions as the viable bacteria assays (Fig. 6.1). Comparison of KM survival curves between these assays indicates the survival of larvae with viable *C. helveticus* and *C. upsaliensis* is mildly worse than with inactivated cell lysates (except for a three day delay in deaths in the cold-inactivated assay) but in larvae inoculated with live *C. jejuni* survival is much worse than in larvae inoculated with cell lysates. This pattern suggests that the metabolic activity of viable *C. upsaliensis* and *C. helveticus* cells contributes less to the death of larvae compared to their cell components than does the activity of *C. jejuni* cells. In light of the results of the culture of larvae that showed the disappearance of *C. upsaliensis* and *C. helveticus* from the haemolymph early in infection, and no successful isolation from haemocytes, a possible explanation is that the bacteria were dying. Whether the bacteria were being killed by the larval immune system response or were dying merely due to the unsuitable environment of the larval haemocoel cannot be determined from this study. Another possible explanation is that due to the adverse environment the bacteria entered a viable-non-culturable state, in which they remain metabolically active with preserved integrity of the cell wall but do not replicate (523) thus, the negative culture results. A study by Senior et al. (452) documented survival of *C. jejuni* in larval haemocytes using green fluorescent protein-tagged cells and showed that the pattern of survival is broadly similar between insect and mammalian macrophage cell lines. In addition, the numbers of intracellular *C. jejuni* cells were increased at 24 hours compared to 4 hours post-inoculation, which suggests *C. jejuni* cannot only survive but also replicate in larvae cells. This is in line with results of haemolymph and haemocytes cultures of *C. jejuni*-inoculated larvae, which demonstrated successful isolation up to 8 days post-inoculation in the present study.

Insect haemocytes recognise pathogens and phagocytise foreign material in a similar manner to mammalian neutrophils, with the killing of ingested microbes achieved in both cell types by the production of superoxide and by the release of enzymes in the process of degranulation. (524) While there are a variety of cellular and humoral immune mechanisms that may also play a role in larvae (451) the survival of *C. jejuni* in haemocytes is likely to be due to possession of genes involved with oxidative and aerobic stresses. Studies have shown the MarR-like transcriptional regulator genes, *rrpA* and *rrpB*, are involved in the regulation of the catalase (*KatA*), the alkyl hydroperoxide reductase (*AhpC*) and the superoxide dismutase (*SodB*) genes. These genes are involved in the tolerance of oxidative and aerobic stresses and mutants of these genes and their regulators were reported to be associated with significantly increased survival of larvae compared to the wild-type isolate, a pattern not seen for mutants of the peroxide-sensing regulator (*PerR*), and *rrpA* and *rrpB* double mutants. (525, 526) These genes have not been described in *C. upsaliensis* and *C. helveticus*, and phenotypically they are catalase weakly positive (101) and catalase negative, (516) respectively. However, the higher survival of *C. upsaliensis*-infected than of *C. helveticus*-infected larvae in the present study suggests more than just catalase activity is needed to explain the phenomenon. Reports of intracellular survival of *C. jejuni* in mammalian macrophage monocyte cell lines are somewhat conflicting as some reported no ability to survive (181) but more studies reported intracellular survival. (527-529) More studies are needed to elucidate these issues but also, to the extent the larvae are a model of innate immunity, the role of the metabolic capacity and virulence of microbe compared to merely its cellular composition in disease development.

The assays with altered environmental conditions in this study showed improved survival for larvae infected with all *Campylobacter* spp. as the conditions become less favourable for *Campylobacter in vitro* growth requirements. As an intestinal bacterium, coping with various adverse environmental conditions is important for *Campylobacter* spp. transmission between hosts, and temperature and atmospheric changes were shown to significantly reduce survival of *Campylobacter* spp. (160) Therefore, the increased survival of larvae infected with *Campylobacter* spp.

incubated in decreasingly favourable conditions could be expected to be due to decreased numbers of viable *Campylobacter* spp. in the larvae. The larval cuticle acts as a physical barrier (530) and although the extent of change in gaseous levels in larvae due to the incubating atmosphere was not investigated, the change in atmospheric conditions did obviate the differences in larvae survival between *C. jejuni* and the other two *Campylobacter* spp. (Fig. 6.2). *C. jejuni* may grow in certain aerobic conditions (531) but the aerotolerance can vary significantly between strains. (532) Whether the difference of *C. upsaliensis* and *C. helveticus* from *C. jejuni* seen in optimal conditions was lost in partial conditions due to aerosensitive strains cannot be determined. Temperature-dependent mortality of larvae with all *Campylobacter* spp. was more pronounced than atmosphere-dependent mortality in this study. *C. jejuni* does not grow at temperatures below 31°C but may survive in temperatures as low as 4°C, although the activity of all vital cellular processes is significantly reduced. (77) The infected larvae at room temperatures did show signs of morbidity that were not seen in the control groups, but survival of larvae was not different between the infected and control groups.

A peculiar finding is the clearing of melanisation from day two of observation in adverse environments observed only with *C. jejuni.* The results of the inactivated bacterial assays indicated that melanisation occurred in response to antigenic stimulation but subsided over time. The melanisation occurs when the larval immune system recognises a pathogen and activates the phenoloxidase cascade leading to the melanisation of haemolymph around the pathogen. (451) Thus, if *C. jejuni* were dying rather than evading the larval immune system, it would be expected for cellular components to become accessible to the larvae immune system and melanisation to appear as observed with *C. upsaliensis* and *C. helveticus*. The few deaths observed in adverse conditions could be associated with a gradual decline in viable bacteria over time whereas with inactivated bacteria the immediate antigen loading may have overwhelmed the immune system and the resulting damage leads to more deaths of larvae. The observation of melanisation only for one day would suggest that some portion of the *C. jejuni* had died but that they were quickly cleared compared to the other two *Campylobacter* species. This is another indication of the ability of *C. jejuni* to survive in larvae compared to *C. upsaliensis* and *C. helveticus*. Alternatively, the

antigens of *C. upsaliensis* and *C. helveticus* may be more able to provoke the larval immune system than that of *C. jejuni* or that the death rate in adverse conditions is faster with the former two species. Cultures of haemolymph and haemocytes would be beneficial in these assays but were not performed.

A review of the literature identified this study as the first to report the use of different *Campylobacter* spp. and with regard to comparison of *C. jejuni* results in this study with the reported literature several limiting factors need to be addressed. All seven studies identified in the literature review used the right foreleg as route of injection with Hamilton syringes, reported no details of incubating atmospheric conditions and means of assessment of death of larvae, all studies used broth-grown isolates and evaluated the survival of larvae at 24 hours post-inoculation. (452, 519, 525, 526, 533-535) The exceptions were the report of survival at 48 hours by Humphrey et al. (534) and Elmi et al. (533) noted no further deaths at 48 and 72 hours compared to 24 hours post-inoculation. More importantly, all the above studies evaluated the death of larvae by macroscopic colour changes only as reported by Champion et al. (519) whereas in the present study responses to stimuli were also included as reported by Cotter et al. (536) Additionally, all of the above studies focused on the evaluation of mutants vs. wild-type isolates for the investigation of genes affecting the survival of larvae and used one (525, 526, 533) or five or less (519, 534, 535) isolates for comparisons, except for the study by Senior et al. (452) that evaluated variation between STs with 67 strains. Therefore, many results are not directly comparable between the studies. During preliminary testing in the present study, in our experience inoculation via the last left pro-leg, as described by Kavanagh et al. (451) was easier than via the right foreleg. Both sites serve as access to haemocoel but whether the site of injection causes a different effect needs further investigation. The report of no additional deaths at 48 and 72 hours compared to 24 hours post-inoculation by Elmi et al. (533) could be attributed to the use of only one strain and its mutants. With regard to temperature-dependent survival of larvae only the study by Champion et al. (519) reported complete killing of larvae with ~10^6 CFU of the *C. jejuni* 11168H isolate at both 25°C and 37°C incubation temperature at 24 hours post-inoculation. This is in stark contrast with results of this study, as with ~10^8 CFU only a few deaths were observed with all three *Campylobacter* species in partial

conditions with a H_2-MA atmosphere and room temperature (unlike partial conditions with 37°C and an aerobic atmosphere), and in the adverse environment with both room temperature and an aerobic atmosphere. The study by Champion et al. (519) was also the only one to report a dose-dependent survival of larvae of 30%, 10% and 0% for doses of ~10^2, 10^4 and 10^6 CFU of the *C. jejuni* isolate 11168H, respectively. This is also in stark contrast from the dose-response survival seen in this study, especially considering that this study used 100-fold higher doses. All other studies of *C. jejuni* in larvae used only a dose of ~10^6 CFU (using turbidity adjustments and no quantification with cultures) of the *C. jejuni* isolate 11168H as a standard to which other *C. jejuni* isolates or mutants are compared. (452, 525, 526, 533-535) In these studies the survival of larvae infected with 11168H varied from 10% to 70%; larger than with the dose response according to Champion et al. (519) Given the results of this study, the variation in dose response and dose-matched survival in reported studies are attributable to the differences in the supplier's batches of larvae, and variations both between strains and within their biological replicates.

As this was a large study, the evaluation of the random effects on survival of larvae was possible. The little variation estimated for the technical replicates and their insignificant contribution to the regression model shows consistent, reproducible results are obtained within the experimental runs. This suggests that internal validity for an observed difference is achieved in controlled experiments. On the other hand, the significant variation in survival of larvae between batches suggests caution needs to be used in comparisons and extrapolation of survival estimates of different studies. *Galleria mellonella* is a relatively new invertebrate model compared to the fruit fly *Drosophila melanogaster*, or the nematode *Caenorhabditis elegans*, which have stock centres and community databases maintained by joint international funding approaches, such as Flybase and WormBase respectively. (537) The lack of sources of genetically defined larvae strains and reference populations, different breeding and maintenance practices between suppliers, and maintenance and non-standardised experimental procedures between research laboratories have been frequently raised as limitations of experimental comparability. (518, 537, 538) Considering that in this study only one supplier was used, the significant batch effect could be expected to be

larger for differences between suppliers than within suppliers, which is a problem when comparing between studies.

The effect of biological replicates both with and without accounting for the bacterial load is intriguing, as three studies of *C. jejuni* employed biological triplicates (each with three technical replicates) but none reported variation thereof, and from the graphical display of the combined results of the mean larval survival does not seem to have varied more than 15 – 20% within each study. (525, 526, 533) The reason for the variation between biological replicates could be explained by unmeasured variables that played a role during the culturing of the bacteria for preparing inocula. *C. jejuni* usually produced sufficient growth on blood agar with one day of incubation, whereas *C. upsaliensis* and *C. helveticus* usually took an additional one and two days, respectively, to give a similar amount of growth. Whether these observations reflect different growth phases of isolates at the time of preparation of inocula is unknown. No study of *Campylobacter* spp. using larvae assessed the influence of the growth phase on the survival rates. With other microorganisms, the inoculum growth-phase dependent survival of larvae has been reported for group A streptococci (539) and *Legionella pneumophila*. (540) Apart from the differences in growth phase, the culture conditions and protocols may also affect the pathogenic properties of the strains. The study by Champion et al. (519) reported a higher mortality of larvae with broth-grown rather than plate-grown *C. jejuni* 11168H. Similarly, the influence of culture and storage conditions in the laboratory on the virulence properties of *C. jejuni* 11168H compared to the original clinical isolate has been reported. (154) In this study, Mueller-Hinton broth was not used for growing of *Campylobacter* spp. due to the very poor growth of *C. upsaliensis* and *C. helveticus* in this medium. Other sources of variation between biological replicates may be related to the variable expression of genes. The genome of *C. jejuni* contains many hypervariable regions characterised by homopolymeric DNA tracts. (541) These regions function as contingency loci that frequently undergo slipped-strand mispairing during replication, resulting in frame-shifts and phase variation. This phenomenon induces genetic heterogeneity resulting in a spectrum of phenotypes thought to be important in the host adaptation, enhanced virulence, and immune evasion of *C. jejuni*. (542, 543) *C. upsaliensis* was reported to contain nearly three times as many variable

homopolymeric repeats than *C. jejuni,* encoding a combination of hypothetical, cell envelope, and virulence-associated genes, (21, 544) but no data exists for *C. helveticus*. Whether the population of *Campylobacter* in an inoculum is, although originally derived from a single colony, diverse due to mutations induced by the homopolymeric tracts and phase variation and thus, responsible for variation in biological replicates would be interesting for further studies.

However, the variation between strains of species is to be expected to a certain extent for comparative studies given the species/isolate (or its mutant) in question is able to kill larvae. Several studies of *C. jejuni* reported strain-related variation in the survival of larvae. Excluding the data for mutant isolates, the ranges in survival of larvae were reported at 10 – 20%, (535) 25 – 43%, (519) 3 – 36% (534) in studies comparing less than five strains and 50 – 100% in a study comparing 67 strains. (452) These studies were matched by the dose used (which corresponds to medium loads in this study), and all evaluated the survival at 24 hours except for Humphrey et al. (534) who evaluated survival at 48 hours post-inoculation. The overall survival of larvae in this study is much higher for the corresponding time of observation and dose, apart from the study by Senior et al. (452) but the variation due to strains as well as the magnitude of the random effect of strains on larval survival in this study corresponds in relative terms with the reported data. With regard to strain variation of *C. jejuni* according to ST/CC designation the results of this study are discordant with the reported data. The study by Senior et al. (452) reported survival of larvae inoculated with ST-21 to be significantly higher than that of larvae inoculated with ST-257 and, though higher, not significantly different from survival of ST-403, ST-45 and ST-48 inoculated larvae, the latter two STs were significantly different from the results seen with ST-21 inoculated larvae in this study. One additional study, using three isolates reported larvae inoculated with ST-21 to have a higher survival than those with ST-257 and ST-137 (CC-45) isolates (534). The differences between the studies could be due to the analytical methods used, as CoxPH models evaluate the survival curve over time and the two reported studies (452, 534) used one point estimates. The lesser survival of CC-48 inoculated larvae than those inoculated with CC-21 in this study is likely to be due to the use of ST-474 and ST-3609 in addition to ST-48 as members of CC-48 whereas the study by Senior et al. (452) only used ST-

48 which was also not significantly different from ST-21 in this study. *C. jejuni* ST-474 is rarely reported outside of New Zealand where it was described as the most frequent ST occurring in human clinical cases and was poultry-associated. (260, 312) The relationship between ST/CC designations and virulence in larvae and its (cor)relation to virulence in humans remains to be further studied. For instance, CC-21 was reported to survive better in both heat and chill stress models than CC-45, which in turn survived better in oxidative, and freeze stress models than CC-21. (545) In contrast, CC-21 dominated CC-45 in the number of aerotolerant and hyper-aerotolerant strains. (532) The discordant results of larval survival could be due to variations between STs within the CCs, as confounding within CC was observed in this study within CC-42. Also, this study showed different hazard rates between animal and human isolates of *C. jejuni* suggesting another possible source of confounding the estimates of the *C. jejuni* isolates. For the larval model, inconsistency on the CC level may be due to variations in lipooligosaccharide classes reported between and within CC, (196) as deletion of the lipooligosaccharide biosynthesis region was reported to attenuate survival of larvae compared to the wild type strain. (519) Further work is needed to evaluate the importance of source, ST/CC and other genotypic, phenotypic or epidemiological data of isolates in the *Galleria mellonella* model.

The present study showed the critical importance of the evaluation of dose-dependency in the survival of larvae when used as an infection model. For the use of the survival of larvae as an end-point for pathogenic features of an isolate being studied, the dose requires sufficiently altered survival of larvae to allow the relative comparison with control larvae groups or some other isolate. (538) If one considers only one dose to have been evaluated in this study, the inference about the survival between infected and uninfected larvae as well as between *Campylobacter* spp. within infected larvae would have been awry (Fig. 6.1). This is further supported by the CoxPH regression modelling showing no significant effect for the interaction of dose and species, which would mean the effect of dose varies between each (or some) species. Therefore, the different survival rates between *Campylobacter* spp. is independent of the dose but the difference may not be observable below a certain dose (Fig. 6.1). The dose-dependant survival of larvae has been documented for

many pathogens used with *Galleria mellonella* larvae and differences in the 50% lethal dose can be as extreme as several thousand-fold within species and within bacterial genera. (518) The dose-response is also useful for determination of the levels that enable differentiation of the survival of control larvae groups and infected larvae. All of the above studies of *C. jejuni* in larvae with observations at 24 hours had 100% survival of control groups and survival was over 98% at 72 hours, the longest observation reported. (534) This is in line with this study as the ~10% mortality of control larvae groups was reached around the fifth day of observation and remained constant until at least 10 days (Fig. 6.2). This relatively low mortality rate of the control groups in this study shows that the observation period can be prolonged and that more information about infection dynamics can be revealed, since the manipulation of larvae and environmental conditions in this study seem to not be a major cause of deaths. The CoxPH models showed violations of proportionality of the hazard rates. The violations were not severe enough to invalidate the model, as no inverse relationships occurred, as shown by no crossing of KM survival curves (Fig. 6.1). However, these violations caution us that the magnitude of the estimates of species differences is not constant over the observation period. This finding raises questions about the choice of observation time for comparisons. For instance, what would it mean if in the reported studies of *C. jejuni* a certain mutant was significantly different from the wild type on day one but not day five? The mutant adapted and caught up and what should the inference be on the gene knocked out/inserted? This is, indeed, speculative but the possibility should be considered.

This study confirmed the ability of both *Campylobacter* spp. cells and cellular components to cause morbidity and mortality of *Galleria mellonella* larvae. A study of the *C. jejuni* 11168H isolate reported no melanisation and no death in larvae at 24 hours post-inoculation with heat-killed cells, (519) this difference could be related to the dose (100-fold difference) and the strain used as previously discussed for other assays. The different KM survival curves between methods for inactivation of bacteria indicates that both heat- and cold-labile compounds are present. Similarly, a study of *C. jejuni* also reported the heat-inactivation of cellular compounds to affect larvae survival. The outer membrane vesicles that are used by bacteria for the delivery of virulence factors into host cells were shown to induce a dose-dependent

mortality in larvae, but upon heat-treatment, irrespective of the dose, all larvae survived. (533) In that study, the exact quantification of outer membrane vesicles was performed and although only high doses were used in this study, it is possible that certain subcellular components had not reached a sufficient concentration for death of larvae to occur. The results of assays with inactivated bacteria in this study indicate both heat- and cold-inactivated soluble components as well as secreted compounds provide little contribution to the mortality of larvae (Fig. 6.3). Considering the procedure for the preparation of these inocula, the concentration of soluble materials may have been equivalent between soluble and whole-cell lysates whereas the concentration of insoluble material may have been higher in insoluble than in whole-cell lysate due to the separation through centrifugation. Notwithstanding these limitations, the results of assays with inactivated bacteria indicate comparative studies of *Campylobacter* cellular components may be conducted, and would be beneficial to evaluate their role in infection dynamics. The larval immune system possesses several similarities in humoral and cellular defence mechanisms and pathogen recognition receptors for peptidoglycan structures, and lipopolysaccharides. (451) The recently published transcriptome of *Galleria mellonella* massively expanded the known spectrum of immunity and stress related genes of this model and will greatly facilitate forthcoming research (546).

6.5. Concluding remarks

The present study demonstrated that the larval model can be extended to *C. upsaliensis* and *C. helveticus* and that these bacteria induced significantly lower levels of mortality in larvae compared to *C. jejuni*. Histopathological changes were indistinguishable between the three *Campylobacter* spp., but cultures of infected larvae showed survival of only *C. jejuni* in larvae haemocytes for up to 8 days post-inoculation. During this time *C. jejuni* was also grown from larvae haemolymph whereas *C. upsaliensis* and *C. helveticus* could only be isolated in first two days post-inoculation. To the extent of larvae as a model of mammalian innate immunity this phenomenon suggests rapid clearance of *C. upsaliensis* and *C. helveticus*, which would support these species as less pathogenic than *C. jejuni* due to inefficient

evasion of the primary immune response during the course of disease development. The dose-dependent, temperature- and atmosphere-dependent survival of larvae was confirmed with all three *Campylobacter* spp., and further demonstrated the varied infection dynamics between the species. Mixed effects CoxPH regression modelling showed significant variation in the hazard rate between batches of larvae, strains of *Campylobacter*, and biological but not technical replicates as random effects, and confirmed species differences and bacterial dose as independent fixed effects on larvae survival. The random effects indicate the consistent results of technical replicates supporting internal validity of experimental runs but suggest limitations for comparisons between experiments, which is in line with the variability in reported studies on *C. jejuni* in larvae. The model diagnostics showed the variation in proportionality of hazards between species, which emphasises the need for caution in interpreting the observed magnitude of the effects with respect to the time of observation of larvae survival. Heat- and cold-inactivated whole cells and cellular components induced sickness and the death of larvae, with different survival of larvae between cellular components and *Campylobacter* species. Based on the larvae model, the data suggests that *C. upsaliensis* and *C. helveticus* are pathogenic species but less so than *C. jejuni*, which could be related to the inability of the former two species to survive within larvae haemocytes and evade immune responses in larvae haemolymph, and that the death of larvae was associated with damage due to antigen-induced immune response rather than viable bacteria.

6.6. Materials and methods

6.6.1. Strains and cultures

Isolates of *C. jejuni* were selected to include frequent, common and rarely occurring STs isolated from humans with clinical signs of gastroenteritis (n = 17), food (n = 4) and animal faeces (n = 12) from the ^{m}EpiLab collection (Table 6.2). The ^{m}EpiLab collection contains over 3,500 isolates from the Manawatu *Campylobacter* sentinel site surveillance, a source attribution study of campylobacteriosis using concurrent sampling of sick people, animal, food and the environment over ten years. (333) The

genotypes of *C. upsaliensis* and *C. helveticus* isolates were unknown and were pseudo-randomly selected from a previous study in dogs (10 *C. upsaliensis* and one *C. helveticus*) and cats (four *C. upsaliensis* and nine *C. helveticus*). Additionally, for *C. upsaliensis*, five isolates from the Auckland Zoo (two each from meerkats and golden lion tamarins and one from a cheetah), and two isolates from humans with clinical signs of gastroenteritis from New Zealand were included. For *C. helveticus*, two strains from cats from international culture collections were also used. The type strains of all three *Campylobacter* spp. were included. Overall, there were 34 strains of *C. jejuni* belonging to 15 different STs from nine different clonal complexes, 22 strains of *C. upsaliensis* and 13 strains of *C. helveticus*.

Table 6.2. Details of *Campylobacter* species isolates used in the study.

***C. jejuni* isolates**	**Source of isolation**	**MLST[a] group**
NCTC 11351	Cattle	ST-605 / CC-43
ACP17c	Dog	ST-474 / CC-48
ACP50a	Cat	ST-48 / CC-48
ACP57b	Dog	ST-45 / CC-45
ACP75a	Dog	ST-21 / CC-21
ACP90c	Dog	ST-474 / CC-48
ACP103c	Dog	ST-45 / CC-45
ACP103d	Dog	ST-45 / CC-45
ACP117c	Cat	ST-696 / CC-1332
ACP176d	Dog	ST-474 / CC-48
ACP191a	Dog	ST-61 / CC-61
BD12f2b	Dog	ST-3676 / CC-42
BD13d3a	Dog	ST-137 / CC-45
H450	Human	ST-61 / CC-61
H550	Human	ST-42 / CC-42
H1763	Human	ST-50 / CC-21
H1792	Human	ST-520 / CC-21
H1796	Human	ST-45 / CC-45
H1799	Human	ST-48 / CC-48
H1804	Human	ST-48 / CC-48
H1849	Human	ST-137 / CC-45
H1878	Human	ST-21 / CC-21
H1884	Human	ST-474 / CC-48
H1910	Human	ST-21 / CC-21
H1924	Human	ST-45 / CC-45
H1969	Human	ST-61 / CC-61
H1972	Human	ST-42 / CC-42
H1978	Human	ST-50 / CC-21
H1987	Human	ST-50 / CC-21
H22082	Human	ST-474 / CC-48
P110b	Poultry	ST-474 / CC-48
P525a	Poultry	ST-2381 / NA
P836a	Duck	ST-3609 / CC-48
P970a	Duck	ST-3961 / NA

***C. upsaliensis* isolates**	**Source of isolation**
ACP5b, ACP18a, ACP19b, ACP64a, ACP72b, ACP144b, ACP136a, ACP149b, BD16e4a, ESR3675	Dogs
ACP9b, ACP135c, ACP170a, ACP170b, ACP179b	Cat
LR128, LR129, LR130, LR131, LR132	Wildlife
ERL103233, ERL112092	Humans

***C. helveticus* isolates**	**Source of isolation**
ACP102a, ACP105a, ACP105b, ACP108a, ACP110b, ACP114b, ACP123b, ACP175a, ACP183a, CCUG30563, CCUG30682, CCUG30683	Cats
ACP141a	Dog

[a] Multi-locus sequence typing scheme: sequence type (ST) / clonal complex (CC)

For the preparation of inocula, all isolates were recovered from 15% (weight/volume) glycerol in nutrient broth vials stored at 80°C by plating to Columbia horse blood agar (Fort Richard, Auckland, New Zealand) incubated in a H_2-enriched microaerobic atmosphere (H_2-MA) (82% N_2, 10% CO_2, 5% H_2, 3% O_2) in variable atmosphere incubators (Don Whitley Scientific, West Yorkshire, UK) at 37°C. Species identification was previously confirmed using PCR assays targeting the *hipO* gene for *C. jejuni* (491) and the 16S rRNA gene for *C. upsaliensis* and *C. helveticus.* (490)

6.6.2. *Galleria mellonella* assays

Fifth instar larvae were obtained from Biosuppliers (Auckland, New Zealand) and kept in a mixture of wood chips and honeycomb at room temperature (approx. 20°C) and used at the latest within four days of arrival. Inocula of approximately 10-15 µL were injected using manual restraint of larvae into the haemocoel via the last left pro-leg using 33G insulin syringes (Becton Dickinson Co., Auckland, New Zealand) in sets of 10 larvae. Upon inoculation the larvae were kept in Petri dishes with a moist tissue provided but with no food. Larvae inoculated with phosphate-buffered saline pH 7.3 (Difco Laboratories Inc., Detroit, MI, USA) (PBS-inoculated or PBS-ctrl) and undisturbed larvae were used as controls for the experimental procedure and the conditions respectively, in all assays. All assays were performed in duplicate. The majority of assays were performed in H_2-MA at 37°C denoted as 'optimal environment' as these conditions are used for the isolation of *Campylobacter* species. Combinations with a normal atmosphere and room temperature were also evaluated with the environmental conditions categorised on their ability to support the growth of *Campylobacter* species. The use of room temperature and H_2-enriched MA or aerobic atmosphere and 37°C was considered a 'partial environment', whereas the use of room temperature and an aerobic environment was designated as an 'adverse environment'. All larvae were observed daily for up to 12 days with a minimum of five days of observation. The morbidity and mortality assessment consisted of evaluation of macroscopic colour changes (melanisation), righting reflex and responses to physical stimuli. The absence of the righting reflex and responses to stimuli were considered signs of death (mortality) whereas melanisation and either reduced

responses to stimuli or absence of righting reflex as signs of morbidity. A subset of larvae was used to culture *Campylobacter* spp. from haemolymph and haemocytes. Whole larvae were ethanol (70% vol/vol) washed, flamed and then the bottom ~2 – 3 mm of the body was aseptically removed to drain the haemocoel into a sterile microcentrifuge tube which was centrifuged at 200 *g* for 5 min. The haemolymph (supernatant) was transferred to a sterile microcentrifuge tube, plated on blood agar and cultured as above. The pelleted haemocytes were resuspended in 1 ml sterile MilliQ water (in-house prepared), and pipetted up and down ten times to lyse the cells and thereafter, cultured as the haemolymph. Cultures of the haemolymph and haemocytes were also performed by plating onto C.A.T. agar (Fort Richard, Auckland, New Zealand).

6.6.3. *Campylobacter* inocula

One-hundred and thirty-five inocula were prepared by suspending one to two whole agar plate's growth of *Campylobacter* spp. in PBS followed by one or two 100-fold dilutions in PBS. For quantification of bacterial concentrations, the inocula were further diluted in PBS and spiral plated (Don Whitley Scientific, West Yorkshire, UK) to blood agar and incubated as above. Bacterial colony counts were performed manually and/or using a plate reader (aCOLyte, Synbiosis, Cambridge, UK), depending on the growth, and expressed as number of colony forming units (CFU) per mL. Subsequently, 18 inocula were adjusted to 0.05 light transmittance at 590 nm (OD590) using a turbidometer (Biolog, Hayward, CA, USA) from which dilutions for the inoculation of larvae and the quantification of bacterial concentrations were performed as before. These estimates of bacterial concentration were used for the subsequent 72 inocula of viable bacteria with the turbidity adjusted accordingly because colony counting was not further performed. Assays using inactivated bacteria or cellular material, and varying environmental conditions were performed with only high doses adjusted by turbidometry as described above. Assays using the inactivated bacteria or cellular material were performed in the optimal environment. Heat-inactivation of bacteria was performed by heating the inocula at 100°C for 10 minutes in sterile microcentrifuge tubes placed in a heat-block. The suspension of

heat-killed bacteria was briefly vortexed and used as a whole-cell assay and an aliquot was placed in a sterile microcentrifuge tube and centrifuged at 12,000 g for 3 minutes. The supernatant was pipetted into a sterile microcentrifuge tube, briefly vortexed and used as a heat-inactivated soluble assay, while the pelleted cellular material was re-suspended in one mL of PBS, briefly vortexed and used as a heat-inactivated insoluble assay. The cold-inactivation of bacteria was performed by a triple freeze-thaw procedure between -80°C and 42°C in three cycles of 30 minutes duration. The suspensions of cold-killed whole-cell and soluble assays were performed as for the heat-killed assays. The cold-inactivated insoluble assay was omitted due to a visible inhomogeneity of samples that could not be improved by vortexing. For the heat- and cold-inactivated assays inactivation was confirmed by culturing as described above. For the secretory products assay, bacteria were subcultured to 6 mL of Mueller-Hinton broth (Fort Richard, Auckland, New Zealand) and incubated in H_2-MA at 37°C in air-tight boxes at 200 rotations per minute until turbid (usually two days for *C. jejuni* and *C. upsaliensis* and four days for *C. helveticus*). The broth was centrifuged as for the heat-inactivation assay with the supernatant pipetted into a sterile microcentrifuge tube and used as the secretory products assay while control larvae were injected with sterile Mueller-Hinton broth.

6.6.4. Histopathology

Larvae were placed in neutral-buffered 10% (vol/vol) formalin and fixed for a minimum of two days. Longitudinal and transverse sections were processed routinely into paraffin for examination under a light microscope. Paraffin-embedded 4µm sections were stained with haematoxylin and eosin. The abundance of haemocytes, haemolymph, pigment, nodules, bacteria and adipose tissue (fat body) were scored semi-quantitatively over all sections of a given specimen. All evaluations were conducted blinded to treatment group. Haemocytes were scored as 1 (low number of individual haemocytes), 2 (low to moderate number of clusters) and 3 (numerous clusters or sheets of haemocytes), and haemolymph and fat body as 1 (fat body taking ≤ 25% of cross sections), 2 (~ 50% of sections) and 3 (≥ 75% of sections). Pigment was scored as 0 (no pigmentation), 1 (faint pigmentation at 4x

magnification), 2 (moderate pigmentation visible at 1.25x magnification) and 3 (obvious dark pigmentation at 1.25x magnification). Nodules as aggregations of pigmented haemocytes (520) were scored as 0 (none observed), 1 (few per section) and 2 (over 10 per section). Bacteria were scored as 0 (none observed), 1 (filling of ~ 25% of gut lumen) and 2 (filling over 25% of gut lumen) in gut tissue and as presence/absence in haemolymph.

6.6.5. Statistical analysis

Statistical and exploratory data analyses were performed using R v3.2.2 (R: A language and environment for statistical computing. R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). Analysis of survival data was performed using log-rank tests of Kaplan-Meier (KM) survival curves, Cox proportional hazards (CoxPH) regression models using the "survival" package and CoxPH mixed effects modelling using the "coxme" package as appropriate for the data. The bacterial dose was $\log_{10}$ transformed and the functional form evaluated for the purposes of survival regression modelling as a continuous covariate. Functional form was evaluated by the plotting of beta estimates against the categorical transformation of dose and by loess regression of martingale residuals against the dose of models with and without the dose included. With the assumptions being satisfied, the bacterial dose was centred to the mean dose and used as a continuous covariate. Models were built using the forward step procedure and selected based on ANOVA tests. CoxPH model diagnostics included evaluation of coefficients as a function of time using scaled Schoenfeld residuals for assumption of constant proportional hazards, DFBETA residuals for influential observations, and martingale residuals and component+residual plots for evaluation of functional form (linearity) of covariates. CoxPH mixed effects model diagnostics included evaluation of error in the Laplace approximation of the integrated partial log-likelihood using a Monte Carlo refinement (n = 1000) and profiling the difference between the integrated and the null log-likelihood over a range of fixed values of random effect variance for the estimation of uncertainty in the random effects modelling. Kruskal-Wallis non-parametric tests were used for testing of the histopathology scores

between the infected and uninfected larvae, and between *Campylobacter* spp. within the infected larvae. Spearman's rank correlation test was used for testing of correlations between ordered factors. All statistical analysis used the level of significance at $\alpha = 0.05$.

Disclosure of potential conflicts of interest

The authors declare no conflict of interests.

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CHAPTER 7

7. Whole Genome Comparison of *Campylobacter upsaliensis*, *C. helveticus* and *C. jejuni*

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7.1. Abstract

Campylobacter-enteritis is one of the most common causes of bacterial gastroenteritis in humans and is primarily associated with *C. jejuni* infection. *C. upsaliensis* and *C. helveticus* are commonly detected in faeces of dogs and cats worldwide, hence frequent exposure of humans is expected. However, the pathogenicity of these species is uncertain due to reports of low occurrence in sick people. In this study, genomes of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates were compared to describe their general, and pathogenicity-associated genome features. *C. helveticus* had the largest genome (an average size of 1,829,321 bp), followed by *C. upsaliensis* (1,667,333 bp) and lastly by *C. jejuni* (1,665,234 bp). Functional analyses of the genomes showed 1,500 unique Clusters of Orthologous Groups (COG) in the pan-genome of *C. jejuni* with 972 core COGs representing ~83% of the strain's genome. Comparable numbers for *C. upsaliensis* were 1,433 with 528 core COGs (~47% of the strain's genome), and for *C. helveticus* 1,243 with 1,083 core COGs (~93% of the strain's genome). Around 93% of COGs were assigned to 22 defined COG functional groups that showed many significant differences in the number of genes between the three *Campylobacter* species on the pan-genome, core and accessory genome levels. Similar patterns of COG functional groups associated with pathogenicity features of bacteria were observed between all three species. Using MP3 and PathogenFinder *C. jejuni* had a higher number of predicted pathogenic proteins than *C. upsaliensis* and *C. helveticus*. An analysis of genes reported as involved in the pathogenicity of *C. jejuni*, showed *C. upsaliensis* and *C. helveticus* to be comparable to *C. jejuni* with the most notable difference being the lack of genes associated with oxidative stress response and iron uptake. The results of this study support the pathogenic potential of *C. upsaliensis* and *C. helveticus*.

Keywords: genome, companion animals, zoonosis, *Campylobacter*, emerging pathogens

7.2. Introduction

The most common presentation of campylobacteriosis is an acute gastrointestinal illness but a number of complications, extra-intestinal infections, and rare long term sequelae such as Guillain-Barré syndrome and reactive arthritis have been recognized (9). Campylobacteriosis is mostly foodborne and data suggest that 24 – 29% of human cases can be attributed to handling, preparation and consumption of chicken meat. Other major risk factors include other meat sources, travel, contact with animals, and untreated drinking water (309). *C. jejuni* is responsible for the majority of confirmed infections, followed by *C. coli* (9).

On the other hand, the last few decades of intensive research has identified many other *Campylobacter* spp. as human pathogens, including *C. upsaliensis* but very little evidence exists for *C. helveticus* (13, 17). The relatively infrequent occurrence of non-*jejuni/coli Campylobacter* spp. is not surprising, as detection methods in diagnostic laboratories are optimised for recovery of *C. jejuni/coli* (17, 18). However, geographical differences, differences in exposure and the lesser virulence of non-*jejuni/coli Campylobacter* spp. may also explain a lower prevalence in people with clinical signs of gastroenteritis. The study of the pathogenesis of campylobacteriosis has mostly focused on *C. jejuni* and the main features of virulence, consisting of motility, chemotaxis, adhesion, invasion, and toxin production (22, 160). However, the exact mechanisms of *C. jejuni* pathogenesis are still not clear and large strain-to-strain variations exist (547). While some strains are not associated with clinical disease, others are associated with more severe outcomes. Population based genetic studies of *C. jejuni* showed various genotypes commonly observed in wildlife that are not associated with disease in humans, which could be linked to deletions of regions associated with virulence (265, 548). In contrast, *C. jejuni* sequence type (ST) 677 has been associated with bacteraemia (549). The degradation of the cytolethal distension toxin operon and similarities between the capsular polysaccharide synthesis loci were observed as shared genomic features between *C. jejuni* ST-677 and *C. jejuni* subsp. *doylei*, a subspecies frequently reported in patients with bacteraemia but not gastroenteritis (549). Pathogenicity and virulence features may also vary depending on the model of investigation used which hinders a clear

establishment of the determinants of virulence. For instance, considerable heterogeneity in the infection biology of *C. jejuni* in avian, mammalian and alternative models of disease was reported though some isolates can exhibit an invasive and virulent phenotype (534). On the other hand, genetic studies between species in the *Campylobacter* genus have been helpful in delineating their biology. For instance, genomic studies of *C. jejuni* and *C. coli* provided insights into their phylogenetic relationship and genetic exchanges on a species level (269, 550) and their relation to the niche and host associations important for epidemiology of human *Campylobacter* infections (272, 282, 340, 551).

As genome sequencing has become more available, genomic studies on non-*jejuni/coli Campylobacter* spp. have been performed. Genomic studies of *C. lari* (552), *C. ureolyticus* (553), *C. rectus* (554), *C. fetus* (555), *C. concisus* (556) have all reported variable levels of sharing and/or similarities of genes between species and unique features within each species that could be related to their pathogenic potential, and disease or niche associations. In this aspect, comparative studies are invaluable for delineating *Campylobacter* pathobiology. The present study focuses on *C. helveticus*, a *Campylobacter* species most frequently detected in cats and, despite a low occurrence, has been implicated as a possible emerging *Campylobacter* pathogen in humans (13, 17). The genome of *C. helveticus* has not been published to date and the species is reported as being most closely related to *C. upsaliensis* (516) which is considered one of the leading emerging *Campylobacter* pathogens in humans and is most commonly detected in dogs and cats (13, 18). Therefore, humans are likely to be exposed to *C. helveticus* and *C. upsaliensis* relatively frequently, as cats and dogs are common pet animals worldwide. The overall aim of this study was to investigate the potential pathogenicity of *C. upsaliensis* and *C. helveticus* using genomic data in comparison with *C. jejuni* as the established major pathogen of the genus.

7.3. Materials and methods

7.3.1. Bacterial genomes sources

Overall, 84 whole genome sequenced (WGS) isolates of *C. jejuni* (n = 42), *C. upsaliensis* (n = 33) and *C. helveticus* (n = 9), including species type strains, were used in the study. Of these, 45 isolates were from publicly available databases, 38 isolates were obtained locally from previous projects and William Miller (USDA, Albany, CA, USA) provided the *C. helveticus* type strain. Fifty-six strains were isolated from different animal sources, 26 from humans, and two from water. The complete list of isolates and related metadata is available in Supplemental Table 7.1.

7.3.2. Genomic DNA preparation

Locally sourced isolates were recovered from glycerol broth stored at -80°C and grown in a H_2-enriched microaerobic atmosphere at 37°C on Columbia Horse Blood agar (Fort Richard, Auckland, New Zealand). Initially, these isolates were obtained using mCCDA and C.A.T. *Campylobacter* selective agar plates (Fort Richard, Auckland, New Zealand). Genomic DNA was extracted using a Qiagen QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for Gram-negative bacteria. Genomic DNA was checked for quality using Qubit dsDNA HS/RNA/Protein assay kits (Life Technologies, Auckland, New Zealand) and visually checked for fragmentation and smearing using gel-electrophoresis. Genomic DNA was stored at -20°C prior to sequencing at New Zealand Genomics Ltd (Massey University, Palmerston North, New Zealand) using an Illumina MiSeq instrument (Illumina, Scoresby, Victoria, Australia) according to the manufacturer's instructions with paired read lengths each of 250 base pairs. The prepared libraries were normalized to equal molarity, diluted to 2nM and pooled in libraries of 20 samples. A flow cell was prepared for each pool and sequencing reactions using nine pmoles of the pooled libraries were performed with the MiSeq Reagent Kit v2 (Illumina, Scoresby, Victoria, Australia) to give approximately 12 – 15 million clusters per run.

7.3.3. Genome assembly, curation and annotation

The algorithm package Velvet (557) was used for *de novo* genome assembly of locally sourced genomes. After standard on-machine MiSeq demultiplexing of the sequence reads into their constituent libraries, the sequences from each isolate were analysed using a suite of tools for sequence quality control. For each library this involved the removal of any PhiX loading control through a mapping to the PhiX genome using the mapper BWA (version 0.7.12). The resulting SAM files were converted to fastQ files using the SamToFastq.jar program from the Picard suite (http://picard.sourceforge.net/). These fastQ files were used as input for any adaptor removal using the "fastq-mcf" program from the ea-utils suite of tools (http://code.google.com/p/ea-utils/; version 1.1.2-621)). Next, the libraries were run through a pair of quality control tools (FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; version 0.11.3) and SolexaQA++ (http://solexaqa.sourceforge.net/; version 3.1.3)) to assess the quality, and to give an overview of the reads from the run. Finally an analysis with FastqScreen (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/; version 0.4) was performed to check primarily for the presence of vectors, Illumina adapters and the PhiX loading control. The resulting fastQ files were used for genome assembly.

For each isolate, short read sequences of 250 base pairs (bp) were broken into shorter sequences (k-mers) and used for construction of a de Bruijn graph. The sequences were assembled across a range of k-mer lengths in increments of 10 between 35 and 225 bases. The best genome assembly was chosen based on the number and size of contiguous sequences (contigs), assembly length and the N_{50} score using QUAST software (558). Concatenated contigs from this best assembly were annotated with Prokka software (559) using the default settings. In addition, FASTA nucleotide files for all the publicly available WGS isolates were also annotated with Prokka to ensure consistency in the annotation.

7.3.4. Comparative genomics

Four forms of analysis were performed on the predicted genes form Prokka; three on the amino acid sequences (Cluster of Orthologous Groups (COGs), a pan-genome analysis and a core genome analysis) and one on the nucleotide sequences (ribosomal MLST). Further analyses were performed using webserver tools as described below.

For the functional analysis of genomes the predicted amino acid sequences from the isolates of *Campylobacter* spp. were searched against the updated 2014 version of the COG database (http://www.ncbi.nlm.nih.gov/COG/; 1,785,722 sequences across bacteria and archaea grouped into 4,632 unique COGs) using COGsoft (version 201204) software (560) with a comparison of presence/absence and gene enrichment in orthologous gene groups. Custom in-house Perl scripts were used to parse the output from these processes for further analyses. Functional genome features were also compared using RAST/SEED subsystem technology (561). For further investigation into pathogenicity related genomic features, *in silico* bioinformatics prediction software PathogenFinder (562) and MP3 (563) were used. Bioinformatics prediction tools were also used to identify clustered regularly interspaced short palindromic repeats (CRISPRs) (564) and phages (565).

The pan-genome analyses were performed on presence/absence of COG data output from the functional analysis of genomes using Roary software (566) and the 'micropan' R package (567). Phylogenetic relationships were presented using NeighborNet trees visualized in SplitsTree (version 4.13.1) (568) based on ribosomal multi locus sequence typing (rMLST), core and pan-genomes (569). Full length nucleotide sequences for 52 of the 53 genes (the order Campylobacterales does not possess the *rpmD* gene) encoding the bacterial ribosomal protein subunits (*rps*) were used for rMLST (271). Custom Perl scripts were used to extract the *rps* genes from the Prokka predictions and also to generate rMLST allelic profiles from the resulting unique sequences. Core genome analyses were performed using OrthoMCL software (version 2.0.9) with default parameters (192, 570), and using a MySQL database (version 5.5.37). Gene clusters were only considered to be core if a single

representative gene came from each of the 84 isolates under investigation. Sequences were further analysed using only amino acid predictions that were the same length, and also where the length range of a cluster was within 20% of the longest predicted gene in the cluster.

7.3.5. Statistical analysis

Statistical and exploratory data analyses were performed using R v3.2.2 (R: A language and environment for statistical computing. R Core Team (2013). R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/). The differences in the number of genes between *Campylobacter* spp. by their genome compartments, COG functional groups, toxin-antitoxin modules, secretion system types and restriction-modification systems were analysed using one way analysis of variance (ANOVA). For pairwise comparison of the means of genes, the Tukey's Honestly Significant Difference adjustment of p values for multiple comparisons was used. The Fisher's exact and Chi square tests for differences in the proportions of genes between *Campylobacter* spp. were used with adjustment of p values by dividing the standard level of significance, 0.05, by the number of the multiple comparisons made.

7.4. Results and Discussion

This study investigated 84 genomes comprising of *C. jejuni* (n = 42), *C. upsaliensis* (n = 33) and *C. helveticus* (n = 9) of which 17 genomes were sequenced in this study, including the newly sequenced species *C. helveticus*. The quality of all genome assemblies is presented in Supplemental Fig. 7.1 – 3. *C. helveticus* harboured the largest genome with an average size of 1,829,321 bp with on average 1,963.3 coding sequences (CDS), followed by *C. upsaliensis* with a genome size of 1,667,333 bp and 1,755.6 CDS whereas *C. jejuni* genomes were on average 1,665,234 bp in size, and had 1,731.0 CDS. The distribution of size, contigs, CDS, guanine and cytosine (GC) percentage, RNA features, and signal peptides as general genome features,

and the number of CRISPRs and phages detected in *Campylobacter* spp. genomes are presented in Fig. 7.1. These results are in agreement with the reported basic genome features of *C. jejuni* and *C. upsaliensis* (55, 228, 571). The *C. upsaliensis* and *C. helveticus* genomes had a consistently higher GC content that is more similar to *C. fetus* (~33.3%) but distinctively lower than those reported in non-thermophilic *Campylobacter* spp. (>39%) (55). It was suggested that differences found in amino acid usage among *Campylobacter* spp. were attributable to adaptive evolution driven by niche-specific environmental conditions, an association also evident when analyzing the global GC content and genome sizes with respect to different niche preferences, especially for oral *Campylobacter* spp. (*e.g., C. curvus*, *C. rectus*, *C. gracilis* etc.), which were distinguished by bigger genomes and higher values of GC content (55). However, all *Campylobacter* spp. in the present study are considered to share the same niche, the vertebrate intestinal tract, thus the variation in genome size and GC content between them may not be explained by their niche preferences unless potentially due to niche adaptation to the particular intestinal environment of their reservoir animals. Across bacterial classes and phyla there is a trend for a reduction of genome size and GC content from free-living organisms to host-associated to host-dependent and to intracellular bacteria (572) and within the *Campylobacter* genus variation in this regard remains to be further studied.

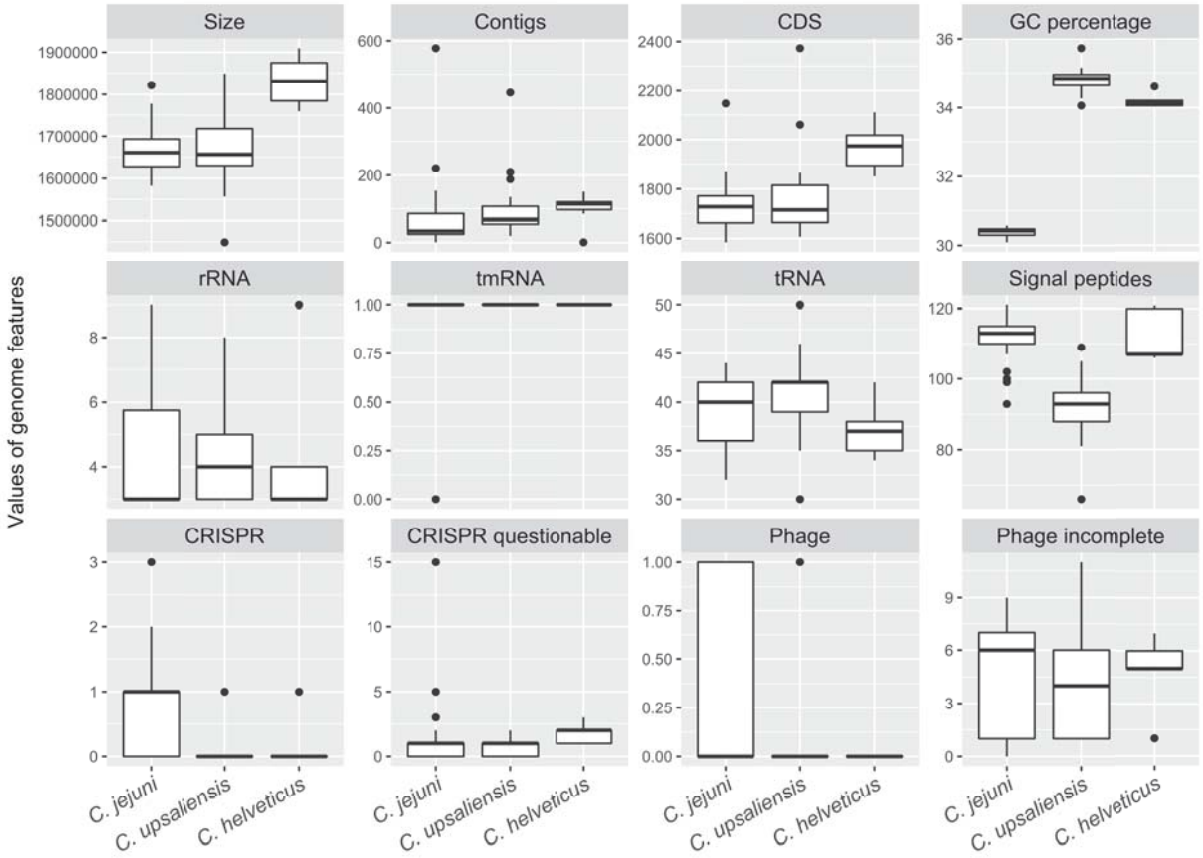

Fig. 7.1. Genome features of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*.

CRISPRs were detected in only one *C. upsaliensis* (RM3195) and one *C. helveticus* (ACP114b) genome. All *C. helveticus* genomes had between one and three questionable CRISPRs detected, similar to 18 of the *C. upsaliensis* genomes, which contained one or two questionable CRISPRs, while the remaining 15 *C. upsaliensis* genomes had no CRISPRs, questionable or otherwise. In contrast, 20 out of 42 *C. jejuni* genomes had one, four had two, one had three, and 17 had no CRISPRs detected. *C. jejuni* genomes had variable numbers of questionable CRISPRs ranging from one to 15, but five genomes had no questionable CRISPRs. CRISPRs have been frequently detected in *C. jejuni*, but rarely in *C. coli* (573, 574), and were reported as absent in *C. lari* RM2100 and *C. upsaliensis* RM3195 (228), and are variably present in other species associated with the *C. lari* group (552). The detection of CRISPR sequences in *C. upsaliensis* RM3195 in this study unlike in the investigation by Fouts et al. (228) is most likely to be due to different methods of

detection. However, as the vast majority of non-*jejuni Campylobacter* spp. in both studies did not contain confirmed CRISPRs, both studies are in agreement on the limited usefulness of CRISPRs for comparing *Campylobacter* species. Comparative aspects of the presence and diversity of bacteriophages were also limited. That is, only *C. upsaliensis* RM3195 contained one intact phage virion of 9.6 kb in length and only 12 (29%) *C. jejuni* genomes examined here contained phages, in contrast to approximately 65% of *C. jejuni* strains reported to contain phages (575).

After aligning the total of 148,671 CDS against the COG database, 126,192 (84.9%) CDS were assigned to 1,770 unique COGs. Of these, 753 COGs (42.5%) had more than one gene copy (repeat genes) with a range from two to 19, and an average of 2.7 gene copies per genome. A study of 34 genomes in 11 *Campylobacter* spp. (using 64,686 genes that clustered to 13,167 gene families) reported the average number of repeat genes at the genus level to be between 2.1 and 2.8, but only 27 to 86 gene families as containing repeat genes (571). The dissimilarity in the number of gene families with repeat genes between this study and that of Zhou et al. (571) is likely to be due to two major factors: 1) the different number of genomes and the species analysed; 2) the different method for clustering the CDS, in which genes are clustered amongst themselves in a “fifty-fifty” rule, compared to an assignment to a reference database using psiBLAST. It would therefore be expected that the former method would produce more families, and furthermore that the families described by Zhou et al. (571) would show a one-to-many relationship to COGs.

Counting of COGs with only one gene copy gave 1,709 unique COGs compared to 753 unique COGs when counting more than one copy. Therefore, 61 unique COGs had more than one gene copy between all the genomes, but there were 956 unique COGs with one gene copy in some genomes and more in others. The 42 *C. jejuni* genomes harboured a total of 64,011 genes belonging to 1,500 unique COGs, the 33 *C. upsaliensis* 48,110 genes belonging to 1,433 unique COGs and the nine *C. helveticus* 14,071 genes belonging to 1,243 unique COGs. The different numbers of isolates per species confounds interpretation of these numbers in terms of what it may indicate for *Campylobacter* spp., in as much as an analogy to a rarefaction analysis may be appropriate. In other words, given that there are only nine *C.*

helveticus isolates, the genomic diversity of these strains might be less than the genomic diversity of the other species if only nine of their isolates were analysed in a similar way. If the diversity was different, this might have implications for the repertoire of the *C. helveticus* genome.

The sharing of COGs between *Campylobacter* spp. by an absence/presence categorisation (the number of gene copies per COG is unaccounted for in this analysis) on the species level is depicted using a Venn diagram in Fig. 7.2 (data are available in Supplemental File 1.). It is important to note that the presence/absence categorisation was performed at the species level, irrespective of how many strains of each species have/lack each COG. Therefore, COGs depicted as shared by all species include core genes of all species, but also variable accessory and singleton genes that one or more strains of each species possessed. Similarly, COGs unique to each species may belong to the core, accessory or singleton genomic compartment of that species. In using this term, we refer to multiple artificial portions of the genome – for comparative genomic analyses – that can be described as core genome, pan-genome and accessory genome and singleton genes, depending on the kind of analysis being performed. The majority of COGs (62.8%, 1,111/1,770) were shared by all three species which represented 74.1%, 77.5% and 89.4% of the total *C. jejuni*, *C. upsaliensis* and *C. helveticus* COGs per species, whereas COGs unique to each species represented 17.1%, 11.8% and 3.9% of the total species COGs, respectively. A study by Zhou et al. reported 1,074 gene families shared between 22 *C. jejuni*, two *C. coli*, one *C. lari* and two *C. upsaliensis* strains that represented 26.9%, 54.13%, 71.74%, and 52.67% of their total gene families and the unique gene families represented 55.28%, 15.63%, 14.96%, and 29.72% of the total gene families respectively (571). However, as stated above, their definition of a family is not the same as the mapping to COGs described here. The differences in proportions between the species tested by the two studies are most likely to be due to the methodological differences mentioned earlier, but the absolute number of shared gene families is strikingly similar to the number of shared COGs in this study. Further work would be required to map these gene families to COGs, and then reanalyse the resultant data. With the consideration that the five different *Campylobacter* spp. evaluated by the two studies are all in the thermophilic

Campylobacter spp. group, these shared gene families are likely to fall into three categories: genes required for basic cell functioning and replication (minimal gene set), genes present across all members of the genus, and those genes required for or associated with the gastrointestinal niche. Conversely, the COGs unique to each species may possibly represent genes associated with adaptation to particular host reservoirs of each species or may be responsible for different outcomes such as (sub)clinical disease, colonization or transient intestinal passage within the human and other hosts.

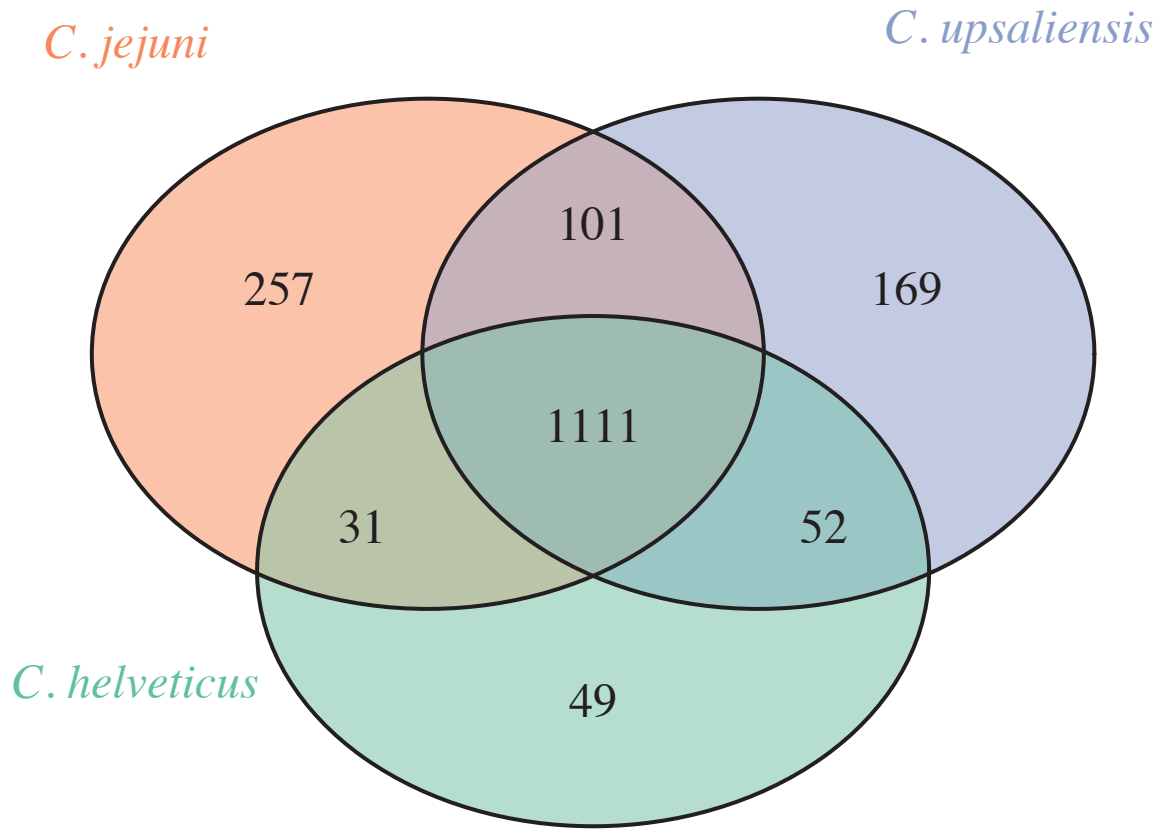

Fig. 7.2. Venn diagram of number of Clusters of Orthologous Groups (COG) shared between 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes.

Three genome studies of the *Campylobacter* genus reported the grouping of *C. coli*, *C. lari* and *C. upsaliensis* with the *C. jejuni* lineage and this group (thermophilic,

gastrointestinal) to be distinctively separated from the *C. fetus* (genital) group and the *C. curvus*, *C. gracilis*, *C. showae* and *C. concisus* (oral) group (55, 571, 576). The current study is the first genome study of *C. helveticus* at the time of writing but (i) the species was initially described as a thermophilic species most closely resembling *C. upsaliensis*, (ii) 16S rRNA studies showed it is the most closely related species to *C. upsaliensis* in the *Campylobacter* genus (251) and (iii) considering it is most frequently detected in faeces of cats and dogs (13), *C. helveticus* is likely to be a member of the thermophilic *Campylobacter* group too.

The strain variation in COGs present in all, and unique to each *Campylobacter* spp. by the functional groups is depicted in Supplemental Fig. 7.4. The figure shows little variation in genes within strains of species and between species for functional groups associated with general cell functioning such as cell cycle control (D), translation and ribosomal structures (J), and transcription (K), plus general metabolic features of *Campylobacter* spp. such as having a few genes associated with carbohydrate (G) and lipid (H) metabolism and transport. These findings are in line with the study by Friis et al. (577) reporting the core genes of *C. jejuni* as more related to DNA replication, transcription, translation and metabolism using the gene ontology terms method. However, large variations exist between the *C. jejuni* strains as compared to *C. upsaliensis* and *C. helveticus* strains in functional groups associated with energy production and conversion (C), amino acid (E) and nucleotide (F) metabolism and transport, cell wall and membrane (M), post-translational modification (O), inorganic ion metabolism and transport (P), general function prediction (R), and unknown functions (S) which in turn show little strain variation in COGs shared by all three *Campylobacter* species. Genes associated with secondary metabolites synthesis (Q), intracellular trafficking, secretion and vesicular transport (U) and defence mechanisms (V) show considerable strain variation amongst those shared by all three species. Interestingly, genes associated with motility (N) had minimal strain variation in genes shared by all species and only two strains of *C. helveticus* were observed to possess a unique gene, the c-di-GMP-binding flagellar brake protein YcgR. This gene has not been reported in *Campylobacter* spp. but in *E. coli* and *Salmonella* spp. it is involved with inhibition of motility and chemotaxis, and researchers suspected that the inhibition of tumbling together with the reduction in

motor torque provides cells with a longer window of opportunity to interact with the surface, setting up the platform for early stages of biofilm formation (578).

The COG data have shown several features to be similar for all the *Campylobacter* species examined. On average *C. jejuni* genomes contained 1,172 unique COGs with 1.3 gene copies per genome, *C. upsaliensis* 1,132 and 1.31 and *C. helveticus* 1,169 and 1.34, respectively. Similarly, taking into account only COGs with more than one gene copy, the average copy number per genome was 2.73 for *C. jejuni* and *C. upsaliensis* and 2.77 for *C. helveticus*. Again considering a rarefaction approach, it is expected that one would observe more different COGs with an increasing number of genomes analysed, but these results also indicate there is very little variation between *Campylobacter* spp. in the number of unique COGs and their copies contained in a single genome. This could suggest similar constraints on genome size between species and that the higher number of CDS observed in *C. helveticus* compared to *C. upsaliensis* and *C. jejuni* is likely to be due to the slightly higher average number of gene copies in a given COG. Furthermore, there was strain-dependent variation in gene copies within *Campylobacter* spp., as 45.1% (676/1500) of the COGs had the same number of copies between the *C. jejuni* strains whereas *C. upsaliensis* and *C. helveticus* had only 21.1% (303/1433) and 28.7% (357/1243), respectively. Of the total of 1,770 unique COGs, 1,637 (92.5%) were assigned to 22 COG functional groups. The proportion of COGs that could not be assigned to a specific functional group category was similar between species: 8% (99/1243), 8.6% (123/1433) and 7.9% (119/1500) for *C. helveticus*, *C. upsaliensis* and *C. jejuni*, respectively. These results are slightly higher than those reported by Zhou et al. (571) for *C. jejuni* (3%) and *C. upsaliensis* (4%) gene families and are most likely to be due to 44 *C. jejuni* and 33 *C. upsaliensis* isolates being analysed in the present study compared to 21 and two, respectively in the reported study. Collectively, these results suggest the distribution of COG functional groups and gene copies should be evaluated at the species and even at the strain level. The distribution of gene copies in COG functional groups between *Campylobacter* spp. is presented in Fig. 7.3 and strain variation in Fig. 7.4. The boxplots in Fig. 7.3 show that the majority of COGs in each functional group have one gene copy, though there are some COGs with multiple gene copies. Combined with the large strain-to-strain variation in gene

numbers by COG functional groups in Fig. 7.4, further analysis was performed on what can be regarded as various “genome compartments”.

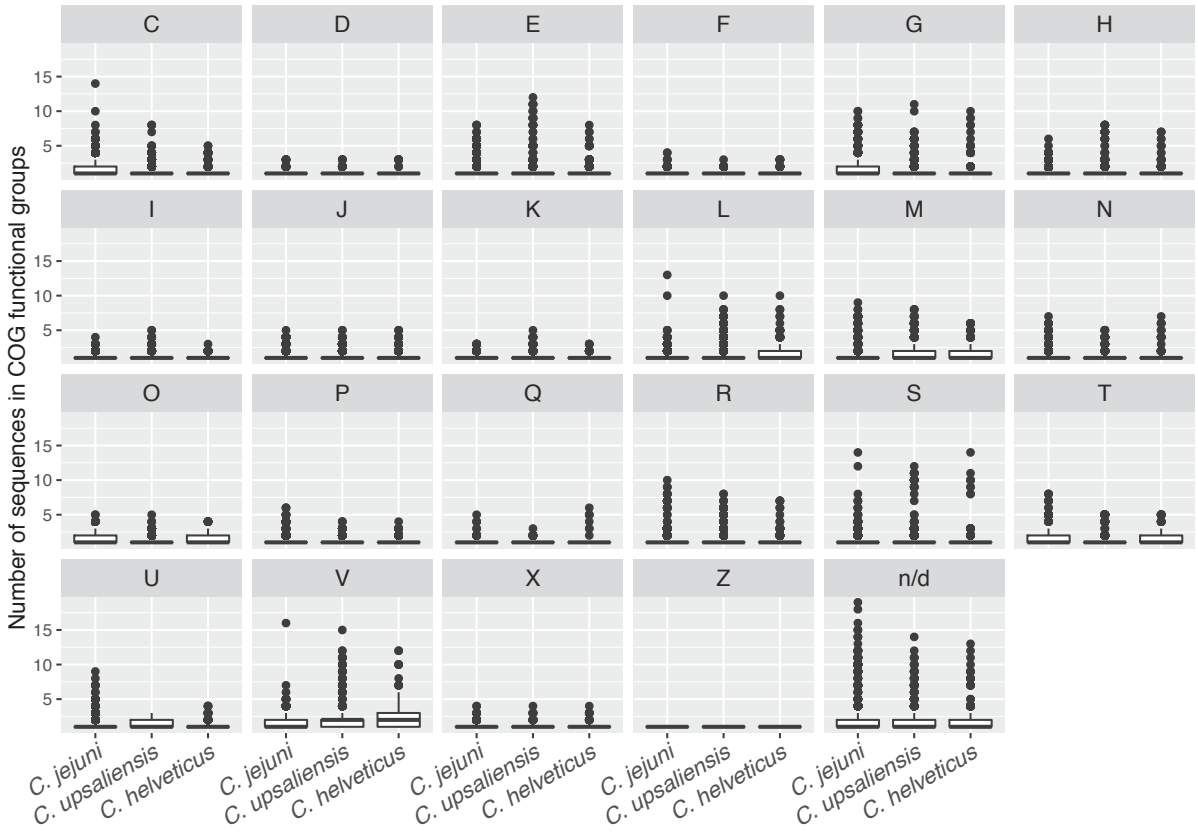

Fig. 7.3. Number of gene copies in Clusters of Orthologous Groups (COG) functional groups between 42 *C. jejuni*, 33 *C. upsaliensis* and 9 *C. helveticus* genomes.

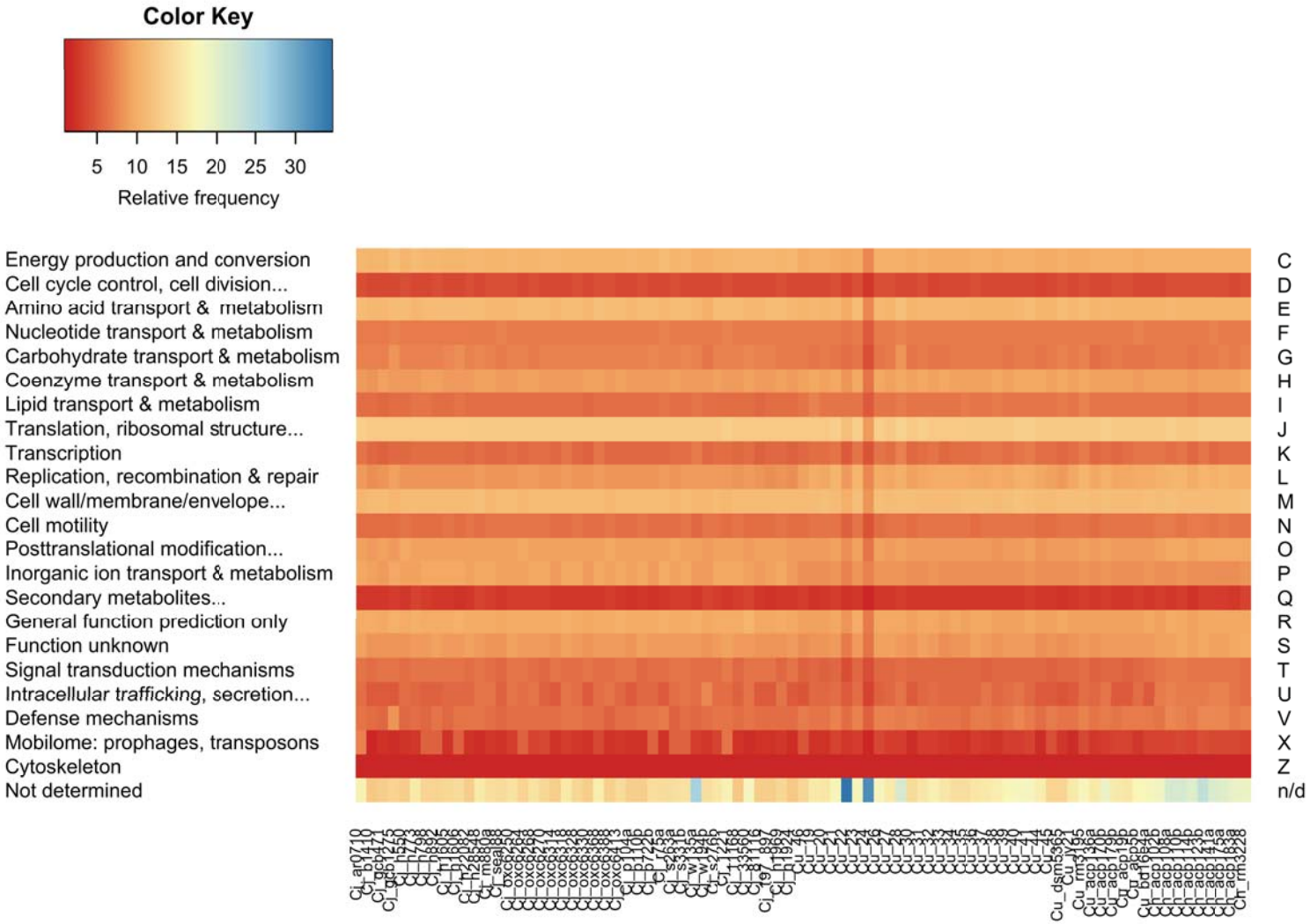

Fig. 7.4. Heat map of the number of gene copies in Clusters of Orthologous Groups (COG) functional groups between the strains of three *Campylobacter* species. Number of gene copies has been square root transformed.

COG data were used for pan-genome analysis separately for each species and for all species together as '*Campylobacter* spp.'. The summary of the pan-genome analysis is presented in Table 7.1. The core and pan-genome of *C. jejuni* has previously reported by others to be 947 and 3,648 genes with 130 genomes (579), 847 and 3,221 with five (580), 1,001 (no pan-genome) with 13 (270) and 1,295 and 2,427 with 13 genomes (577). The results of this study are in line with these reports, considering that studies differed in the number and selection of strains and methods used. The study by Friis et al. (577) reported from population estimates that about a third of the *C. jejuni* genome is attributable to auxiliary (non-core) genes, which is in line with this study's estimate of 64.8% for unique core COGs, but different when accounting for total gene copies which increased the proportion of core genes to >80% (Table 7.1).

Table 7.1. Number of unique gene clusters (and total genes) in Clusters of Orthologous Groups by genome compartments of *Campylobacter* species.

Genome feature	*C. jejuni* (n = 42)	*C. upsaliensis* (n = 33)	*C. helveticus* (n = 9)	*Campylobacter* spp. (n = 84)
A) Population level				
Core	972 (54,334)	528 (26,727)	1,083 (13,153)	472 (60,642)
Accessory	444 (9,590)	754 (21,227)	134 (892)	1,136 (65,384)
Singletons	84 (87)	151 (156)	26 (26)	162 (166)
Pan-genome	1,500 (64,011)	1,433 (48,110)	1,243 (14,071)	1,770 (126,192)
Core genome/ pan-genome ratio %	64.8 (84.9)	36.8 (55.6)	87.1 (93.5)	26.7 (48.1)
B) Average number per strain of species				
Core	972 (1,294)	528 (809)	1,083 (1,461)	472 (722)
Accessory	198 (228)	584 (643)	82 (99)	676 (778)
Singleton	4 (4)	7 (7)	3 (3)	4 (4)
Pan-genome	1,172 (1,524)	1,117 (1,458)	1,169 (1,563)	1,150 (1502)
Core genome/ pan-genome ratio %	82.9 (84.9)	47.3 (55.5)	92.6 (93.5)	41.0 (48.1)

The core genome of *Campylobacter* spp. was reported at 647 genes (17 genomes of eight species) (576), 608 (21 genomes of nine species) (577), 660 (192 genomes of two species) (579), 482 (18 genomes of six species) and 348 (34 genomes of 11 species) gene families (571). While the absolute numbers are quite different between studies that is likely to be due to variation in methods and strains used, as mentioned earlier, the reported relative proportion of *Campylobacter* spp. core genes represented in a given genome is more similar to the present study; reported at 18.83% (using BLAST clustering according to the “fifty-fifty” rule) (571), 39% (using

OrthoMCL clustering) (576) of the *Campylobacter* spp. genome and between 55% and 70% (using Chao lower bound estimates and OrthoMCL clustering) (270, 577) of the *C. jejuni* genome. A study of seven genomes of *C. jejuni* and *C. coli* reported a core genome of 1,035 genes accounting for 59% of the genes in each isolate using BLAST clustering according to the 70% identity and the 50% length rule (579). Analysis of COG data in particular, reported ~370 COGs to be shared when comparing three lineages (species) which also supports the results of the present study (581). For comparison, the pan-genome analysis of each *Campylobacter* spp. using Roary software (566) is presented in Supplemental File 1. and rarefaction curves of pan and core genomes are presented in Fig. 7.5, which suggest an open pan genome in all species due to the absence of the plateauing of curves.

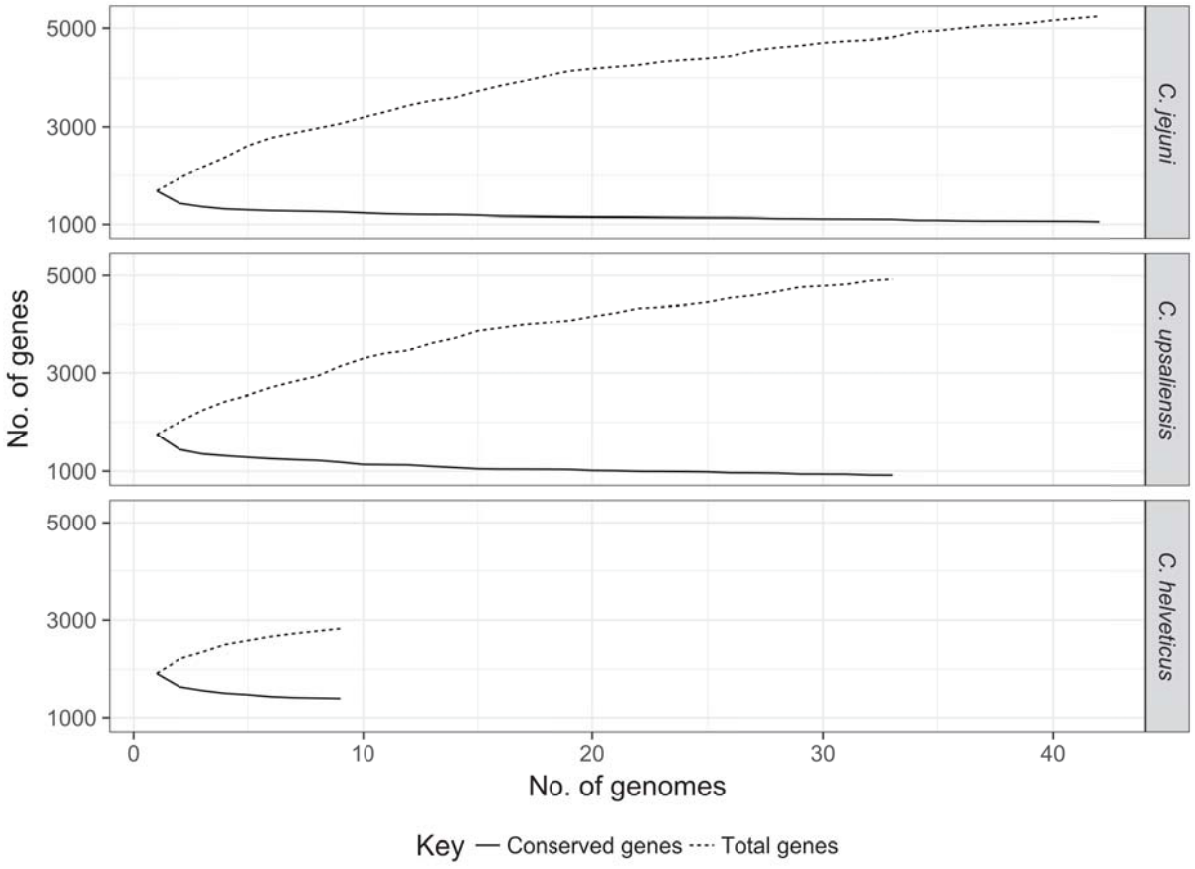

Fig. 7.5. Pan-genome and core genome rarefaction curves of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes.

Comparative analysis of multiple strains designate the pan genome as closed if no new genes, or open if many new genes, occur compared to the core genome for that pathogen species (275). The core/pan-genome ratio of *C. jejuni* in this study is similar to 76% reported in analysis of 14 *C. jejuni* genomes, and less than 89% in all *Campylobacter* spp. in this study, indicating an open pan-genome typical of a sympatric lifestyle (277) which is expected for the intestinal niche of *Campylobacter*. However, using the Heaps law type of model (276) the pan-genome of *C. helveticus* was shown as closed (α >1) and the other two species as open (α <1). The closed pan-genome of *C. helveticus* could be a false finding due to the potential bias of fewer genomes used compared to the other two species and the reduced diversity of *C. helveticus* isolates, as all except one were isolated from one region of New Zealand. Interestingly, *C. helveticus* and *C. jejuni* genomes on average had a much higher proportion of core genes in a given genome as compared to *C. upsaliensis.* The pattern is maintained, though increased, when comparing the genome compositions at the population vs. strain level (Table 7.1) suggesting that the strains of the former two species constrict the flexibility of the genome by preferentially keeping core genes. This differential composition of the genome between species in light of the results of sharing of COGs between strains of species (Supplemental Fig. 7.4) and strain-to-strain variation (Fig. 7.4) prompted further analysis of the genome compartments by COG functional groups.

The distribution of the number of genes between strains in pan-genome, core, accessory, and singleton genome compartments of *Campylobacter* spp. by COG functional groups is presented in Fig. 7.6 – 8. and Table 7.2, respectively. The study by Merhej et al. (582) showed a consistent genome reduction across COG functional groups as bacteria change lifestyle from free-living to facultative host-associated to host-dependent and to obligate intracellular lifestyle across 317 genomes, including two genomes of *C. jejuni*. As that study consisted of many different phyla the precision in the number of genes is less than in this study. Nevertheless, the distribution of genes in the pan-genome of all three *Campylobacter* spp. (Fig. 7.6) corresponds to the genomes of facultative host-associated tending toward host-dependent organisms for the COG groups C, D, E, F, H, M, N, O, P, and T and obligate intracellular organisms for the COG groups I, K, L, Q, R, S, and U and free-

living organisms for the COG groups J, O, and V. It could be expected that *Campylobacter* spp., as intestinal bacteria would group with facultative host-associated and host-dependent species, and to a certain extent with obligate intracellular bacteria as the pathobiology of *C. jejuni* is associated with invasion and intracellular survival in host cells and it has been described as a facultative-intracellular pathogen (41). Conversely association with free-living organisms is unexpected but could be a particular feature of *Campylobacter*, as the study by Zhou et al. (571) also reported the core genome of *Campylobacter* genus to be enriched with genes belonging to the J (translation) functional group. A study by Merhej et al. (582) showed genes involved in DNA replication, recombination and repair (L), RNA processing and modification (A), translation (J), post-translational modification (O), and intracellular trafficking and secretion (U) had significantly increased representation in all the host-dependent compared to free-living bacteria, whereas genes involved with transcription (K), defence mechanisms (V), transport and metabolism of amino acids (E), inorganic ions (P), and secondary metabolites (Q) significantly decreased in their percentage of genome representation.

Table 7.2. Average number of genes[1] in three *Campylobacter* spp.[2] (42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*) by COG functional groups and genome compartments.

COG group	Pan-genome			Core genome				Accessory genome and singletons			
	C. jejuni	*C. upsaliensis*	*C. helveticus*	*C. jejuni*	*C. upsaliensis*	*C. helveticus*	*Campylobacter* spp.	*C. jejuni*	*C. upsaliensis*	*C. helveticus*	*Campylobacter* spp.
Total	1,524.1	**1,457.9**	1,563.4	**1,293.7**	809.9	1,461.4	721.9	**230.4**	648.0	102.0	780.4
C	**114.6**	98.8	102.4	**105.2**	63.7	99.7	66.4	**9.5**	35.1	2.8	40.7
D	15.2	14.8	**16.8**	15.1	**5.0**	14.7	5.2	1.2	**9.8**	2.4	10.0
E	**123.5**	113.8	111.8	**105.5**	64.8	109.3	57.9	**18.0**	49.0	2.4	60.5
F	47.1	45.1[a]	46.3	**44.7**	19.9	43.7	19.4	2.4	**25.2**	2.7	26.8
G	**52.5**	43.5	46.2	**37.2**	26.8	42.6	21.5	15.4	16.8	**3.7**	26.8
H	77.8	82.0[a]	81.6	**69.0**	44.0	79.0	35.3	**8.8**	38.0	2.6	44.6
I	**37.0**	41.7	41.6	**33.1**	25.3	38,1	20.7	3.9	**16.4**	3.4	18.7
J	163.6	162.2	169.2	**154.8**	69.2	165.1	68.2	8.8	**92.9**	4.1	95.5
K	34.2	37[a]	36.4	**26.7**	16.1	29.7	15.0	7.5	**20.9**	6.8	20.5
L	**70.0**	91.5	105.3	**60.0**	54.1	96.3	36.1	10.0	**37.4**	9.0	46.1
M	125.0	128.7	128.2	**109.2**	76.2	119.2	70.3	**15.8**	52.5	9.0	56.5
N	38.7	38.6[a]	43.6	**34.8**	20.0	43.3	20.1	4.0	**18.5**	1.0	19.0
O	**80.8**	74.4	85.7	**76.7**	44.2	83.3	44.3	4.1	**30.1**	2.3	34.5
P	**83.6**	64.0	64.6	**73.3**	43.5	62.6	40.0	**10.3**	20.5	2.0	33.8
Q	8.3	7.7	8.9	**3.5**	2.0	8.8	1.0	4.8	**5.7**	1.0	7.2
R	**95.5**	82.7	86.4	**71.2**	39.4	78.7	36.8	**24.3**	43.3	7.8	52.8
S	**74.3**	70.6	85.2	**54.5**	37.4	75.9	33.2	**19.8**	33.2	9.3	40.9
T	**38.5**	31.1	41.4	**30.2**	17.9	37.0	16.6	**8.3**	13.2	4.4	19.3
U	34.0	30.8	**46.1**	**24.4**	13.0	38.2	10.9	9.6	**17.9**	8.9	23.2
V	**37.7**	45.1	52.2	**22.1**	32.7	46.4	13.5	**15.5**	12.4	5.8	28.6
X	12.5	9.2	13.6	1.0	1.1	6.7	0	11.5	8.1	6.9	11.3
n/d	**158.6**	143.4	148.9	140.6	**92.4**	142.2	88.5	**18.0**	51.0	6.7	63.0

[1] Not sharing the type of font used for the average number of genes between *C. jejuni*, *C. upsaliensis* and *C. helveticus* (excluding *Campylobacter* spp.) denotes significant difference using Tukey's Honest Significant Difference of means; [2] *Campylobacter* spp. in core, and accessory and singleton genes columns denotes joint analysis of the three species; [a] only a significant difference between *C. upsaliensis* and *C. jejuni*.

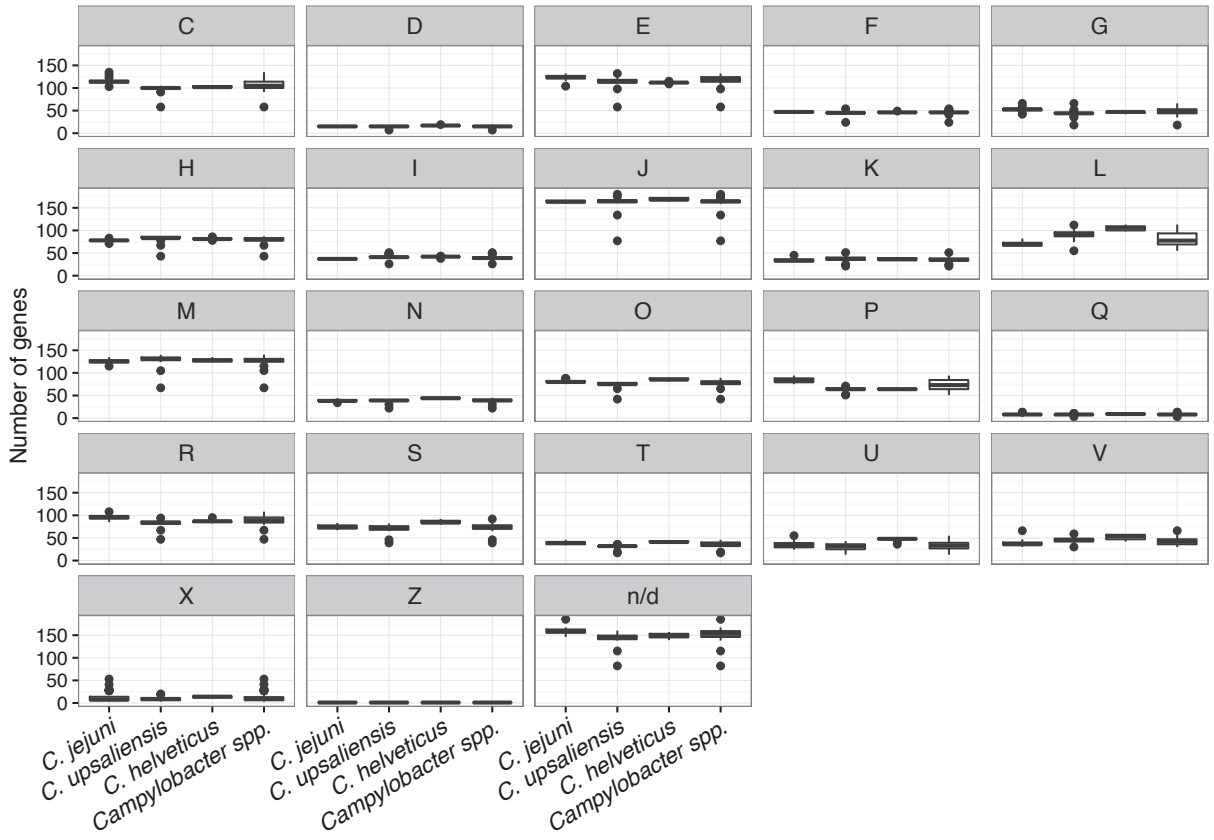

Fig. 7. 6. Distribution of the number of genes per strain in the pan-genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

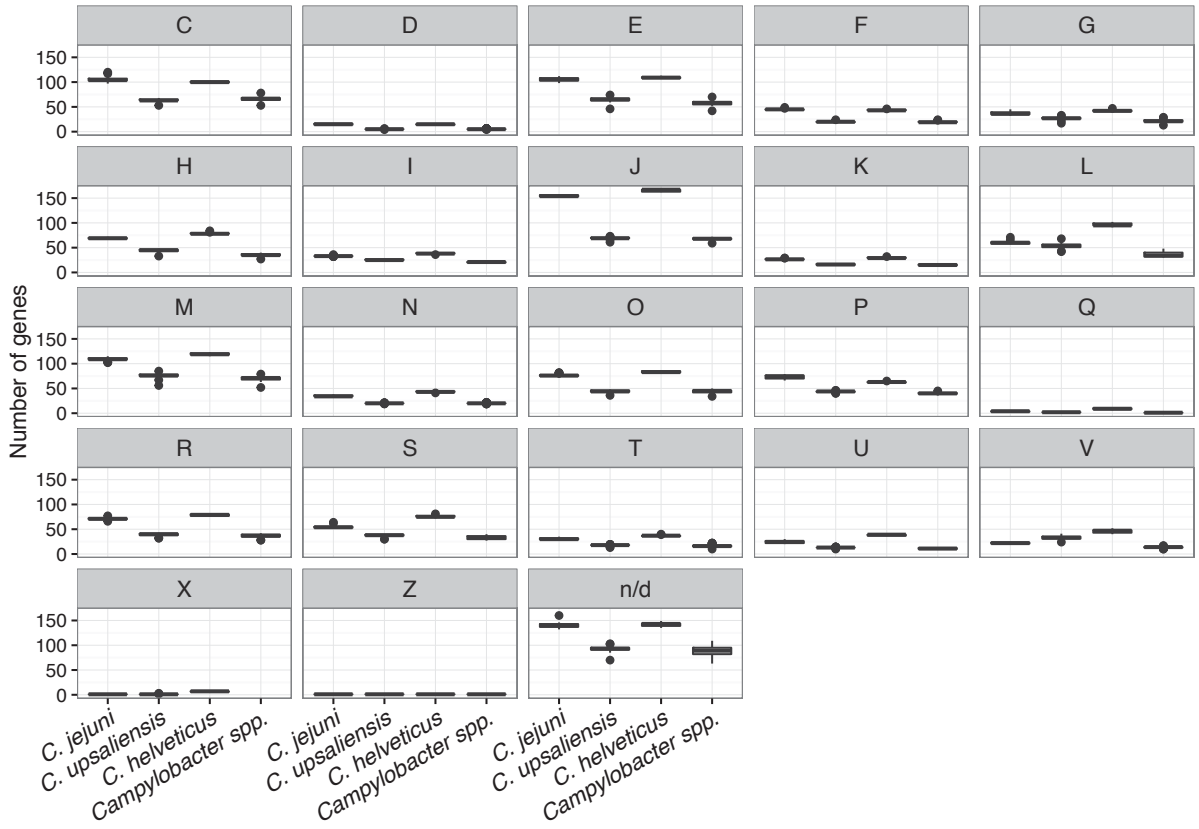

Fig. 7.7. Distribution of the number of core genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

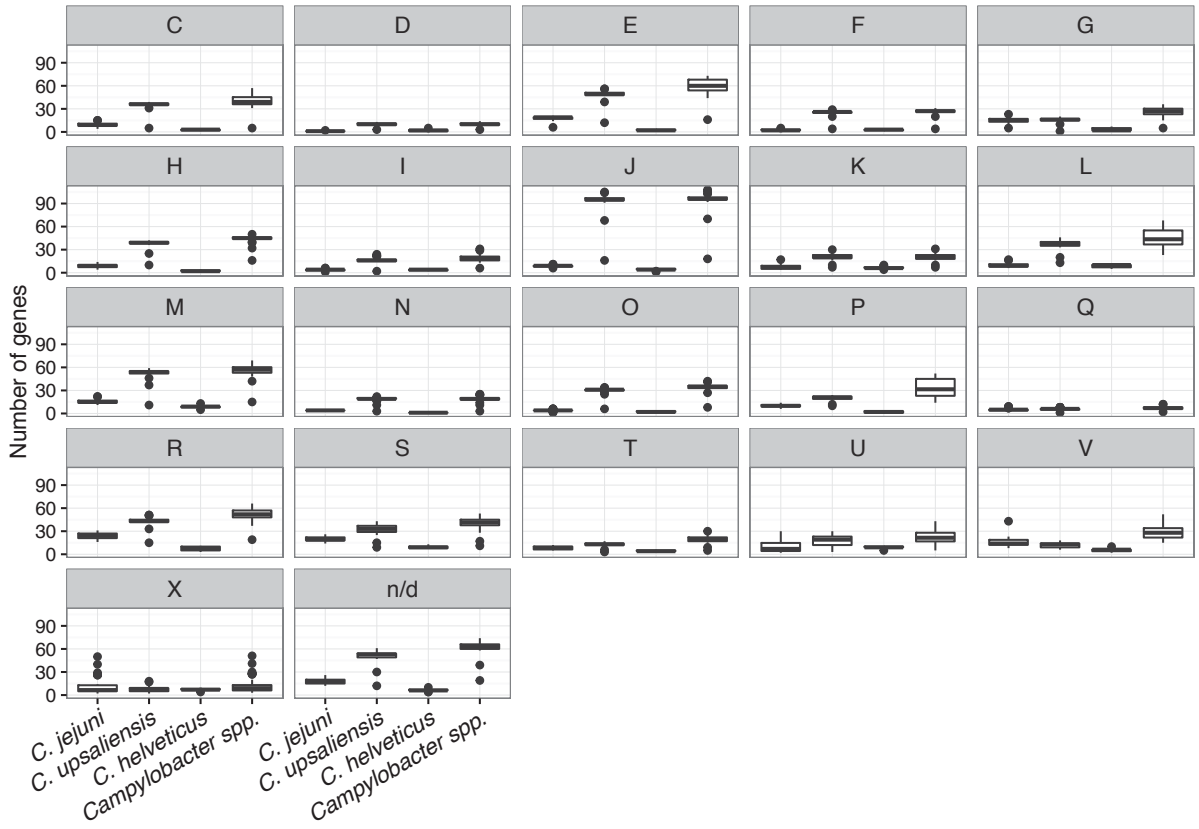

Fig. 7.8. Distribution of the number of accessory genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

Apart from the association with the lifestyle of bacteria, several of these COG functional groups were reported to be also differently represented in comparative genomic studies of pathogens vs. non-pathogenic or commensal species. The studies by Georgiades et al. (583) and Merhej et al. (584) showed that pathogens have fewer genes involved in transcription (K), energy production and conversion (C), metabolism and transport of amino acids (E), nucleotides (F), carbohydrates (G), lipids (I), inorganic ions (P) and coenzymes (H), secondary metabolites (Q) and signal transductions (T) compared to closely related non-pathogenic species. The study by Merhej et al. (584) also reported fewer genes involved in cell wall/membrane biogenesis (M), intracellular trafficking, secretion and vesicular transport (U), post-translational modifications, protein turnover and chaperones (O),

general predictions (R) and hypothetical/unknown functions (S) in pathogenic than in non-pathogenic species. On the other hand, pathogenic bacteria have more genes involved with DNA replication, recombination, and repair (L) than non-pathogenic species (584) though within this functional group Georgiades et al. (583) report that “bad bugs” have fewer recombination repair genes whereas “good bugs” have fewer mismatch excision repair genes. The genes involved in DNA replication, recombination and repair group (L) are considered to be needed by pathogenic bacteria for protection from the host immune response (279).

In the present study there was no significant difference between the three *Campylobacter* spp. in the number of recombination repair genes occurring in strains but *C. helveticus* had a significanly lower number of mismatch excision repair genes than *C. jejuni* ($p = 0.02$) and *C. upsaliensis* ($p < 0.01$). Although, *Campylobacter* spp. were not included in the above studies of pathogenic and non-pathogenic species, the comparison of the trends may be applicable, as the three *Campylobacter* spp. investigated in this study are closely related species and *C. jejuni* is an established pathogen. Considering *C. jejuni* to be more pathogenic than *C. upsaliensis* and *C. helveticus,* the agreement, with the former having fewer genes than the latter two was observed for groups K, V, H and I, but *C. jejuni* was placed in between the latter two for groups T, U, O, N and S and with regard to groups L, P, C, G, E and R, *C. jejuni* would be actually considered less pathogenic. Accordingly, *C. helveticus* would be considered more pathogenic than *C. upsaliensis* by group L and *vice versa* for groups O, S, T, U, and V whereas the two species were not significantly different between each other in the remaining COG functional groups (Fig. 7.6, Table 7.2).

These discordances in patterns expected could be indicative that all three *Campylobacter* spp. in this study are at least potentially pathogenic and a closely related non-pathogenic species is lacking in the present study. Alternatively, there may be confounding effects within species due to the presence of pathogenic and non-pathogenic strains or these reported patterns may not hold for the *Campylobacter* genus. Another source of variation of the gene content profile between species and within their strains was shown to be dependent on the genome compartments. *C. jejuni* and *C. helveticus* strains have more core genes in virtually

all COG functional groups than *C. upsaliensis*, which compensates for these genes by having more accessory COGs (very few with singletons) than the former (Fig. 7.6 – 8 and data for singletons in Supplemental Fig. 7.5). As the genomes of *C. upsaliensis* strains are mostly composed of the accessory gene pool, it is conceivable that the gene content profile pertaining to the general and also to the pathogenicity-associated patterns above may differ more between *C. upsaliensis* strains than between both *C. jejuni* and *C. helveticus* strains. The study by Merhej et al. (584) suggested that the core genome of the genus includes most genes in highly pathogenic bacteria, whereas the pan-genome is expanded by gene repertoires of the bacteria with low pathogenicity, and highly pathogenic bacteria have a lower proportion of lineage-specific genes in their genomes. Their results support the hypotheses of gene reduction in pathogenic bacteria with highly pathogenic bacterial genomes as subsets of less pathogenic and commensal bacteria species. Furthermore, the authors suggest the lower proportion of lineage-specific genes in the genomes of the highly pathogenic bacteria as indicating that genes are not acquired via horizontal gene transfer as often as in their closely related bacteria with low pathogenicity (584). The numbers of COGs unique to each *Campylobacter* spp. shown in Fig. 7.2 do not agree with this, although bias due to the number of genomes used per species is possible. However, the results presented in Supplemental Fig. 7.4, show that *C. jejuni* unique COGs have a larger strain variation than COGs unique to *C. upsaliensis* and *C. helveticus* and imply that a different selection of strains or use of only a few strains may, indeed, result in discordant associations. However, the average number of genes belonging to COGs unique to each *Campylobacter* spp. occurring in the strains of species was significantly higher in *C. jejuni* (99.95) than both *C. upsaliensis* (27.03) and *C. helveticus* (27.78) in the present study ($p < 0.001$). Therefore, the high number of *C. jejuni* unique COGs and the number of these genes in species strains compared to *C. upsaliensis* and *C. helveticus* in the present study is more suggestive of *C. jejuni* having had more frequent horizontal gene acquisition than *C. upsaliensis* and *C. helveticus*.

Despite the variable patterns of pathogenic features in COG groups, *C. jejuni* was shown to have smaller genomes and a lower GC content than *C. helveticus* and a lower GC content than *C. upsaliensis* genomes (Fig. 7.1). Having a smaller genome,

lower GC content and fewer rRNA genes are features that support the hypothesis of reductive evolution of pathogenicity through processes of gene loss, genome degradation and decay (275, 279, 584). On the other hand, the virulence of pathogenic bacteria is also related to possession of virulence factors in their genetic repertoire (585). The genomic study of *Campylobacter* genus, indeed, reported smaller genomes and lower GC content in established vs. putative pathogens of the *Campylobacter* genus and that putative pathogens (mostly the 'oral group' of *Campylobacter* spp.) have a significantly decreased number of virulence genes (55). Furthermore, by phylogenetic analyses, that study suggested that most recent common ancestor of *Campylobacter* was non-pathogenic and the species of *C. jejuni*-lineage group (and the *C. fetus* group) acquired its virulence armament through horizontal gene transfer from other bacteria. Using the analysis of COGs, several studies reported that toxin proteins and toxin-antitoxin (TA) modules are also enriched in pathogenic, compared to closely related non-pathogenic species (583, 584, 586) and with regard to bacterial lifestyle, that free-living have more TA modules than host-dependent prokaryotes (587). Toxin-antitoxin modules are small two-component genetic elements; a toxin that inhibits cell growth and an antitoxin that contains a DNA-binding motif and autoregulates transcription of the TA operon. The TA modules are associated with pathogenicity by enabling cells to persist in the presence of antibiotics, and environmental and nutritional stresses (588, 589). In this study the association of TA modules with pathogenicity was shown as an additional discordant feature between *Campylobacter* species (Table 7.3). That is, *C. jejuni* genomes harboured only three TA genes compared to seven in *C. upsaliensis* and nine in *C. helveticus*. Furthermore, TA genes in *C. jejuni* and *C. upsaliensis* were mostly part of the accessory genome unlike the majority of *C. helveticus* TA genes which belonged to the core genome. These results are in line with a study reporting strains of *E. coli* may vary in presence/absence of TA genes (590). In the present study, the inspection of TA modules showed incomplete modules, having only toxin or antitoxin present, occurring in *Campylobacter* species. On average the *C. helveticus* strains had 12.11 TA genes which is significantly higher than the 1.62 TA genes seen in *C. jejuni* ($p < 0.001$) and 2.91 TA genes in *C. upsaliensis* strains ($p < 0.001$); the latter also had a significantly higher number of TA genes than *C. jejuni* ($p = 0.002$).

Table 7.3. Presence of toxin-antitoxin modules in *Campylobacter* spp. genomes.

Feature components	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)	
	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.
Toxin-antitoxin modules						
MazF toxin	6	1.0	3	1.0	9	3.3
MazE antitoxin			2	1.0	9	1.8
YafO toxin						
YafN antitoxin	42	1.0	33	1.3	9	1.4
YafQ toxin	8	1.1	16	1.3	9	1.7
YafQ-DinJ antitoxin					9	1.1
YwqJ toxin						
YwqK antitoxin	19	1.1				
RelE toxin			21	1.7	5	1.8
RelB antitoxin					9	1.1
HipA toxin			3	1.7	9	2.7
HipB antitoxin						
Phd/YefM antitoxin			9	1.0	7	1.0

Restriction-modification systems are bacterial defense mechanisms against bacteriophage or other foreign DNA that act by cleaving the DNA at specific sites (591) and the endonucleases when extracted from bacteria have been reported to have a toxic potential for mammalian cells by promoting DNA mutations in the host (592). The number of restriction-modification genes was reported as not being significantly different between highly pathogenic and non-pathogenic bacteria (586). In the present study, *C. helveticus* strains possessed on average 17.78 and *C. upsaliensis* strains 14.7 genes involved in restriction-modification systems and were not significantly different between each other ($p = 0.06$) but both species had a significantly higher average number of these genes than the average 8.24 restriction-modification genes in the *C. jejuni* strains ($p < 0.001$). The restriction-modification genes were shown to be linked with phase-variation affecting adaptive and virulence traits (593) and may be associated with niche differentiation in *C. jejuni* (594). The preponderance of these genes in *C. upsaliensis* and *C. helveticus* is an interesting feature but further studies to explain their roles are needed.

Given the variation in the presence of genes between strains and species observed in the present study and their contribution to the pathogenic phenotype of the isolate, the investigation of potential antivirulence genes in *Campylobacter* spp. would be beneficial. An antivirulence gene is a gene whose expression in a pathogen is incompatible with the virulence of that pathogen and must be inactivated, deleted, or differentially regulated to prevent its expression from interfering with the pathogen's virulence as described, for instance, in *Shigella*, *Salmonella*, *E. coli* and *Yersinia pestis* (595, 596).

With regard to toxin proteins, all genomes in this study contained the cytolethal distending toxins (*cdt*) operon with only two genomes of *C. jejuni* and one of *C. upsaliensis* having an incomplete tripartite operon (Supplemental Table 7.2). These results are similar to the study reporting the presence of the *cdtB* component, used for PCR detection of species, in all strains of the three *Campylobacter* spp. (193). Supplemental Table 7.2 presents a list of genes reported to be associated with pathogenic features and mechanisms of *C. jejuni* compiled from several reviews and studies and the number of strains having these genes, and their copy number, occurring in genomes in the present study. The results in Supplemental Table 7.2 show very little variation in the number of strains containing genes related to motility, chemotaxis, adhesion, and invasion mechanisms between *Campylobacter* species. Notable exceptions were presence of a negative regulator of flagellin synthesis (anti-sigma 28) present in all *C. upsaliensis* and *C. helveticus* but only 14 *C. jejuni* strains, though six *C. jejuni* strains had an antagonist of the anti-sigma factor $SpoII^{AA}$. With regard to genes related to invasion, the *VirK* gene reported as being important for resistance to antimicrobial peptides and virulence in a mouse infection model (185) was absent in *C. upsaliensis* yet it was present in all *C. jejuni* and *C. helveticus* strains. Gluconate dehydrogenase (cj0415), reported as important for colonization of avian, but not mammalian hosts (597), is present only in *C. jejuni*, which could imply that *C. upsaliensis* and *C. helveticus* do not have the ability to colonize avian hosts. Similarly, the majority (40/42) of *C. jejuni* strains harboured an outer membrane protein belonging to the filamentous hemagglutinin family (p95) involved in an adhesion mechanism (598) in contrast to only 18/33 *C. upsaliensis* and none of the *C. helveticus* strains. On the other hand, *C. helveticus* had significantly more genes

involved in secretion systems than both *C. jejuni* ($p < 0.001$) and *C. upsaliensis* ($p < 0.001$); the latter two were not significantly different ($p = 0.2$). There are at least six types of protein secretion systems (types I–VI) in gram-negative bacteria to export proteins to the periplasmic space or the environment (599). Interestingly, there is little difference in secretion systems type II and III between *Campylobacter* spp. unlike the differences seen in types IV, V and VI (Supplemental Table 7.2). At most, 16 out of 42 *C. jejuni* strains possessed genes in the type IV secretion system in contrast to approximately three quarters and almost all of *C. upsaliensis* and *C. helveticus* strains, respectively. Gram-negative bacteria use the type IV secretion system to translocate a wide variety of virulence factors into the host cell and to mediate horizontal gene transfer (600). The results show genes in the type VI secretion system as belonging to the core genome of *C. helveticus* whereas only ~10% of *C. jejuni* strains and almost none of *C. upsaliensis* strains harbour these genes. The study by Bleumink-Pluym et al. (601) also reported that around 10% of *C. jejuni* strains possessed genes of the type VI secretion system and that these enable contact-dependent cytotoxicity towards red blood cells but not macrophages. The study by Georgiades et al. (583) reported species of low pathogenicity as possessing significantly more proteins in secretion systems than their closely related pathogenic species except for an epidemic *Shigella dysenteriae* strain. Accordingly, the results of this study would suggest that *C. helveticus* has low pathogenicity compared to *C. upsaliensis* and *C. jejuni*.

The results in Supplemental Table 7.2 show that largest diversity between *Campylobacter* spp. occurs for genes involved with iron uptake and aerobic/oxidative stress responses, namely in outer membrane ferric receptors (*cfrA/B*), and catalase (*katA*), NADPH-quinine reductase (*cj1545c*), the *yurZ* antioxidant family, thioredoxin-related protein (*soxW*) and transcriptional regulators of oxidative stress response (*rrpA/B* and *lysR*), respectively. The regulation of iron homeostasis and oxidative stress in *C. jejuni* has been shown to be linked (203-206) and that these regulators are also involved in flagellar biosynthesis (205). The genes involved in the antioxidant defence system are induced and their activity increased when *Campylobacter* are exposed to reactive oxygen species that may be generated due to an aerobic environment, aerobic respiration or those produced by the host immune

system against the invading pathogen (209). The possession of catalase by *C. jejuni* and not *C. upsaliensis* and *C. helveticus* may be a crucial difference regarding pathogenicity. That is, for conversion of hydrogen peroxide to water and oxygen catalase is needed when hydrogen peroxide concentrations in the cytoplasm are relatively high, whereas at low concentrations, alkyl hydroperoxide reductase (AhpC) is primarily responsible for detoxification (209). Therefore, *C. upsaliensis* and *C. helveticus* may be potentially pathogenic, indeed, but the lack of a catalase gene impedes their survival (either by evasion or counteraction) of the host immune responses. In support of this hypothesis are several studies that reported catalase activity and/or its transcriptional regulator as important for survival of *C. jejuni* in phagocytic cell lines (527, 528), as well as insect (525, 526), avian (206, 602) and mammalian (603, 604) infection models. Similarly, Iraola et al. (55) reported genes involved in the oxidative stress response separate established from putative pathogens in the *Campylobacter* genus. Furthermore, the researchers showed the catalase gene to be present in all of the *C. jejuni* subspecies, clades of *C. coli*, *C. lari*, and *C. fetus* subspecies but not *C. upsaliensis,* and by this phylogenetic analysis (the divergence of these species from the most common recent ancestor), it is possible that *C. upsaliensis* has lost catalase or the other species have acquired it through horizontal gene transfer.

To further investigate the virulence factors in *Campylobacter* spp., the genome assemblies were annotated using the SEED subsystem technology database. On average, *C. jejuni* genomes contained 61.0 (range 55 – 65) genes in the “Virulence, disease and defence” category, which was significantly higher than both of *C. upsaliensis* genomes (52.5 genes on average with a range of 40 – 55; $p < 0.001$) and *C. helveticus* genomes (52.6 genes on average with a range of 52 – 53, $p < 0.001$) with the latter two species not significantly different from each other ($p = 1$). Out of ten genes present in *C. jejuni* but absent in both *C. upsaliensis* and *C. helveticus*, five were associated with the metabolism of metals (four arsenic and one mercuric) and the others were a filamentous hemagglutinin domain protein, the tetracycline resistance gene *tetO*, a multidrug-efflux transporter from the major facilitator superfamily, FAD-dependent NAD(P)-disulphide oxidoreductase and a CopG protein of unknown function. Additionally, *C. helveticus* did not possess another two genes

associated with metabolism of metals present in both *C. jejuni* and *C. upsaliensis*; copper homeostasis protein CutE and magnesium and cobalt efflux protein CorC. *C. helveticus* did not harbour any virulence-associated genes that were not present in *C. jejuni* and *C. upsaliensis*. On the other hand, *C. upsaliensis* had a MerR family transcriptional regulator and the MtrF multidrug efflux pump component not present in both *C. jejuni* and *C. helveticus*. The 47 virulent genes present in all three species were mostly associated with copper metabolism, cobalt-cadmium-zinc resistance, resistance-nodulation-cell division efflux system proteins, macrolide efflux proteins, multidrug resistance transporters, beta-lactamase, and genes associated with DNA/RNA processes.

For a further exploration of pathogenicity associated features, the bioinformatics predictive software MP3 (563) and PathogenFinder (562) were employed. The overall number and proportion of predicted pathogenic proteins in *Campylobacter* spp. by their genome compartments is given in Supplemental Table 7.3. Using the predicted proteins as a proxy of the potential virulent genetic repertoire the results show *C. jejuni* to consistently have a higher number and greater proportions of predicted pathogenic proteins than *C. upsaliensis*, with *C. helveticus* having still fewer. These bioinformatics tools have different methods for prediction of pathogenic proteins and PathogenFinder (562), unlike MP3 (563), does not take all the sequences into account. The results in Supplemental Table 7.3 show PathogenFinder returned very few non-pathogenic proteins, and considering the high proportions of predicted pathogenic proteins, is potentially biased toward *C. jejuni* compared to *C. upsaliensis* and *C. helveticus*. This could be due to training of the software of PathogenFinder based on the species level, which included *C. jejuni* genomes, selected by developers compared to MP3, which is based on the protein level using public databases. The agreement of the two methods on predictions is presented using a Venn diagram in Supplemental Fig. 7.6. PathogenFinder provides the overall prediction of an organism as being pathogenic to humans and all *Campylobacter* strains in this study were predicted as such. The results of MP3 were matched to COG results (data available in Supplemental File 1.) to explore the predicted pathogenic proteins by COG functional groups in genome compartments of *Campylobacter* spp., as performed for a number of genes previously, and are

presented in Fig. 7.9 – 11. (data for singletons in Supplemental Fig. 7.7) and Table 7.4. The results show a similar relationship to the one observed with gene numbers (Fig. 7.9 – 11.) with regard to the genomic composition. That is, a higher number of predicted pathogenic proteins are found in the core genome of *C. jejuni* and *C. helveticus* compared to that of *C. upsaliensis* for COG functional groups M, N, P, O, R and S, which the latter compensates for through accessory and singleton predicted pathogenic genes.

Table 7.4. Average number of predicted pathogenic proteins[a] in three *Campylobacter* spp.[2] (42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus*) by COG functional groups and genome compartments.

COG group	Pan-genome			Core genome				Accessory genome and singletons			
	C. jejuni	*C. upsaliensis*	*C. helveticus*	*C. jejuni*	*C. upsaliensis*	*C. helveticus*	*Campylobacter* spp.	*C. jejuni*	*C. upsaliensis*	*C. helveticus*	*Campylobacter* spp.
Total	**277.7**	211.5	232.2	**218.5**	116.8	204.6	121.9	59.3	94.8	27.7	124.9
C	**12.0**	2.5	3.3	**10.9**	2.3	3.0	4.9	**1.0**	0.2	0.3	2.4
D	3.6	**1.5**	3.2	**3.5**	1.0	2.0	1.6	0.7	0.4	1.4[a]	1.1
E	**14.0**	12.2	10.4	**12.5**	10.4	10.4	10.5	1.5	1.8	**0**	2.4
F	**3.6**	1.1	1.0	**2.5**	0.1	1.0	0	1.2	1.0	**0**	2.3
G	**8.9**	7.2	4.3	**6.3**	5.1	3.8	4.2	2.6	2.1	**0.6**	3.5
H	1.3	1.1	1.1	**0.2**	1.0	1.0	0.2	**1.1**	0.2	0.1	1.0
I	**3.1**	3.8	5.6	**3.0**	1.6	4.9	1.5	0.1	**2.3**	0.7	2.2
J	3.3	4.1[b]	4.1	**2.7**	1.4	3.7	1.6	0.6	**2.7**	0.4	2.1
K	2.1	**4.3**	1.8	0.8	1.2[b]	1.2	0.5	1.3	**3.1**	0.6	2.4
L	**4.8**	6.4	9.8	2.6	3.2	**8.1**	1.4	2.2	**3.3**	1.7	4.5
M	**34.6**	27.9	29.2	29.5	**15.7**	28.3	18.1	**5.2**	12.2	0.9	13.3
N	**26.2**	28.0	28.8	**24.2**	13.0	28.6	13.0	2.1	**15.0**	1.0	14.2
O	**17.2**	10.8	14.1	**16.4**	5.9	11.8	7.5	**0.8**	4.9	2.3	6.8
P	**20.2**	14.5	14.2	**16.5**	10.8	13.6	9.8	3.7	3.6	**0.7**	7.5
Q	**1.9**	0.8	3.0	**0.5**	0	3.0	0	1.4	0.8[b]	0	1.5
R	**22.4**	13.8	13.2	**16.7**	5.3	11.1	8.1	**5.7**	8.4	2.1	9.9
S	**25.2**	15.4	12.9	**17.5**	7.0	11.0	7.5	7.8	8.4	**1.9**	12.5
T	5.8	4.7[b]	5.7	**5.3**	1.9	3.8	2.7	**0.5**	2.8	1.9	2.6
U	**13.7**	10.0	20.2	**6.5**	3.2	14.0	2.6	7.2	6.8	7.0	10.3
V	**16.7**	12.3	12.9	**12.5**	9.3	11.2	8.5	**4.2**	3.0	1.7	6.0
X	4.9	3.4	3.8	**1.0**	0	1.6	0	3.9	3.4	2.2	4.2
n/d	32.0	**26.0**	30.0	27.0	**17.0**	28.0	17.0	**5.0**	8.5	2.0	12.0

[1] Not sharing the type of font used for the average number of proteins between *C. jejuni*, *C. upsaliensis* and *C. helveticus* (excluding *Campylobacter* spp.) denotes significant difference using Tukey's Honest Significant Difference of means; [2] *Campylobacter* spp. in core, and accessory and singleton protein columns denotes joint analysis of the three species; [a] only significant difference between *C. helveticus* and *C. upsaliensis* and [b] only significant difference between *C. upsaliensis* and *C. jejuni*.

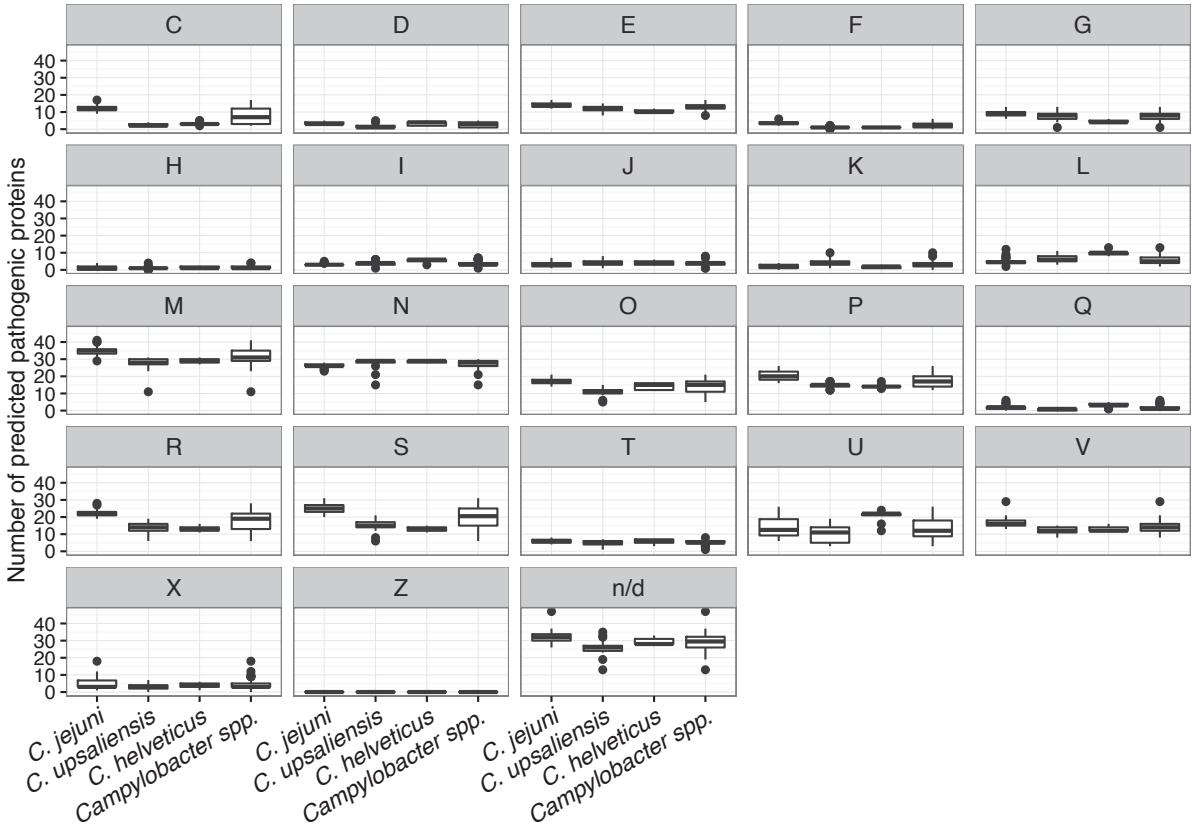

Fig. 7.9. Distribution of the number of MP3 predicted pathogenic proteins per strain in the pan-genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

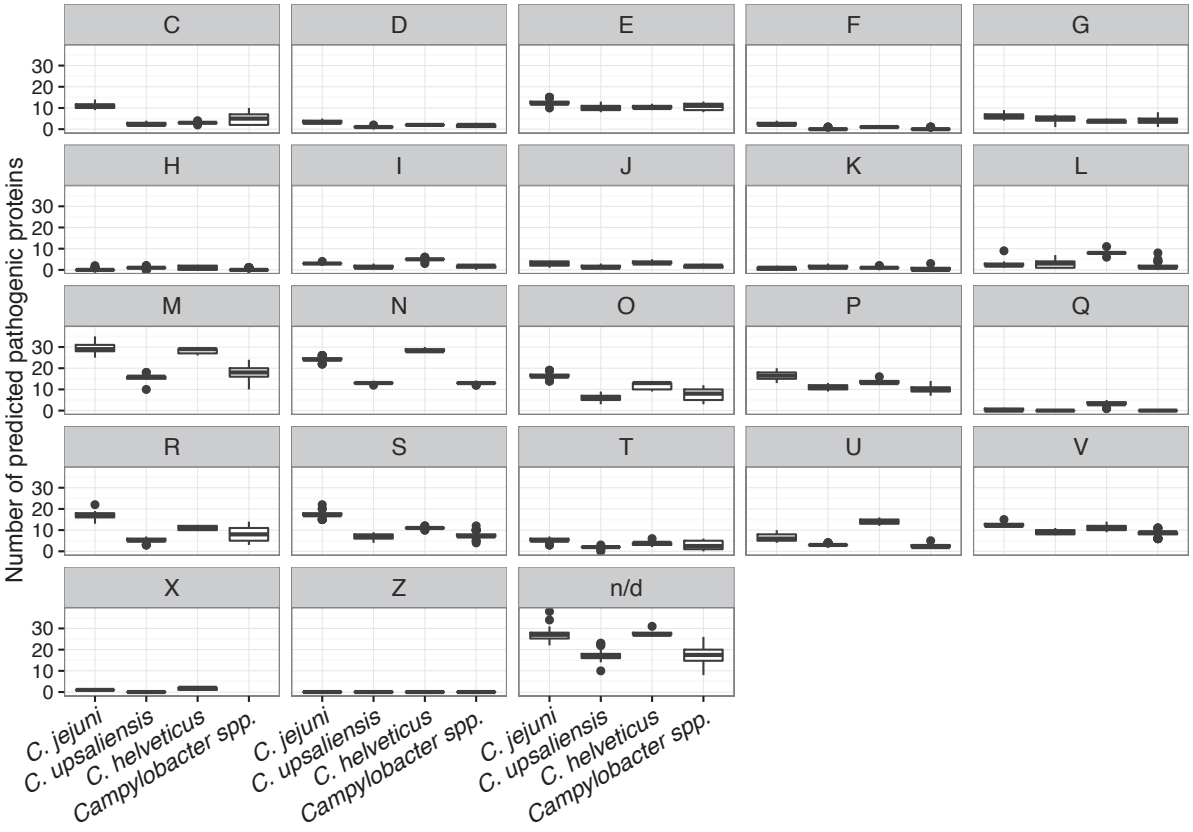

Fig. 7.10. Distribution of the number of MP3 predicted pathogenic proteins per strain in the core genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

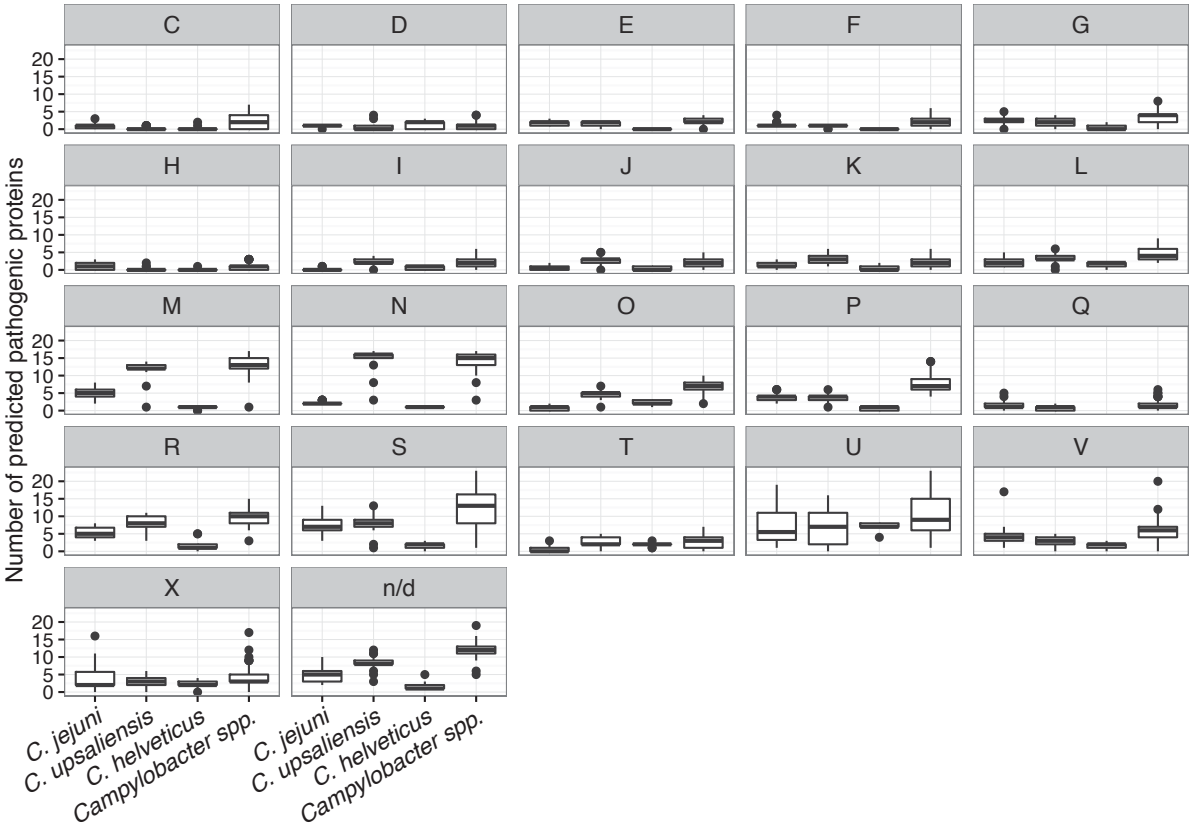

Fig. 7.11. Distribution of the number of MP3 predicted pathogenic proteins per strain in the accessory genome of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.

Overall, the highest number of predicted pathogenic proteins is attributed to COG functional groups E, G, M, N, O, P, R, S and V belonging to the metabolic and cellular processes and signalling groups. The number of predicted pathogenic proteins was the highest in *C. jejuni* (except for group N). With regard to the information storage and processing group, *C. upsaliensis* and *C. helveticus* had a slightly higher number of predicted pathogenic proteins in functional groups K and J, and L (in *C. helveticus* especially), than *C. jejuni*. Apart from the functional groups N and L, *C. helveticus* has the highest number of predicted pathogenic proteins in the functional group U. Overall these results show *C. jejuni* as having the largest virulence-associated genetic repertoire in a majority of COG functional groups. On the other hand, *C. upsaliensis* does not have the highest number of predicted pathogenic proteins in any functional groups except for a slightly higher count in K.

Taking all *Campylobacter* species together, the highest variability in presence of predicted pathogenic proteins was observed in groups C, M, O, P, R, S and U.

The phylogenetic relationships between *Campylobacter* spp. were investigated using Neighbor-Joining analysis based on core genome and rMLST analyses. A histogram for the gene cluster distribution size is shown in Fig. 7.12. This plot shows that across the 84 genomes, there are 2,589 genes that do not cluster and remain as singletons. There are 878 genes that are found in all 84 genomes, and they represent the core genome of these isolates. Of further note is a small peak at 42, which could be due to a subset of 102 genes that are either only found in *C. jejuni* (the number of isolates), and not in a union of *C. helveticus* or *C. upsaliensis*, or *vice versa*. However, there are not clearly identifiable peaks at 33 and 9 for the *C. upsaliensis* and *C. helveticus* species respectively.

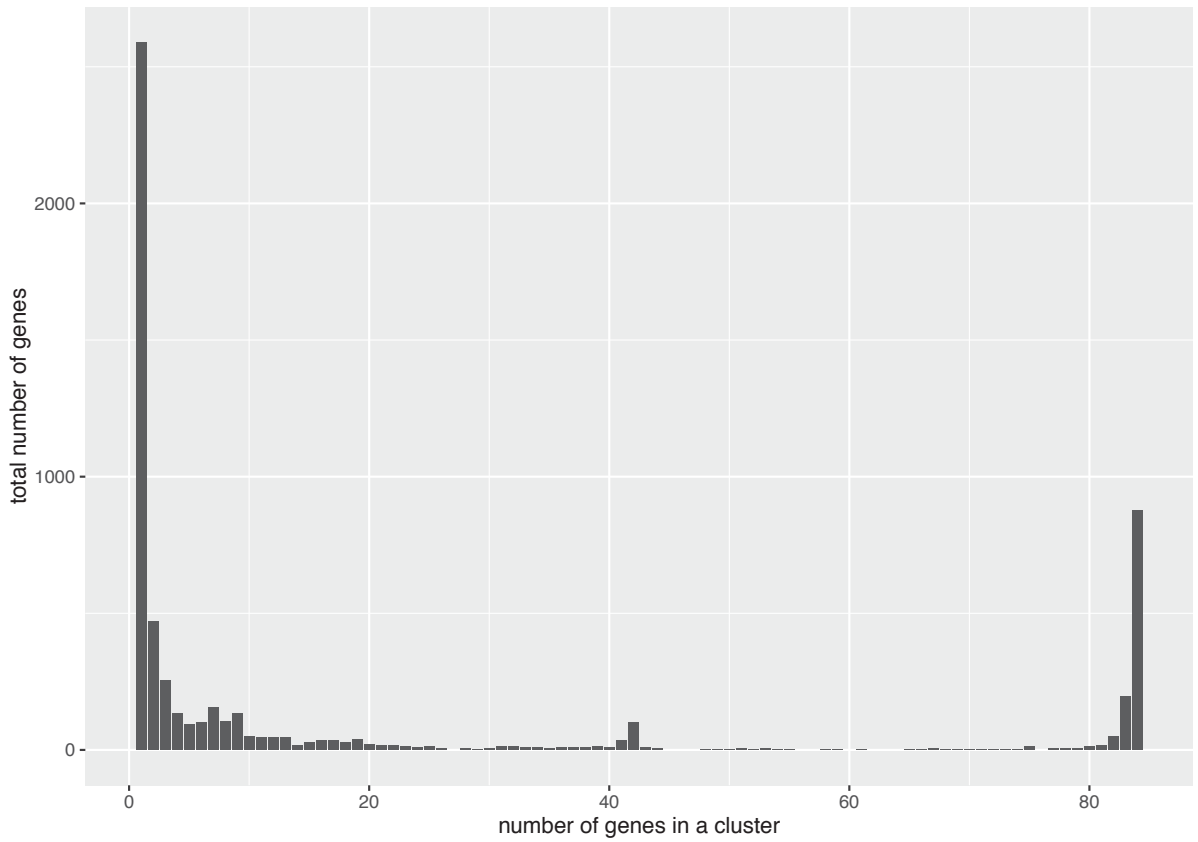

Fig. 7.12. Histogram of gene cluster distribution size by OrthoMCL core genome analysis of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes.

The results of the core genome analyses are presented in Supplemental Fig. 7.8 and 7.9 for clusters of genes of the same length and those within a 20% length range respectively. Using these criteria, for the genes of the same length, 182 genes contributed to an alignment of 45,949 amino acids. Within this alignment, there were 9,840 variable sites (21.42%). Similarly for the case where the length range of the genes in a given cluster were up to 20% of the longest gene, 649 genes contributed to an alignment of 197,053 amino acids and within this alignment, there were 59,486 variable sites (30.19%). This is less than the 878 described above, due to the length range criterion that has been applied. It can clearly be seen from both these figures that the isolates from a given species cluster together, and that on a core genome scale, there is good separation for each of the three species. In addition the NeighborNet is very tree-like, indicating that there is not much recombination evident for these genes within isolates for a given species. This would not necessarily be the case for genes in other genomic compartments. *C. helveticus* shows the tightest cluster, but it does have the fewest isolates. *C. jejuni* and *C. upsaliensis* show a degree of variation in their species clusters, indicating a greater diversity in the underlying amino acid sequences.

When the ribosomal MLST analysis was performed (Fig. 7.13), the resulting alignment of 20,779 nucleotides showed two major differences when compared to the core genome analyses (Supplemental Fig. 7.8 – 9). Firstly, the number of variable sites (6,525; 31.40%) was similar to the level found in the core genome dataset with the length range of 20%. Secondly, whilst the isolates cluster on species as they did for the core genome analysis, there is evidence for some uncertainty in the NeighborNet due to the box-like structures visible, for example with the *C. upsaliensis* isolate Cu_28080_40.

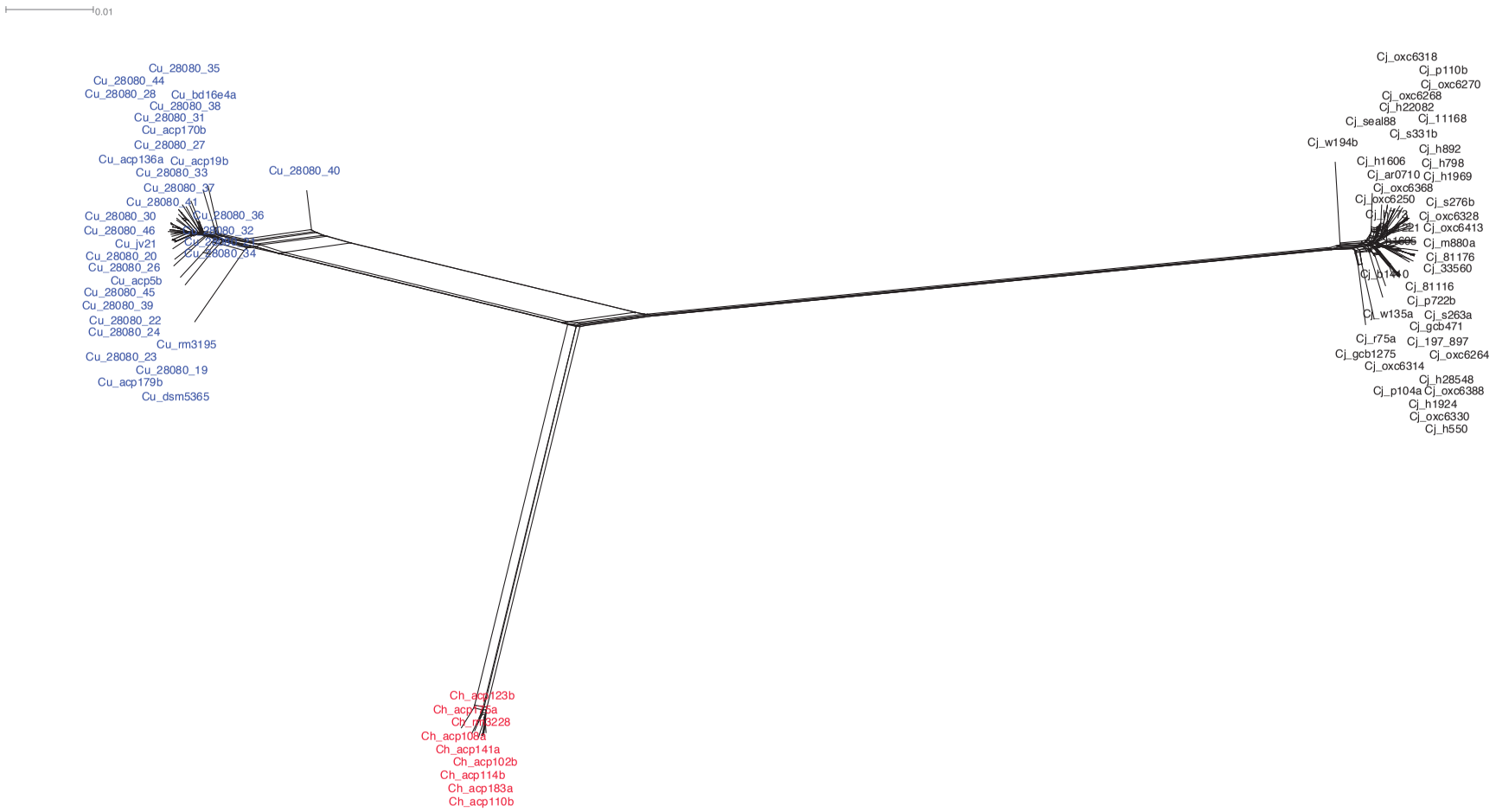

Fig. 7.13. NeighborNet visualized in SplitsTree for the 52 rMLST genes in 84 genomes of *Campylobacter* species. Alignment of 20,779 amino acids in length with 6,525 (31.4%) variable sites excluding the *rpmD* gene that is lacking in the Campylobacterales order; Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus* respectively.

7.5. Conclusions and limitations

This study provided a wealth of information about the genomes of three different *Campylobacter* spp. and has showed many differences between them. There are several limitations in the study, the most important one being the sample size and sources/selection of the genomes. Apart from the relatively few genomes of *C. helveticus* that were mostly isolated in New Zealand as mentioned earlier, the further main limitation is the lack of *C. helveticus* isolates from human clinical cases. *C. helveticus* isolates from human clinical cases are not publicly available and only one study reported occurrence in two out of 442 diarrhoeic samples using PCR (347). Similarly, only two *C. upsaliensis* isolates in this study were from human clinical cases. Reported studies of *C. upsaliensis* isolates from humans and dogs showed little clustering of genotypes between the two sources by 16S rRNA typing and plasmid profiling (236), amplified fragment length polymorphism (244) and multi-locus sequence typing (267). The limitations to these studies are small number of isolates from human cases (267) and geographically distant sources of human and canine isolates (236, 244). Nevertheless, their results suggest that unidentified sources of exposure of humans to *C. upsaliensis* may exist. Therefore, the lack of *C. upsaliensis* and *C. helveticus* strains from sources other than pet animals in the present study limit extrapolation of results for these species as a whole and are more relevant to the (sub)population associated with pets.

In conclusion, this study is the first to report a detailed functional analysis of the genome of *C. helveticus*. These species' genomes were clearly separated in the phylogenetic analysis of core genome and also, though to a lesser extent, in the rMLST gene analysis. The *C. helveticus* genome was shown to be larger with less variation in size between strains than genomes of *C. upsaliensis* and *C. jejuni. C. upsaliensis* was shown to have the highest GC content and lowest number of signal peptides. The pan-genome analysis indicates an open pan-genome in *C. jejuni* and *C. upsaliensis* and closed in *C. helveticus*, although fewer and more geographically clustered genomes may have biased the latter. The genome of *C. jejuni* and *C. helveticus* strains are composed of ~ 85% and higher of core genes of their respective species in contrast to ~ 55% in *C. upsaliensis*, indicating the largest strain-

to-strain diversity in the latter. However, metabolic features and capacities need not necessarily be as different as gene content profiles, because organisms may accomplish the same set of cellular processes with different sets of genes through non-orthologous gene displacement; the role of one gene is replaced by an unrelated gene accomplishing the same function (605). The variation in the number of genes between the different *Campylobacter* spp. by their genome compartments showed large differences between COG functional groups and that *C. upsaliensis* compensates for reduced gene content in the core genome with an enlarged accessory gene pool and *vice versa*. These results indicate that a comparison of genome compartments on the species level may distort the actual gene profile occurring at the strain level. To what extent the metabolic pathways are maintained so that the genome of an isolate results in consistent phenotypic features needs further investigation. Profiles of gene representation in most COG functional groups suggest a host-associated to host-dependent lifestyle of *Campylobacter* spp., which gravitates toward intracellular for transcription, replication, recombination and repair, intracellular trafficking, secretion and vesicular transport, secondary metabolites biosynthesis, transport and catabolism, carbohydrate and lipid transport and metabolism, and in poorly characterised COG groups. However, the COGs for post-translational modification, protein turnover and chaperones, translation and ribosomal structures, and defense mechanisms are more like those seen associated with free-living organisms. The association of the number of genes in COG functional groups with pathogenic potential of the species were discordant in the present study, as the emerging pathogen *C. upsaliensis* and the very infrequently pathogenic *C. helveticus* were designated as more pathogenic than the established pathogen *C. jejuni*. Similarly, *C. helveticus* and *C. upsaliensis* possessed more genes in TA modules than *C. jejuni*, and *C. helveticus* more genes in secretion systems than *C. jejuni*. On the other hand, using bioinformatics tools for the prediction of pathogenicity of proteins as a proxy of virulence armament, *C. jejuni* possessed a significantly larger genetic repertoire than *C. upsaliensis* and *C. helveticus*. Higher numbers of predicted pathogenic proteins were mostly associated with genes in energy production and conversion, motility, biogenesis of cell walls, membranes and envelopes, posttranslational modification, protein turnover and chaperones, inorganic ion transport and metabolism, intracellular trafficking, secretion, and vesicular transport

and defense mechanisms. With regard to evaluation of the presence of genes documented and associated with pathogenesis of *C. jejuni*, the vast majority of genes are similarly present in all three *Campylobacter* species. However, genes involved in iron uptake and stress response, especially oxidative stress and transcriptional regulators thereof, are missing in *C. upsaliensis* and *C. helveticus*. Protection from various reactive oxygen species plays a crucial role in the ability of *C. jejuni* to survive the attack by the host's immune system and development of the disease. Overall, the results of this study imply *C. upsaliensis* and *C. helveticus* to be almost on par with *C. jejuni* by many features reported to be associated with pathogenicity. Therefore, the lack or possession of a few particular genes may play a pivotal role and could explain the lesser incidence of *C. upsaliensis* and *C. helveticus* than *C. jejuni* in human clinical cases. Potentially, these vital genes are those associated with resistance to oxidative stress, most notably catalase. These genes and gene groups should be investigated in future studies of pathogenicity of the *Campylobacter* genus using gene insertion/deletion in infection models or phenotypic characterisation.

Disclosure of potential conflicts of interest

The authors declare no conflict of interests. The newly sequenced genomes in this study will be deposited to PubMLST before submission.

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CHAPTER 8

8. General discussion and future research

The research projects in this thesis have provided a wealth of information and new data on *Campylobacter* spp. in dogs and cats. While the research aims have been fulfilled, the data also generated new research questions and hypotheses to investigate. A series of experiments showed several *Campylobacter* spp. to be present as gastrointestinal flora in New Zealand dogs and cats and two types of their raw meat diets. In addition, it has shown the importance of suitable diagnostic methods to provide us with the ability to detect and isolate the desired range of bacterial species. The research also reported the first epidemiological associations with the carriage of *Campylobacter* spp. in New Zealand pets without clinical signs. The isolates obtained enabled investigation of the analytical sensitivity for the detection of *Campylobacter* spp. by a common antigen test used in human medicine (Chapter Five), and studies on the pathogenic potential using a larval disease model (Chapter Six) and whole genome research (Chapter Seven). In Chapter Five, *C. upsaliensis*, *C. helveticus*, and *C. hyointestinalis* subsp. *hyointestinalis* isolates obtained from the faeces of pet animals and farmed deer were shown to be detected by a common antigen-based test routinely employed by human diagnostic laboratories for the detection of *C. jejuni* and *C. coli* in faecal samples. The pathogenic potential of strains of *C. upsaliensis* and *C. helveticus* has been further characterised in Chapter Six by using an insect model of disease and was shown to be less than that of *C. jejuni*, but the strains were not non-pathogenic. Many variables were also shown to affect the survival parameters in experimental procedures that

are relevant for the reproducibility of results. The last of the research projects, Chapter Seven, compared the general and functional genome features of *C. upsaliensis* and *C. helveticus* strains to *C. jejuni* strains, and using patterns of gene presence and distribution associated with pathogenic traits in their genomes and also genomic compartments, suggested that there is the pathogenic potential to humans from *C. upsaliensis* and *C. helveticus*.

In Chapter Three the New Zealand household populations of dogs and cats were shown to frequently harbour *Campylobacter* spp., with approximately a third of dogs and one sixth of cats being positive in a cross-sectional sample. *C. upsaliensis* and *C. helveticus* in dogs and cats respectively, were more frequently isolated than *C. jejuni*. These results provided new data for New Zealand dogs and cats, as previous work only focused on *C. jejuni* (356) and undifferentiated *C. upsaliensis/C. helveticus* were only anecdotally reported by veterinary clinical laboratories (606). This study also provided the first data on epidemiological associations with faecal carriage of *Campylobacter* spp. in New Zealand dogs and cats, some previously reported worldwide and others newly observed. The relatively few epidemiological associations observed are likely to be due to the small sample size of the study.

In Chapter Four, New Zealand working dogs were also shown to be frequent carriers of various *Campylobacter* species. Therefore, exposure of humans to *Campylobacter* spp. through contact with household pet animals and working dogs is possible. The research aim for Chapter Four was to test a variety of culture conditions and methods to detect as many different *Campylobacter* spp. as possible and as a pilot study with only 50 dogs, epidemiological investigations were not performed. Future studies in New Zealand could further investigate other dog and cat populations such as shelter or kennelled and clinically ill populations to expand our knowledge of the epidemiology of *Campylobacter* sp. in New Zealand dogs and cats.

C. upsaliensis was shown to be the most commonly isolated *Campylobacter* spp. from faeces of two different populations of New Zealand dogs in this thesis. An overall comparison of household and working dog populations showed a significantly higher prevalence of *C. upsaliensis* ($p = 0.03$) but not *C. jejuni* ($p = 0.5$) in working

dogs compared to household dogs using Fisher's exact test. Although a study in Denmark showed city dogs to have significantly higher odds for carriage of *Campylobacter* spp. than rural dogs (139), the working farm dog population may have a higher exposure to potential sources than both rural and city dogs. The farm animal lifestyle could be the cause for a higher faecal carriage of *C. upsaliensis* in working dogs due to a higher probability of their direct exposure to faeces of wild and farm animals and access to outdoor water sources that would not be expected for household dogs. Access to outdoor water sources was reported as a risk factor for carriage of *C. upsaliensis* in dogs frequenting city parks in three cities in Ontario, Canada (292). In that study, mixed effects modelling did not show any clustering by park and visit (park and date of visit) either for *Campylobacter* spp. or *C. upsaliensis,* and regression models indicated that water at the parks was not a consistent source where dogs were exposed to *C. upsaliensis* (292). A study in Switzerland reported a general tendency for dogs living close to lakes and rivers to have higher carriage rates of *C. jejuni* (364), with higher carriage rate of *C. jejuni* also observed for working dogs as compared to household dogs in this thesis. While these findings are expected for *C. jejuni* given what is known about it's epidemiology, the results for *C. upsaliensis* are not as straightforward to explain as dogs and cats are the species with the highest prevalence rates and further research is needed. Only a few studies have shown *C. upsaliensis* to be present in animal faeces, other than faeces of dogs and cats, or in meat. *C. upsaliensis* and *C. helveticus* were isolated from 3% of beef meat samples intended for human consumption (61) and *C. upsaliensis* was detected in 3% of ground beef samples by PCR (607). *C. helveticus* was isolated from 15% of porcine caecal contents and 2% of carcass swabs (62). *C. upsaliensis* was isolated from 2% of rodents caught on pig farms (63) and was reported to be present in flocks of ducks (64), broilers (65) and chicken neck skin (608), guinea fowl (609), and meerkats (95). No other reports could be identified in the literature and it is clear that these reported prevalences are significantly lower (also likely for anecdotal reports of mere presence) than in dogs and cats (Table 2.7). Therefore, while farm and wild animals may be a source of exposure to *C. upsaliensis* in dogs, it is not known if wild and farm animals can also carry *C. upsaliensis* for prolonged periods as they can *C. jejuni*. Importantly, longitudinal studies of intestinal carriage and quantification of *C. upsaliensis* bacterial loads would be beneficial to support the hypothesis of *C.*

upsaliensis having a reservoir status. Long term colonisation of the intestinal tract, frequently in high numbers, without clinical illness is a feature of amplifying or reservoir hosts. Dogs could be hosts that maintain the population of bacteria in the environment (139). In the absence of more data on the carriage of *C. upsaliensis* in other animals, dogs are perhaps the most likely source of exposure to other dogs. This is supported by genetic similarity of *C. upsaliensis* isolates from dogs within the same household (364) and the report of living with a dog that was positive for *C upsaliensis* to increase the likelihood of a dog being positive, whereas living with a dog that was not positive appeared to be protective compared with being the only dog in a household (396). Similarly, high *Campylobacter* prevalence rates in kennelled or shelter populations of dogs (362, 395) support this hypothesis of dog-to-dog transmission but one cannot rule out the possible confounding of a shared environment, lifestyle, diet etc. of dogs living in the same household, shelter/kennel, or farm. Future epidemiological studies would require molecular studies to discern the connections.

Both Chapters Three and Four have shown the influence of the detection method on the perceived prevalence rate of *Campylobacter* spp. and the species distribution profile obtained from faeces of dogs, cats and raw meat pet food. Comparison of seven different culture methods in Chapter Four was responsible for the first isolation of *C. rectus*, *C. volucris*, *C. lari* subsp. *concheus* and *Helicobacter winghamensis* from dog faeces, all species reported to be of significance to human health. These results will contribute to the active research of emerging *Campylobacter* and related organisms. Whether the aim of future studies would be to merely further search for the presence of *Campylobacter* spp. in animals, food, the environment or other samples, or if the aim would be also focused on *Campylobacter* epidemiology, the detection methods will be an important factor limiting inferences from results. Other sources of limited comparability may be also present. For instance, two studies in the same region of Canada used the same detection methods and mainly attributed the differences in *C. upsaliensis* prevalence rates detected to the types of populations sampled and study design; dogs in city parks during summer (292) compared to dogs attending veterinary clinics over one year (393). Conversely, a study in Swedish dogs reported a lower prevalence of *C. jejuni* (382) compared to a previous study in the

country (610) that, despite other variables such as targeting a younger dog population, was mainly attributed to the culture methods used; only mCCDA versus three different culture methods, respectively. In this thesis, the higher prevalence of *C. upsaliensis* in working dogs compared to household dogs is not likely to be due to the five additional culture conditions used because the culture method that is identical in the two studies (direct plating to CAT agar) was the one to detect all the *C. upsaliensis* positive working dogs and all but one *C. upsaliensis* positive household dogs. Furthermore, when two days of additional incubation in the working dogs study was used, no further *C. upsaliensis* positive dogs were identified.

Therefore, while variations in study designs may explain factors more likely to be involved with differences in prevalence estimates and epidemiological associations observed, detection methods can profoundly impact the internal validity of the classification of positive and negative animals, and cases and controls, respectively. For instance, the use of mCCDA alone in Chapter Three would have precluded any meaningful epidemiological investigations as only one animal was positive for *C. upsaliensis*, and the prevalence would have been significantly underestimated, whereas isolation using CAT alone would draw very different conclusions from the same sampled population. Furthermore, if one considers the comparison of results with the selection of only one method from a panel of culture methods with a range of sensitivities and specificities such as observed in Chapter Four and other studies (54, 402), the results of epidemiological analyses should be expected to show varying levels of discordance. In support of this hypothesis are the results of a recent meta-analysis study of *Campylobacter* spp. prevalence and concentration in household pets (449). In that study, a significantly high level of heterogeneity in the prevalence estimates between studies was shown even with subgroup analysis accounting for the diarrhoeic status, source of animals (*e.g.*, household, clinic, shelter), geographical location (both at country and regional level), and animal species sampled, which indicated that other factors affecting the range of prevalences seen in the literature are yet to be identified. Furthermore, the importance of speciation of *Campylobacter* spp., especially for *C. upsaliensis*, and pet animal species were shown to be significant sources of variation in modelling the prevalence rate estimates by the respective meta-analysis study (449). These findings challenge the

usefulness of reporting results as '*Campylobacter* spp.' for all species together, and grouping different animals such as dogs and cats as 'pets' in reporting of prevalence rates. The authors reported that actually the key finding of the study was the lack of solid research data and that studies lacked a standardised reporting structure for prevalence values: the study locations, sample size (a clearly stated numerator and denominator), inclusion of diarrhoeic animals, and the study periods. The results of investigations of culture methods in Chapter Three and Four support the influence of detection methods as another very important cause of discordant reports between *Campylobacter* prevalence and epidemiological studies.

The increased ability of CAT agar to support the growth of *Campylobacter* spp. compared to mCCDA agar, especially in the isolation of *C. upsaliensis*, but other species too, was shown in both Chapters Three and Four. On the other hand, as discussed in the respective chapters, several studies worldwide have reported both the inverse and no difference between the two agars. More studies are needed to elucidate the reason for the variable success in the isolation of *C. upsaliensis* using mCCDA. Current evidence shows a mildly better analytical sensitivity of culture onto CAT compared to mCCDA agar, but data suggest a possible complex relationship between faecal flora and agars to be affecting isolation of *C. upsaliensis* (99). One study of gastrointestinal microbiota in chicken faeces reported *C. upsaliensis* in 10% of samples and as more prevalent in a gastrointestinal tract substantially dominated by Firmicutes (611). Firmicutes are abundant in the canine gastrointestinal tract (612), which could be linked to the higher prevalence rates of *C. upsaliensis* observed in dogs than in chicken. However, the influence of faecal flora on isolation of *Campylobacter* spp. requires further investigation. In Chapter Five, although using an antigen test, the effect of faecal samples, both in terms of consistency and the random effect of the individual faecal sample, on analytical sensitivity estimates was quantified and shown to be statistically significant. Perhaps there are similar effects of faecal samples on culture methods when used in spiked experiments that may explain the variable reports. A large heterogeneity of *C. upsaliensis* strains across the world (473) might be another important factor to consider. By this token, the most fruitful design of such a future study would be a double-blinded inter-laboratory study with random effects modelling conducted in various worldwide locations to capture as

large a variety of geographical and animal sources of *C. upsaliensis* strains as possible.

Several other influences of culture methods on prevalence rates and species distribution profiles of *Campylobacter* spp. that pertain to the comparability of studies were confirmed in Chapter Three and Four. Most notably these were the duration of incubation, and the incubating atmosphere and temperature. Prolongation of incubation to four and six days and use of 37°C and a H_2-enriched microaerobic atmosphere were important factors associated with the detection of species other than *C. jejuni* and *C. upsaliensis*, and to a lesser extent important for detection of *C. coli* and *C. lari*. Since the isolation of *Campylobacter* spp. is difficult and culture methods and protocols vary widely in their ability to isolate different species, the studies that employ multiple methods are more likely to detect the diversity of *Campylobacter* in faeces (54, 397, 402). Exploring modifications of protocols with CAT and mCCDA agar from Chapter Three and testing of a novel filtration method were the reasons that led to the first report of isolation of *C. rectus*, *C. lari* subsp. *concheus*, *C. volucris*, and *Helicobacter winghamensis* from dogs in Chapter Four. Only a few dogs were positive for these species and these studies cannot reliably infer if the respective prevalence rates are, indeed, low or the methods used are not optimised for isolation of these species. However, detection of *C. rectus* was reported in one out of 70 healthy and two out of 65 diarrhoeic dogs (359) and one in 60 dogs (613) in studies in Canada using qPCR directly in faecal samples with concentrations between 10^3 and 10^5 CFU/g of faeces. Excluding other differences between studies, such as the region and populations sampled, a possibility is that the culture methods used in Chapter Four were of sufficient analytical sensitivity and dogs are truly rare excretors of *C. rectus*. The protocol for the selection of colonies in cultures is another important limiting factor in the ability to profile multiple species and strain variation in samples. A study that picks only one colony (382) inherently cannot detect multiple species whereas studies that will sample any (54), or up to 12 (402) or even 33 (486) colonies from one sample will have different probabilities of success. Therefore, the colony picking protocols in Chapters Three and Four do contribute to the limited comparability to a certain extent between each other and other worldwide studies.

Chapter Four also showed how non-target growth could significantly impede the ability to pick colonies by reducing or obscuring agar surface area indicating the need for further optimisation of these methods. Culture methods for *Campylobacter* spp. are an active research area and building on gathered knowledge and experiences will improve current limitations. An example of such success is the adaptation of the "Cape Town protocol" that was shown to support the growth of 17 *Campylobacter* species from inoculated beef samples (43). In the ensuing application to un-inoculated meat for human consumption, several *Campylobacter* spp. were isolated for the first time (61). Our experience with this method applied to dog faeces and home-kill meat in Chapter Four was not successful; this was most likely due to the high background flora of faeces and, similarly, the highly prevalent faecal contamination of meat samples. The use of that novel method with an additional reduction of enrichment duration from 48h to 24h but with maintainence of the centrifugation of enrichment broth as originally reported (43) had been successfully employed in porcine caecal samples and carcass swabs (62). These modifications were not performed in Chapter Four and might have been helpful in reducing the non-target growth to enable the reading of plates and sampling of colonies for both faecal and meat samples.

The limitations of culture methods and protocols discussed above are also important to consider in comparison with molecular diagnostic studies. The most striking finding of the Canadian qPCR study is the detection of 14 different *Campylobacter* spp. in dog faeces (359) when compared to studies using culture methods as a first step and PCR and/or phenotypic tests were used to confirm culture isolates in Table 2.7. The study by Chaban et al. (359) also showed newly reported associations of diarrhoea in dogs with several *Campylobacter* spp. by their presence/absence, by higher bacterial concentrations, and also by higher richness of *Campylobacter* spp. in faeces. It is not known to what extent this diagnostic superiority of qPCR over culture methods is due to a general inability of methods to isolate these species or the higher analytical sensitivity of qPCR, or the practical limitations of culture protocols such as not testing all the colonies grown or the reduced ability in sampling of colonies due to non-target growth. Nevertheless, as there are no reported studies, so far, in dogs and cats comparing PCR or qPCR with isolation by bacterial culture, it could be considered as

one of the most needed future studies of *Campylobacter* spp. in these animals to address these outstanding issues. As shown in Chapter Four, culture protocols may vary widely in workload requirements and if a wider ability to isolate different species is desired, the selection of several culture methods would be preferable to compare to molecular methods. In order to facilitate practical workload issues for such a study a “molecular screening” approach may be helpful as reported for studies in humans. That is, samples are initially tested by a molecular method, as a more sensitive method, upon which only positive samples are further cultured (137, 481). Therefore, if the aim were to isolate emerging species, more culture methods would be easier to implement on a reduced number of samples. Given the results of Chapter Five, future comparative studies of culture-, antigen-, and molecular-based methods will need to more closely address the influence of analytical sensitivity on laboratory/diagnostic versus clinical sensitivity that is currently under the spotlight due to the effects on the interpretation of results for infectious diarrhoea (614). That is, if there is clinical relevance of a certain bacteria only if it is present above a certain threshold, the choice of method and resulting inference will be strongly dependent on its analytical sensitivity and desired aim. A comparative study of molecular and culture methods could also help elucidate if discordant reports between epidemiological studies employing cultures for factors, *e.g*. age, gender, exposure variables and others as previously discussed, were confounded by the limited ability of cultures to support growth of *Campylobacter* spp. or whether discordance was related to bacterial loads and species richness. Similarly, this kind of study could be implemented in a longitudinal design to provide data on the dynamics of the carriage of *Campylobacter* spp. in dogs and cats, especially for the improvement of our understanding of the association with diarrhoea that is limited by cross-sectional studies with only one sampling.

The results of Chapter Three and Four also support the need for joint longitudinal studies in dogs, cats and people in order to further address the potential health risks to humans through exposure to *Campylobacter* spp. from these animals or the risks associated with other factors related to owning pets or having contact with them. This is supported by the first report of contamination of commercially available raw meat pet food, and of raw meat home-kill diets for working dogs with *Campylobacter*

species, and the results of *C. jejuni* MLST typing from meat, dog, and cat samples. The MLST typing was a secondary aim of the research, as source attribution studies were not intended in this thesis, although results were used to add to the current ^{m}EpiLab database. Similarly, rather than attempting a more extensive investigation, the screening of raw pet food was piloted due to the successful experience in isolation of *Campylobacter* spp. from food, faeces and environment in ^{m}EpiLab (67, 312, 346). As presented in Chapter Three no other study has identified *Campylobacter* spp. in raw meat pet food. This is most likely due to the methods used, although the possibility of sampling error exists too. Raw meat diets are becoming more and more popular (615, 616) and with regard to the epidemiology of *Campylobacter* we reasoned that raw meat food could be a possible source to test as well. These proved to be valuable findings. Classical case-control epidemiological studies report contact with, or having a pet animal, as a risk factor for campylobacteriosis in people (14). It cannot be discerned whether people actually get exposed to *Campylobacter* from pets or that the behaviour, practices and lifestyle associated with having pets causes higher exposure from other sources and transmission vehicles relative to people not having pets. For the former, the data on the risk exposure to *Campylobacter* spp. from e.g. hair coat of pets, saliva or possible genitourinary excretions are lacking (449). Raw food for animal consumption contaminated with *Campylobacter* spp. can be considered as such a source of exposure for both animals and people. In the molecular epidemiology approach, genotyping of the human and pet isolates in that scenario would show the same genotype yet the actual pathway and direction of transmission remains unknown. A study in the Netherlands showed dog, particularly puppy, owners at an increased risk of infection with pet-associated *C. jejuni* STs, and isolation of identical strains in humans and their pets to occur significantly more often than expected by chance, but common sources of infection and the direction of transmission were unknown due to the cross-sectional design (300). MLST results of *C. jejuni* isolates in Chapter Three and Four show that STs common in dogs, cats, and raw pet food are common in human disease too, most notably the STs also associated with farmed animals. As discussed in the respective Chapters, reported studies worldwide are in agreement with this observation of shared STs between dogs and people (300, 386).

In considering the design of a longitudinal study in people and pets, the commonalities of *C. jejuni* ST profiles within the two groups would require accounting for scenarios that may varyingly affect the exposure of people of the same household to *Campylobacter* spp. from their pets too. For instance, children may play more often with family dogs than other family members and perhaps share the bed with them, while parents may have relatively less frequent direct contact but are more involved in feeding pets. In a study in the United Kingdom, dog-human contacts were found to be highly variable and were affected by the size, sex and age of the dog, individual dog behaviours, human behaviours and human preferences in the management of the dog and important situations in relation to zoonoses such as sleeping areas, playing behaviours, greeting behaviours, food sources, walking, disposal of faeces, veterinary preventive treatment and general hygiene (480). Similar findings with regard to pet husbandry and infection control practices were also reported in Canada (350).

The limitations of both classical and molecular epidemiological studies could thus be overcome by a combined approach in a longitudinal study that would follow-up behaviour and exposure variables with concurrent sampling of human and animal members, as well as food and the environment of the household. A targeted approach such as that reported in a study of people commencing work in poultry abattoirs (617) may be adopted for people not owning dogs or cats but having contact with them through their work. A combination of epidemiological approaches may provide important information for the implementation of pathway-specific control strategies as reported by a study that showed young children in rural areas had a higher risk of infection with ruminant strains than their urban counterparts (260). There is a general need for accessible zoonotic disease information for both pet- and non-pet owning households, with additional efforts made by veterinary, human and public health personnel to make the general public aware of potential risks and measures to prevent transmission (351).

The dynamics of carriage of *Campylobacter* spp. in pets present another issue for source attribution studies. As reviewed in Chapter Two, *C. jejuni* as a human pathogen, is an “accidental tourist” (23) that has reservoirs in various animals. The

source attribution framework cannot ascertain the contribution of particular transmission pathways, but attempts to estimate the relative contributions from amplifying animal hosts as sources from whence the observed genotypes in human patients have originated. That is, the animals are the primary amplifying hosts of *C. jejuni* and water is considered as a transmission vehicle contaminated by, to varying degrees, different animals (312). Longitudinal studies in dogs showed faecal excretion of *C. jejuni* to be short in duration (139, 395) and also that the genotypes between these episodes were diverse (139). The varying degree of association of *C. jejuni* and diarrhoea in dogs is to an extent similar to humans in developing countries. In developing countries where campylobacteriosis is hyper-endemic, the disease is mainly confined to young children who, through repeated exposure to infection, develop immunity early in life and diarrhoea is rarely seen in adults (2, 9, 618). In light of a short duration of carriage of varying genotypes of *C. jejuni* in dogs, it is could be that dogs are not a natural reservoir of *C. jejuni* and more alike to humans “accidental tourists”. As such *C. jejuni* STs observed in dogs (for cats there is a lack of data) cluster to the amplifying hosts and no ST has been observed as a pet “specialist” in reported research to date (300, 450). For these reasons future molecular studies in dogs and/or pets and people would benefit from combining the epidemiological metadata and longitudinal design.

The consideration that dogs may not be a reservoir of *C. jejuni* in the framework of source attribution studies in people, suggests it would be useful to perform source attribution studies for *C. jejuni* in dogs. That is, the analysis would be directed to attribute relative contributions of the amplifying hosts to *C. jejuni* excretion in dogs, and humans and water could be considered transmission vehicles; the methodology could be similar to that performed in human source attribution studies. Currently there is a lack of information on the range of both *C. jejuni* and *C. upsaliensis* STs in pets worldwide (264, 267, 477) and this is an emerging field of research compared to reported research of *Campylobacter* spp. in farm animals, food, and water. Until more data are available, a source attribution study of *C. jejuni* in dogs may be attempted retrospectively using non-local, non-recent MLST data as previously illustrated in humans (341). Current projects of *C. jejuni* surveillance such as those performed in the UK (619) and New Zealand (307) are an excellent base to expand

the current knowledge of the epidemiology of both *C. jejuni* and *C. upsaliensis* in pets and humans. For *C. jejuni* more data and isolates from pet sources are needed, and for research involving *C. upsaliensis* the current challenge is to identify sources other than pets. Therefore, suggested further research would involve, through the collaboration of ^{m}EpiLab with the regional medical laboratory for the purposes of the Manawatu *Campylobacter* sentinel site project, to screen faeces of human clinical cases, food and environment for the presence of *C. upsaliensis* and *C. helveticus*, and other *Campylobacter* spp., in addition to *C. jejuni*. The studies in Chapters Three and Four have provided a wealth of isolates, especially of *C. upsaliensis*, that can be used to expand the MLST (257) database of ^{m}EpiLab for future molecular epidemiology studies that will contribute to the knowledge of *Campylobacter* spp. worldwide.

Chapter Five showed the commonly employed EIA test for the diagnosis of *C. jejuni* and *C. coli* in humans at the regional Manawatu medical laboratory to be able to detect isolates of *C. upsaliensis* and *C. helveticus* from local dogs and cats, as well as *C. hyointestinalis* from local deer. This supports the possibility of detection of the respective 'emerging' species in human clinical cases that had negative culture results, as presented in the literature review of the New Zealand data. A major current knowledge gap in New Zealand and worldwide is the extent of non-*jejuni/coli Campylobacter* spp. occurring in humans and to which extent these species are a cause of clinical signs. The data in this thesis show the frequent presence of *C. upsaliensis*, and to a lesser extent for *C. helveticus*, in the relatively small population of New Zealand dogs and cats tested. Other studies have reported these species anecdotally in New Zealand patients (348) and that the current diagnostic laboratories do not use suitable methods for their detection (53). Therefore, it is possible the samples positive by EIA at the regional Manawatu clinical laboratory that were negative by culture at ^{m}EpiLab are due to cross-reactions with non-*jejuni/coli Campylobacter* species. There is also a possibility of viable-but-non-culturable bacteria or the analytical sensitivity (*e.g.*, influenced by parameters observed in Chapter Five) of the methods being the cause of their discordant results. The possibility of the EIA test detecting non-*Campylobacter* spp. also exists but as discussed in Chapter Five this is perhaps the least likely as so far the EIA has not

been reported to show such cross-reactions. Future research, both on retrospective data of ^{m}EpiLab and by prospective studies, is needed to explain these outstanding issues. Current data would support the EIA being used as a screening test after which positive results should be confirmed by other methods that enable speciation of results to suit the desired clinical aim of whether only *C. jejuni/coli* are of interest or a wider range of *Campylobacter* species.

The selection of *Campylobacter* spp. of clinical interest to humans for diagnostic laboratories is dependent on the documented pathogenicity, or at least strongly supportive data of association with a pathogenic potential in order to avoid false positive results. Chapters Six and Seven investigated the pathogenic potential of *Campylobacter* spp. commonly detected in dogs and cats. However, the provision of more isolates of *C. upsaliensis* and *C. helveticus*, especially from sources other than dogs and cats would greatly benefit studies that could expand on results from Chapters Six and Seven. As discussed in the respective chapters, the lack of isolates from other sources were the main limitation of both Chapters, as is for the reported previous studies discussed therein. The extension of the *G. mellonella* infection model to both *C. upsaliensis* and *C. helveticus* shown in Chapter Six is promising for future research of the two species but also suggests that inclusion of other emerging *Campylobacter* spp. may be possible. Extending the model to other *Campylobacter* spp. is of importance from the public health perspective, as dogs were previously shown positive for 14 *Campylobacter* species (359) that have been associated with clinical signs in humans (13); most of which were not evaluated in this thesis. With regard to the higher survival of larvae inoculated with *C. upsaliensis* and *C. helveticus* compared to *C. jejuni*, future studies using gene insertion/knockouts could particularly help elucidate causes of these observations. The application of the model to *C. upsaliensis* and *C. helveticus* in Chapter Six was supported by the results of histopathology, cultures of larval haemolymph and haemocytes, and modelling of survival parameters. One of the more interesting findings of Chapter Six was also the violations of the assumption of proportional hazard rate between *Campylobacter* species, which questions the selection of time points for inference of results. These are all newly reported observations, but the results also support more research into other aspects of the larval model such as the comparability of the larvae model with

other models, cell lines and animal (mammalian and avian) models of disease. Finally, the extent to which the results from larval model correlate with and are truly representative of the human host needs to be clarified.

Chapter Six showed how inactivated cellular material may lead to the death of larvae with a similar survival curve to the inoculation of live cells of *C. upsaliensis* and *C. helveticus* but not of *C. jejuni*. The reasons for these differences may be important for the inference of pathogenic potential using the larval model. In light of the overall results of Chapter Six, what should actually be inferred of the pathogenicity of *C. upsaliensis* and *C. helveticus* using the larval model if these species are rapidly and successfully eliminated by the larval immune cells and death is being caused by a mere antigen-induced damage due to an overload of an arbitrary experimental dose used? The application of transcriptome studies (546) may be helpful in elucidating this differential larvae response between *Campylobacter* spp. and to identify which cellular components activate, or suppress, the larval immune system mechanisms (451). *G. mellonella* is a relatively new infection model and Chapter Six also showed important sources of variation in survival estimates, important for the reproducibility of results such as the effects of batches and biological replicates. Further validation studies of the larval model could investigate the inter- and intra-observer variability in the assessment of larval morbidity and mortality. An important limitation of internal validity in Chapter Six and other reported studies of *C. jejuni* using larvae is the lack of double blinding in the experimental procedure, which could be also further addressed by future studies.

The results of Chapter Seven have shown many differences between the genomes of *C. jejuni*, *C. upsaliensis* and *C. helveticus* strains that neatly separated to their designated species shown by phylogenetic analyses using the core genome and the rMLST scheme. Differences were observed in general genomic features such as genome size and the number of genes, and GC content but also in functional analysis of genomes using analysis of Clusters of Orthologous Groups (COGs), presence/absence of genes with a documented involvement in pathogenicity of *C. jejuni*, and in gene content profile related to pathogenicity by RAST/SEED annotation server results and bioinformatics software for the prediction of pathogenic proteins.

Apart from genome-based studies, these results may be used to facilitate further research into pathogenicity using the larval model, and other models of disease. The search for genes involved with pathogenicity by gene insertion/knockout experiments is difficult due to the numerous possible candidate genes that are available. Comparative genomic studies of different *Campylobacter* spp. with varying pathogenic potential should be helpful for prioritising the candidate genes. The results in Chapter Seven suggest that some of the most likely candidate genes could be those involved with the oxidative stress response that were shown to be important to *C. jejuni* both for aerotolerance, and survival from the attack by the innate immune system (160). Therefore, testing this hypothesis further would be interesting research to perform in both the larvae model and human immune cell lines to evaluate the representativeness of larvae as a model of the mammalian innate immune system.

Chapter Seven also showed how various reported pathogenicity features may vary between *C. jejuni*, *C. upsaliensis*, and *C. helveticus* by COG functional gene content profile, toxin-antitoxin systems, restriction-modification systems, and predicted pathogenic protein profiles. Currently, it is not known if these features reported as pathogenic signatures are truly valid for *Campylobacter* spp. but herein were shown to be present at the level of the pan-genome, the core and accessory genomes and singleton genes. The results between the three *Campylobacter* spp. in Chapter Seven have shown that these pathogenicity-associated features may vary; some features show *C. jejuni* strains as the more pathogenic while others show *C. upsaliensis* and *C. helveticus* strains as a more pathogenic species. Therefore, apart from the non-applicability to *Campylobacter* spp., the results may also support both the genome reduction and the virulent gene repertoire theories of the emergence of pathogenicity as present in these *Campylobacter* spp. as well as that all of them have pathogenic potential, and the discordances observed were due to the comparison of a pathogen (*C. jejuni*) with other pathogens (*C. upsaliensis* and *C. helveticus*) rather than a pathogen with non-pathogens. Alternatively, the differences in pathogenicity of these *Campylobacter* spp. to humans may not be due to properties of the bacteria but rather due to the host itself or complex host-pathogen interactions that may include influences of diet (620), microbiome (621) and other factors in the development of disease. It is important to note that these factors cannot be

accounted for by studies using genome data alone. Combining the results of genome studies with phenotypic features related to pathogenic mechanisms within the genome-wide association studies framework in the *Campylobacter* genus might be a promising approach for the investigation of pathogenicity as was shown with *Staphylococcus aureus* (622).

Pathogenicity is a broad concept and it has been argued to be an obsolete term considering that any bacteria may cause a disease given the appropriate circumstances (623). *C*. *jejuni*-associated disease development in humans has been shown to be strain dependent (149) but also dependent on many other factors such as pre-existing exposure and immunity (88), and colonisation by other bacteria such as *E. coli* (149). Therefore, the indications of the pathogenic potential of *C. upsaliensis* and *C. helveticus* for humans in Chapter Six and Seven should be cautiously interpreted in relation to the clinical outcomes in humans upon exposure thereof. Pathogenic potential may be related to other clinical syndromes than gastroenteritis, such as those reported in cases with bacteraemia associated with certain *C. jejuni* STs (549, 624). *C. jejuni* subsp. *doylei* has been associated with bacteraemia in humans, which was attributed to the absence of genes involved with metabolic, transport and virulence functions compared to strains of *C. jejuni* subsp. *jejuni* (625). One study reported functional similarities between *C. upsaliensis* and *C. jejuni* subsp. *doylei*, although only one genome of each species was compared (577). *C. upsaliensis* has been reported to be associated with bacteraemia in humans (626), and other extra-intestinal diseases such as, sepsis (627), haemolytic–uraemic syndrome (628) and Guillain–Barré syndrome (514, 629). However, a study reported all four stool isolates of *C. upsaliensis* as susceptible to the bactericidal activity of normal human serum, whereas seven of eight isolates from blood displayed resistance, which suggests more invasive infections occur only if host immune defences mount a suboptimal response to an infecting strain (626). Future studies whether epidemiological, or genome based or of experimental disease model design all need to take into account various complexities of the pathobiology of *Campylobacter* species.

Similarly to the issues of which clinical outcomes relate to the pathogenic potential, there are also limitations in extrapolation to the host species. The studies in Chapter Six and Seven provided associative data on the pathogenic potential of *Campylobacter* spp. and evidence needed for explaining the results observed will require more targeted approaches. With regard to Chapter Six, it would be unreasonable to claim that the pathogenic potential of the *Campylobacter* spp. shown is applicable to all mammal species because of larvae being a model of the mammalian innate immune system. In Chapter Seven, the validity of inferences of pathogenic potential of *Campylobacter* spp. were shown as potentially compromised because the studies that reported gene content associations with pathogenic signatures are performed on several bacterial genera and did not include the *Campylobacter* genus (582-584, 586). While these studies intended to investigate pathogenicity from a human standpoint, the question is to what extent does this relate to other hosts that may or may not be clinically affected by the bacteria in question.

Evolution of *Campylobacter* spp. is considered (co-)related to their primary animal reservoirs. Thus it is more reasonable to postulate that pathogenicity to humans could be a by-product of unrelated evolutionary processes rather that the result of positive selection for human pathogenicity alone. Therefore, grouping various skin, respiratory and intestinal pathogens in the above pathogenicity studies may be confounded by co-evolution of bacteria with their reservoirs or perhaps with the host's microbiome of the colonized/invaded tissues. For these reasons it would be fruitful for future genome-wide association studies to account for the type of host (reservoirs and transient carriers), and the host's intestinal microbiome and/or immunity in addition to the pathogen in question. A research topic could be formed around the issue as the following: "Which processes lead *C. upsaliensis* to have dogs as reservoirs yet (accidentally) cause disease in humans, compared to processes that lead *C. jejuni* to have poultry as their reservoir while being variably pathogenic between dogs and people?" These questions may help explain differences in the gene content profiles between *Campylobacter* spp., shown in Chapter Seven, when species are compared in general (as in general biology of species and their lifestyles) versus relations to an arbitrary phenotypic feature such as pathogenicity to humans.

Despite these limitations the results and the amount of data provided in Chapters Six and Seven will be of benefit to future research. Important limitations of both Chapters Six and Seven are related to their novelty, as there are no reported studies to compare the results with. In Chapter Six this was due to the first use of *C. helveticus* in disease models, and the sequencing of the *C. helveticus* genome for Chapter Seven. As reviewed in Chapter Two research data on disease models and genome studies for *C. upsaliensis* are also very limited. Recently a power calculator for genome-wide associations study has been published, which can help future research to avoid limitations of reported studies that used a few or an arbitrary chosen number of genomes (630).

In conclusion, the research Chapters in this thesis immensely increased our knowledge of epidemiology of *Campylobacter* spp. in dogs and cats of New Zealand and their significance to public health. The data also confirmed some previous research findings and overall, this thesis is expected to significantly inform and, hopefully, encourage future research.

CHAPTER 9

9. References

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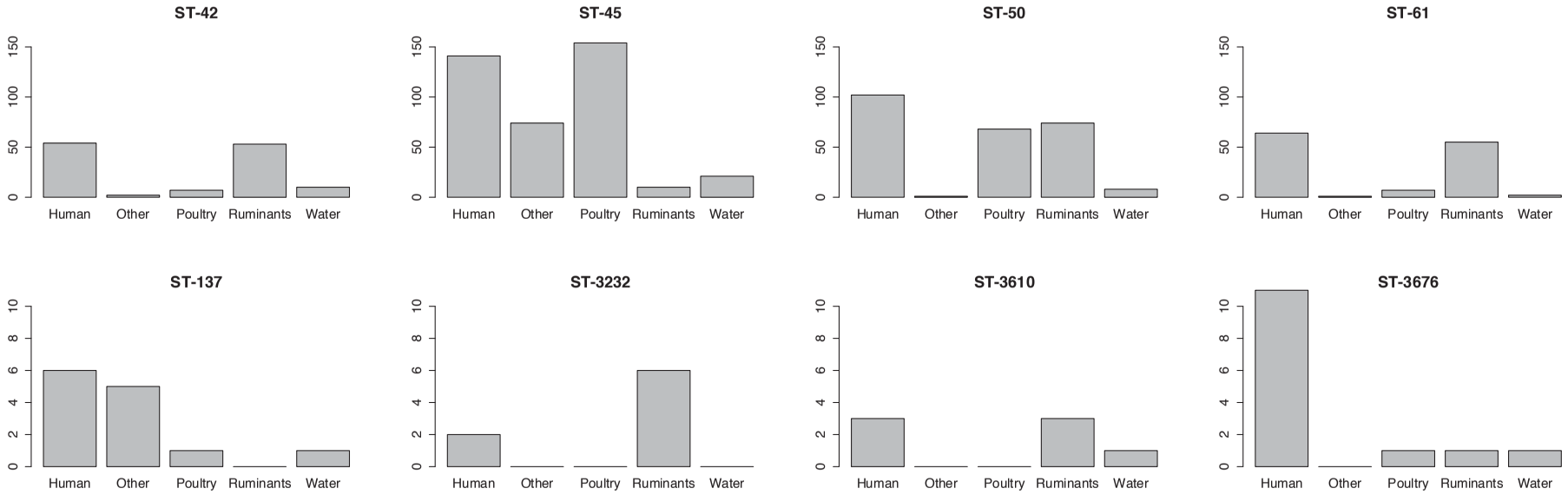
CHAPTER 10

10. Appendix

Supplemental Table 3.1. PCR assays used on isolates from dogs, cats and retail raw meat pet food products.

PCR assay	Primer sequences	Target gene (product size in base pairs)	Amplification conditions[a]	Reference
Campylobacter spp.	C412F: 5′ GGATGACACTTTTCGGAGC 3′ C1228R: 5′ CATTGTAGCACGTGTGTC 3′	16S rRNA (816)	40 cycles annealing at 56°C	(490)[b]
C. jejuni	MapAF: 5′ CTTGGCTTGAAATTTGCTTG 3′ MapAR: 5′ GCTTGGTGCGGATTGTAAA 3′	*mapA* (603)	38 cycles annealing at 60°C	(631)
C. coli	F: 5′ AATTGAAAATTGCTCCAACTATG 3′ R: 5′ TGATTTTATTATTTGTAGCAGCG 3′	*ceuE* (462)	38 cycles annealing at 58°C	(492)
C. upsaliensis / *C. helveticus*	CHCU146F: 5′ GGGACAACACTTAGAAATGAG 3′ CU1024R: 5′ CACTTCCGTATCTCTACAGA 3′ CH1371R: 5′ CCGTGACATGGCTGATTCAC 3′	16S rRNA (878 / 1225 or 1375)	35 cycles annealing at 55°C	(490)
C. fetus / *C. hyointestinalis*	CFCH57F: 5’ GCAAGTCGAACGGAGTATTA 3′ CF1054R: 5’ GCAGCACCTGTCTCAACT 3′ CH1344R: 5’ GCGATTCCGGCTTCATGCTC 3′	16S rRNA (997 / 1287)	35 cycles annealing at 55°C	(490)
C. lari	CL594F: 5’ CAAGTCTCTTGTGAAATCCAAC 3′ CL1155R: 5’ ATTTAGAGTGCTCACCCGAAG 3′	16S rRNA (561)	35 cycles annealing at 55°C	(490)
Arcobacter butzleri	AB959F: 5’ CCTGGACTTGACATAGTAAGAATGA ’3 AB1338R: 5’ CGTATTCACCGTAGCATAGC ’3	16S rRNA (401)	35 cycles annealing at 61°C	(493)
16S rRNA	27F: 5’ AGAGTTTGATCMTGGCTCAG ’3 1492R: 5’ GGTTACCTTGTTACGACTT ’3	16S rRNA (1465)	30 cycles annealing at 58.5°C	(494)

[a] All PCR assays had an initial denaturation at 96°C and a final extension at 72°C for 2 minutes each. All amplification cycle steps were 30 seconds in duration, and all elongation and extension steps were at 96°C and 72°C respectively. [b] (474) reported a typographical error in the paper by Linton et al. (1996): primer C1288R should read C1228R.



Supplemental Fig. 4.1. Occurrence of *Campylobacter jejuni* MLST types isolated in working farm dogs from Manawatu, New Zealand, across all sources from the ^{m}EpiLab database (N = 950).

Supplemental Table 7.1. List of strains and related metadata of *Campylobacter* spp. genomes used in the study.

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
ACP175a	*C. helveticus*	cat	NZ	NA	NA	This study
ACP123b	*C. helveticus*	cat	NZ	NA	NA	This study
ACP102b	*C. helveticus*	cat	NZ	NA	NA	This study
ACP108a	*C. helveticus*	cat	NZ	NA	NA	This study
ACP114b	*C. helveticus*	cat	NZ	NA	NA	This study
ACP183a	*C. helveticus*	cat	NZ	NA	NA	This study
ACP110b	*C. helveticus*	cat	NZ	NA	NA	This study
ACP141a	*C. helveticus*	dog	NZ	NA	NA	This study
RM3228	*C. helveticus*	cat	EU	NA	NA	This study
ACP170b	*C. upsaliensis*	cat	NZ	NA	NA	This study
ACP136a	*C. upsaliensis*	cat	NZ	NA	NA	This study
ACP179b	*C. upsaliensis*	cat	NZ	NA	NA	This study
ACP5b	*C. upsaliensis*	dog	NZ	NA	NA	This study

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
BD16e4a	*C. upsaliensis*	dog	NZ	NA	NA	This study
ACP19b	*C. upsaliensis*	dog	NZ	NA	NA	This study
RM3195	*C. upsaliensis*	human	NA	NA	NA	GenBank
JV21	*C. upsaliensis*	human	NA	NA	NA	GenBank
DSM5365	*C. upsaliensis*	dog	EU	NA	NA	GenBank
28080.46	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.19	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.20	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.21	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.22	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.23	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.24	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.26	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.27	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.28	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.30	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
28080.31	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.32	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.33	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.34	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.35	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.36	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.37	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.38	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.39	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.40	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.41	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.44	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
28080.45	*C. upsaliensis*	dog	EU	NA	NA	RAST/SEED
NCTC_11168	*C. jejuni*	human	UK	ST-43	CC-21	PubMLST
ATCC_33560	*C. jejuni*	cattle	EU	ST-403	CC -403	PubMLST
RM1221	*C. jejuni*	chicken	USA	ST-354	CC -354	PubMLST

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
81-176	*C. jejuni*	human	USA	ST-913	CC -42	PubMLST
81116	*C. jejuni*	human	UK	ST-267	CC -283	PubMLST
H1924	*C. jejuni*	human	NZ	ST-45	CC -45	This study
H1969	*C. jejuni*	human	NZ	ST-61	CC -61	This study
H22082	*C. jejuni*	human	NZ	ST-474	CC -48	This study
H550	*C. jejuni*	human	NZ	ST-42	CC -42	This study
S331b	*C. jejuni*	cattle	NZ	ST-21	CC -21	This study
H892	*C. jejuni*	human	NZ	ST-48	CC -48	This study
OXC6368	*C. jejuni*	human	UK	ST-52	CC -52	PubMLST
H1605	*C. jejuni*	human	NZ	ST-1517	CC -354	This study
H28548	*C. jejuni*	human	NZ	ST-2026	CC -403	This study
OXC6314	*C. jejuni*	human	EU	ST-45	CC -45	PubMLST
OXC6388	*C. jejuni*	human	EU	ST-42	CC -42	PubMLST
OXC6270	*C. jejuni*	human	EU	ST-21	CC -21	PubMLST
OXC6268	*C. jejuni*	human	EU	ST-61	CC -61	PubMLST
OXC6328	*C. jejuni*	human	EU	ST-48	CC -48	PubMLST

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
OXC6250	*C. jejuni*	human	EU	ST-257	CC -257	PubMLST
GCB471_S1_4724	*C. jejuni*	wildbird	NZ	ST-583	CC -45	This study
P104a	*C. jejuni*	chicken	NZ	ST-45	CC -45	This study
OXC6413	*C. jejuni*	human	EU	ST-429	CC -48	PubMLST
S276b	*C. jejuni*	cattle	NZ	ST-61	CC -61	This study
S263a	*C. jejuni*	cattle	NZ	ST-42	CC -42	This study
W194b	*C. jejuni*	water	NZ	ST-2381	CC -2381	This study
Seal_88	*C. jejuni*	seal	EU	ST-696	CC -1332	PubMLST
OXC6318	*C. jejuni*	human	EU	ST-356	CC -353	PubMLST
197.897	*C. jejuni*	dog	EU	ST-1326	CC -45	RAST/SEED
OXC6264	*C. jejuni*	human	EU	ST-22	CC -22	PubMLST
M880a	*C. jejuni*	sheep	NZ	ST-2341	CC -61	This study
GCB1275_S20_4692	*C. jejuni*	wildbird	NZ	ST-1304	CC -1304	This study
OXC6330	*C. jejuni*	human	EU	ST-583	CC -45	PubMLST
W135a	*C. jejuni*	water	NZ	ST-3655	NA	This study

Strain name	Species	Isolation source	Location	Sequence type (ST)	Clonal complex (CC)	Sourced from
H798	*C. jejuni*	human	NZ	ST-50	CC -21	This study
H1606	*C. jejuni*	human	NZ	ST-51	CC -443	This study
B1410	*C. jejuni*	wildbird	NZ	ST-177	CC -177	This study
H773	*C. jejuni*	human	NZ	ST-3711	CC -257	This study
H_15AR0710	*C. jejuni*	chicken	NZ	ST-6964	CC -354	This study
P722b	*C. jejuni*	chicken	NZ	ST-25	CC -45	This study
P110b	*C. jejuni*	chicken	NZ	ST-474	CC -48	This study
R75a	*C. jejuni*	wildbird	NZ	ST-1324	NA	This study

Supplemental Table 7.2. Number of strains (and number of genes) in *Campylobacter* spp. for genes documented to be involved or associated with pathogenicity of *C. jejuni*.

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
CadF	Fibronectin-binding outer membrane protein	COG2885	M	Adhesion	42	3.19	33	2.97	9	3	(160)
Fn3	Fibronectin type III domain	COG3401	S	Adhesion	42	1	33	1	9	1	(160)
PldA	Phospholipase A	COG2829	M	Adhesion	42	1	33	1	9	1	(160)
Peb1A	Periplasmic binding protein	COG0834	E	Adhesion	42	4	33	3	9	3	(160)
Peb3	Transport protein, accessory colonization factor ACFC	COG4588	S	Adhesion	42	2.1	33	1.03	9	1	(160)
Peb4	Chaperon important for export of proteins to the outer membrane	COG0760	O	Adhesion	42	3.12	33	2.94	9	3	(160)
FlpA	Fibronectin-like protein, Fibronectin-binding A domain protein	COG1293	K	Adhesion	42	1	33	1	9	1.11	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
Rac1	GTPases that induce internalisation	COG1100	S	Adhesion	0	0	0	0	0	0	(160)
Cdc42	GTPases that induce internalisation	COG1100	S	Adhesion	0	0	0	0	0	0	(160)
cj0588	Homologue of haemolysin, putative virulence factor	COG1189	J	Adhesion	42	1	32	1	9	1	(21)
p95	Filamentous hemagglutinin family outer membrane protein	COG3210	U	Adhesion	40	2.85	18	1.28	0	0	(21)
MOMP	Major outer membrane protein	COG2885	M	Adhesion	42	3.19	33	2.97	9	3	(21)
VirB11	Type IV secretory pathway component	COG0630	U	Adhesion/ Secretion systems	12	1.33	23	1.26	8	1.88	(160)
KpsE	Capsular biosynthesis gene	COG3524	M	Capsule	42	1	32	1	9	1	(160)
CheA	Chemotaxis proteins histidine kinase	COG0643	n/d	Chemotaxis	42	1	32	1	9	1.11	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
CheB	Chemotaxis proteins	COG2201	n/d	Chemotaxis	42	1.43	32	1	9	1	(160)
CheR	Chemotaxis proteins	COG1352	n/d	Chemotaxis	42	1.88	32	1.78	9	1	(160)
CheV	Chemotaxis proteins	COG0784	T	Chemotaxis	42	2.38	32	1.25	9	1	(160)
CheW	Chemotaxis proteins	COG0835	n/d	Chemotaxis	42	1	32	1	9	1	(160)
CheZ	Chemotaxis proteins	COG3143	n/d	Chemotaxis	42	1	33	1	9	1	(160)
Tlp1/4/10	Methyl-accepting chemotaxis proteins, Tar conserved domain	COG0840	n/d	Chemotaxis	42	11.83	33	7.61	9	9.67	(160)
CheY	Response regulator controlling flagellar rotation	COG0784	T	Chemotaxis	42	2.38	32	1.25	9	1	(160)
CetB	Campylobacter energy taxis system proteins	COG2202	T	Chemotaxis	2	1	0	0	0	0	(160)
LuxS	AI-2 biosynthesis enzyme	COG1854	T	Chemotaxis	42	1	33	1	9	1	(160)
IamA	Invasion associated protein	COG1127	Q	Invasion	42	1	32	1	9	1	(160)
HtrA	Chaperon involved in folding of adhesins	COG0265	O	Invasion	42	2.17	33	2	9	2	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
VirK	Possible protection against antimicrobials and virulence factor in mouse model	COG2990	S	Invasion	42	1.1	0	0	9	1	(160)
cj1496c	Glycoprotein involved in colonization and invasion	COG3334	S	Invasion	41	1	31	1	9	1	(21)
cj1121c	Aminotransferase involved in motility and invasion in chicken	COG0399	E	Invasion	42	3.98	33	3.55	9	3.78	(21)
cj0596	Periplasmic isomerase, involved in invasion and colonization	COG0760	O	Invasion	42	3.12	33	2.94	9	3	(21)
cj0143c / ZnuA	Zinc ABC transport system, involved in colonization	COG0803	P	Invasion/ Adhesion	42	1	33	1	9	1	(21)
GalE	Lipopolysaccharide synthesis, involved in invasion and adhesion	COG1087	M	Invasion/ Adhesion	42	1.24	33	1.09	9	1	(21)
KpsM	Capsular glycan	COG1682	V	Invasion	41	1	33	1	9	1	(21)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
cj0977	Sigma-28 factor, non-flagella gene involved in invasion and disease	COG2050	Q	Invasion/ Adhesion	42	1	33	1	9	1	(21)
HspR/ SoxR	Transcriptional regulator, involved in invasion and adhesion, part of MerR family	COG0789	K	Invasion/ Adhesion	42	2.02	33	2.88	9	1.78	(21)
Ppk	Polyphosphate kinase, involved in intracellular proliferation and colonization	COG0855	P	Invasion/ Adhesion	42	1	33	1.21	9	1	(21)
cj0415	Gluconate dehydrogenase involved in colonization in chicken	COG2303	E	Invasion/ Adhesion	42	1.55	0	0	0	0	(21)
cj1461	DNA methyltransferase involved in adhesion and invasion	COG0742	L	Invasion/ Adhesion	41	1	30	1	9	1	(21)
CsrA	Carbon starvation regulator	COG1551	T	Invasion/ Adhesion	42	1	31	1	9	1	(21)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
ClpP	ATP-dependent protease involved in motility and invasion	COG0740	O	Invasion/ Motility	42	1	32	1	9	1.67	(21)
FlhA	Components of the flagellar Type III secretion system	COG1298	N	Invasion/ Secretion systems	42	1	32	1	9	1	(160)
FlhB	Components of the flagellar Type III secretion system	COG1377	N	Invasion/ Secretion systems	42	1	33	1	9	1	(160)
FliO	Components of the flagellar Type III secretion system	COG3190	N	Invasion/ Secretion systems	42	1	30	1	9	1	(160)
FliP	Components of the flagellar Type III secretion system	COG1338	N	Invasion/ Secretion systems	42	1	33	1	9	1	(160)
FliQ	Components of the flagellar Type III secretion system	COG3190	N	Invasion/ Secretion systems	42	1	30	1	9	1	(160)
FliR	Components of the flagellar Type III secretion system	COG1684	N	Invasion/ Secretion systems	42	1	33	1	9	1	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
FliI	Flagellar biosynthesis/ Type III secretory pathway ATPase	COG1157	n/d	Invasion/ Secretion systems	42	1.02	32	1.06	9	1	(160)
FliH	Flagellar biosynthesis/ Type III secretory pathway protein	COG1317	n/d	Invasion/ Secretion systems	42	1.02	31	1	9	1.56	(160)
FliN	Flagellar motor switch/ Type III secretory pathway protein	COG1886	n/d	Invasion/ Secretion systems	42	2	33	2.03	9	2	(160)
FlhB-like	Type III secretion system substrate exporter	COG2257	U	Invasion/ Secretion systems	42	1	33	1	9	1	(160)
CfrA	Outer membrane ferric enterobactin FeEnt receptor	COG4771	P	Iron uptake system	42	2.57	22	1	0	0	(160)
CfrB/ cj0178	Outer membrane ferric enterobactin FeEnt receptor	COG1629	P	Iron uptake system	24	1.38	2	1	4	1	(160)
CeuE	Lipoprotein involved in iron acquisition	COG4607/ 0614	P	Iron uptake system	42	1.02	33	2.27	9	1.56	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
Fur/ perR	Ferric uptake regulator	COG0735	P	Iron uptake system	42	2	33	2.24	9	2	(160)
ChuA	Outer membrane receptor for haemin and haemoglobin	COG4773	P	Iron uptake system	1	1	13	1	1	1	(160)
FeoB	involved in iron acquisition	COG0370	P	Iron uptake system	42	1.17	33	1	9	2	(21)
DmsA	Presence indicates possible genetic marker for increased pathogenicity in humans	COG0243	C	Marker	42	5.02	33	2.97	9	4	(632)
cj1365 c	Absence indicates possible decreased pathogenicity in humans	COG1404	O	Marker	29	1.14	33	1.09	0	0	(632)
FucP	Absence indicates possible decreased pathogenicity in humans	COG0738	G	Marker	19	1.16	19	1	0	0	(632)
FlaA	Major flagellin protein	annotations		Motility	42	2	33	1.06	9	1.89	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
FlaB	Major flagellin protein	annotations		Motility	23	1.61	33	1.91	7	1.71	(160)
FliM	Flagellar motor proteins	COG1868	N	Motility	42	1	33	1	9	1	(160)
FliY	Flagellar motor proteins	COG1886	N	Motility	42	2	33	2.03	9	2	(160)
FlgI	P-ring in the peptidoglycan	COG1706	N	Motility	41	1	32	1	9	1	(160)
FlgH	L ring in the outer membrane	COG2063	N	Motility	42	1	32	1	9	1	(160)
FlgE	Minor hook components	COG1749	N	Motility	42	2	33	1.97	9	2	(160)
FliK	Minor hook components	COG3144	N	Motility	42	2	31	1.71	9	2	(160)
FliA	Promoter regulating *FlaA* gene expression (sigma factor 28)	COG1191	K	Motility	42	1.02	33	1	9	1	(160)
RpoN	Promoter regulating *FlaB* gene expression (Sigma factor 54)	COG1508	K	Motility	42	1.02	32	1	9	1	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
cj1321	Acetyltransferase involved in flagellin O-linked glycosylation	COG0110	S	Motility	42	2.05	33	3.33	9	2.67	(160)
cj1322	Dehydrogenase reductase involved in flagellin O-linked glycosylation	COG1028	S	Motility	42	3.21	33	4.27	9	4	(160)
cj1324	Protein involved in flagellin O-linked glycosylation	COG0037	D	Motility	42	3.9	33	2.97	9	3	(160)
Anti-sigma 28	Negative regulator of flagellin synthesis	COG2747	n/d	Motility	14	1	32	1	9	1	This study
SpoII^{AA}	Antagonist of anti-sigma factor	COG1366	T	Motility	6	1	0	0	0		This study
FlgR	Regulator of flagellum protein biosynthesis (Sigma 54 specific)	COG2204	T	Motility	42	2	33	1.97	9	2.11	(160)
FlgS/ CprS	Regulator of flagellum protein biosynthesis	COG0642	T	Motility	42	5	33	4.12	9	4.22	(22)
FlgP	Flagellar motility	COG3018	S	Motility	42	1	33	1	9	1	(22)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
FlaC	Protein secreted into the host cells, part of Type III secretion system	COG1344	N	Motility/ Secretion systems	42	4.67	32	4.06	9	4.22	(583)
FliF	Hook-basal body proteins, part of Type III secretion system	COG1766	n/d	Motility/ Secretion systems	42	1.02	33	1.03	9	1	(583)
CmeB	Inner membrane efflux transporter component of efflux pump	COG0841	P	Multidrug resistance	42	2.17	33	2.03	9	2.22	(160)
CmeC	Outer membrane protein component of efflux pump	COG1538	M	Multidrug resistance	42	3.12	33	3.18	9	3	(160)
CmeR	Efflux pump transcriptional repressor	COG1309	K	Multidrug resistance	42	1.02	32	1.91	9	1.67	(160)
VmrA	Na+ Coupled Multidrug Efflux Pumps	annotations		Multidrug resistance	3	1.67	0	0	9	2	This study
VirB10	Type IV secretory pathway component	COG2948	U	Secretion systems	14	1.07	23	1.26	8	1.88	This study

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
VirB9	Type IV secretory pathway component	COG3504	U	Secretion systems	14	1.07	22	1.27	8	1.88	This study
VirB8	Type IV secretory pathway component	COG3736	U	Secretion systems	8	1.25	22	1.27	8	1.88	This study
VirB6	Type IV secretory pathway component	COG3704	U	Secretion systems	13	1.23	22	1.27	8	1.38	This study
VirB4	Type IV secretory pathway component	COG3451	U	Secretion systems	16	1.25	23	1.65	9	2.56	This study
VirB2	Type IV secretory pathway component	COG3838	U	Secretion systems	7	1	0	0	0	0	This study
VirD4	Type IV secretory pathway component	COG3505	U	Secretion systems	16	1.06	26	1.65	9	2.89	This study
VirD2	Type IV secretory pathway component	COG3843	U	Secretion systems	15	1.13	26	1.73	9	2.22	This study
TrbF	Type IV secretory pathway component	COG3701	U	Secretion systems	7	1	0	0	0	0	This study
TrbL	Type IV secretory pathway component	COG3846	U	Secretion systems	7	1	0	0	0	0	This study
TraF	Type IV secretory pathway protease	COG4959	n/d	Secretion systems	7	1	0	0	0	0	This study

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
TrbD	Type IV secretory pathway component	COG5268	U	Secretion systems	7	1	0	0	0	0	This study
PulD	Type II secretory pathway component *GspD/PulD* (secretin)	COG1450	U	Secretion systems	41	1.1	33	1	9	1.33	(583)
PulF	Type II secretory pathway component	COG1459	n/d	Secretion systems	41	1.1	32	1	9	1	(583)
PulG	Type II secretory pathway pseudopilin	COG2165	n/d	Secretion systems	42	2.12	33	2.94	9	3	(583)
PulE	Type II secretory pathway ATPase *GspE/PulE* or T4P pilus assembly pathway ATPase PilB	COG2804	n/d	Secretion systems	41	1.07	33	1	9	1	(583)
PulO	Prepilin signal peptidase *PulO* (Type II secretory pathway) or related peptidase	COG1989	n/d	Secretion systems	42	1.05	32	1	9	1	(583)
hcp/ TssD	Type VI secretion system effector, *hcp*1 family	COG3157	S	Secretion systems	4	1	0	0	9	1	(601)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
VgrG/ TssI/	Type VI secretion component	COG3501	S	Secretion systems	8	1.88	0	0	9	1	(601)
VasA/ TssF	Type VI secretion component	COG3519	S	Secretion systems	4	1	0	0	9	1	(601)
VasB/ TssG	Type VI secretion protein, VC_A0111 family	COG3520	S	Secretion systems	4	1	0	0	9	1.22	(601)
VasD/ TssJ	Type VI secretion component	COG3521	S	Secretion systems	4	1	0	0	9	1	(601)
VasE/ TssK	Type VI secretion protein, VC_A0114 family	COG3522	S	Secretion systems	4	1	0	0	9	1	(601)
VasF/ TssL/ IcmH	Type IV / VI secretion system protein, *DotU* family	COG3455	S	Secretion systems	4	1	0	0	9	1	(601)
VasH	Transcriptional regulator of the Type VI secretion (Sigma-54)	COG3604	K	Secretion systems	0	0	1	1	0	0	(601)
VasJ/ TssA	Type VI secretion-associated protein	COG3515	S	Secretion systems	4	1	0	0	9	1	(601)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
VasK/ TssM/ IcmF	Type VI secretion protein	COG3523	S	Secretion systems	4	1	0	0	9	1.11	(601)
VipA/ TssB	Type VI secretion protein, VC_A0107 family	COG3516	S	Secretion systems	4	1	0	0	9	1	(601)
VipB/ TssC	Type VI secretion protein, *EvpB* VC_A0108 family	COG3517	S	Secretion systems	4	1	0	0	9	1	(601)
gp25/ TssE	Type VI secretion system, lysozyme-related protein	COG3518	S	Secretion systems	4	1	0	0	0	0	(601)
VacA	Outer membrane autotransporter of the Type V secretion system	COG3468	n/d	Secretion systems	40	2.53	3	1.33	0	0	This study
SpoT	Stringent control (also associated with *RelA* and *DksA*)	COG2357	n/d	Stress response	2	1	11	1	9	1	(160)
DksA	RNA polymerase-binding factor	COG1734	J	Stress response	42	1.14	31	1	9	1	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
KatA	Catalase	COG0753	P	Stress response	42	1.02	0	0	0	0	(160)
AhpC	Alkyl hydroperoxide reductase	COG0450	V	Stress response	42	1	30	1	8	1	(160)
TrxB	Thioredoxin reductase	COG0492	O	Stress response	42	2.43	33	1.97	9	2	(55)
MsrA	Methionine sulfoxide reductase	COG0225	O	Stress response	42	1.07	0	0	0	0	(55)
IspH/ HMBPP	Oxidoreductase	COG0761	I	Stress response	42	1	32	1.03	8	1	(55)
CstA	Carbon starvation protein	COG1966	T	Stress response	42	1	33	1	9	1	(55)
Tpx	Thiol peroxidase	COG2077	O	Stress response	42	1	33	1.03	9	1	(160)
SoxW	Thioredoxin-related protein	COG2143	O	Stress response	13	1	0	0	9	1	This study
Bcp	Bacterioferritin comigratory protein	COG1225	O	Stress response	42	1.02	32	1.31	9	1	(160)

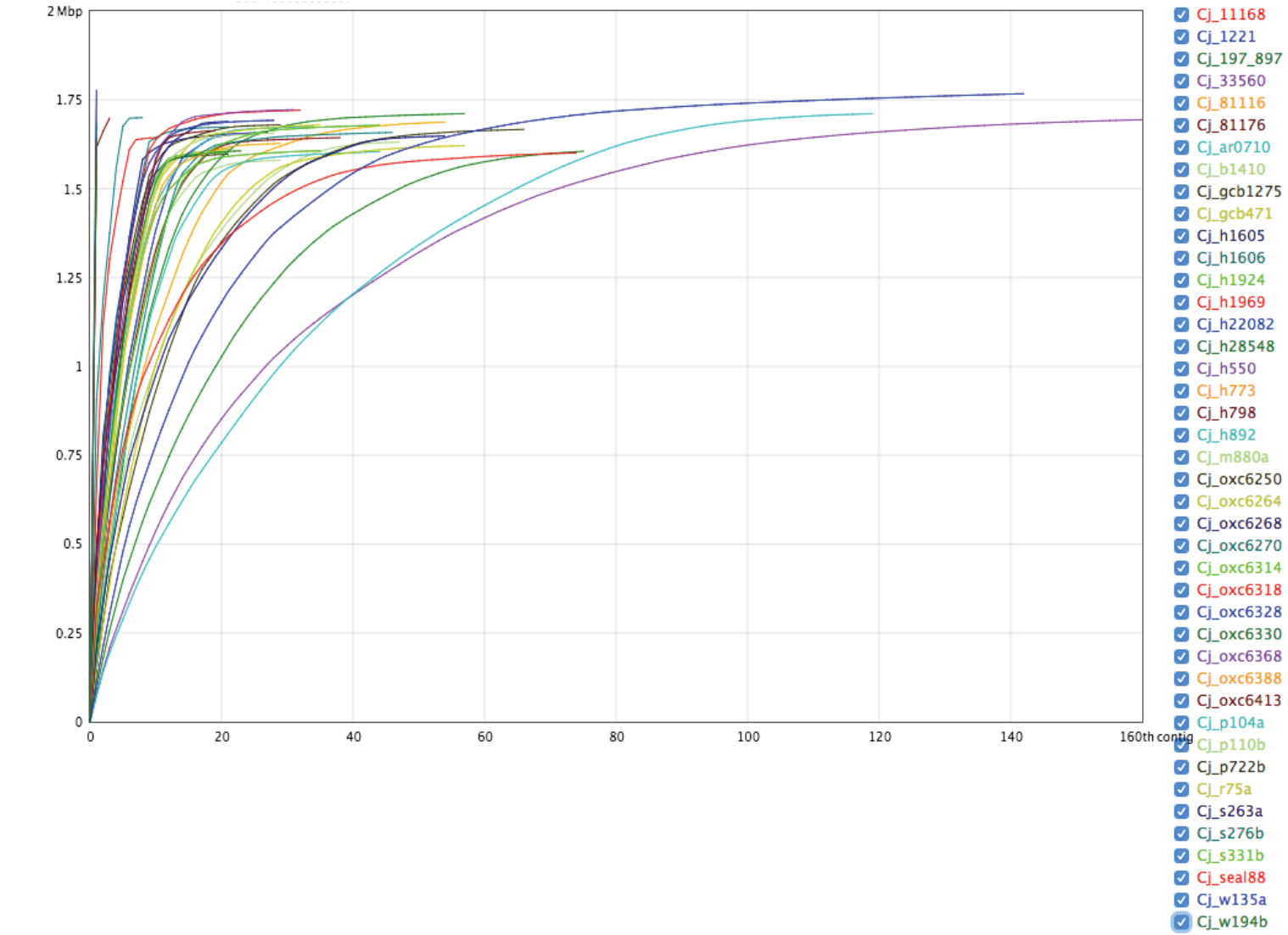
Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
cj0358/cj0020c	Cytochrome c peroxidase, conserved protein family MauG	COG1858	O	Stress response	42	2.05	32	1.06	9	1	(160)
sod	SOD proteins, antioxidant proteins	COG0605	P	Stress response	42	1	32	1	9	1	(160)
YurZ	Alkylhydroperoxidase/ carboxymuconolactone decarboxylase family	COG0599	R	Stress response	30	1.07	1	1	0	0	This study
cj1545c	NADPH quinine reductase	COG2249	R	Stress response	41	1	23	1	9	1	(160)
cj0012c	Rubrerythrin, protein protecting against reactive oxygen species	COG1592	C	Stress response	42	1	32	1	9	1	(160)
cj1371	Lipoprotein, protecting against reactive oxygen species	COG2853	M	Stress response	42	1	33	1	9	1	(160)
DnaJ	Chaperone heat shock protein	COG0484	O	Stress response	42	2.36	33	2.39	9	3	(160)
RpoD	Sigma factor 70	COG0568	K	Stress response	42	1.21	33	1.27	9	1	(160)

Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
LysR	DNA-binding transcriptional regulator (orthologous to *oxyR*)	COG0583	K	Stress response	42	1.29	4	1.25	6	1.17	This study
cj1546/ cj1556	*RrpA/RrpB*, MarR-like transcriptional regulators of response to peroxide	COG1733	K	Stress response	39	1.31	22	2.05	0	0	(525)
GroES	Heat shock protein	COG0234	O	Stress response	42	1	33	1.03	9	1	(22)
GroEL	Heat shock protein	COG0459	O	Stress response	42	1	33	1	9	1	(22)
RacR/ CprR/ CosR	Regulator of temperature-dependent signalling, negative regulation of oxidative stress	COG0745	T	Stress response	42	9.98	33	7.85	9	7.78	(22)
ggt	Gama-glutamyltranspeptidase involved in utilisation of glutamine and glutathione	COG0405	E	Stress response	13	1.15	0	0	0	0	(22)

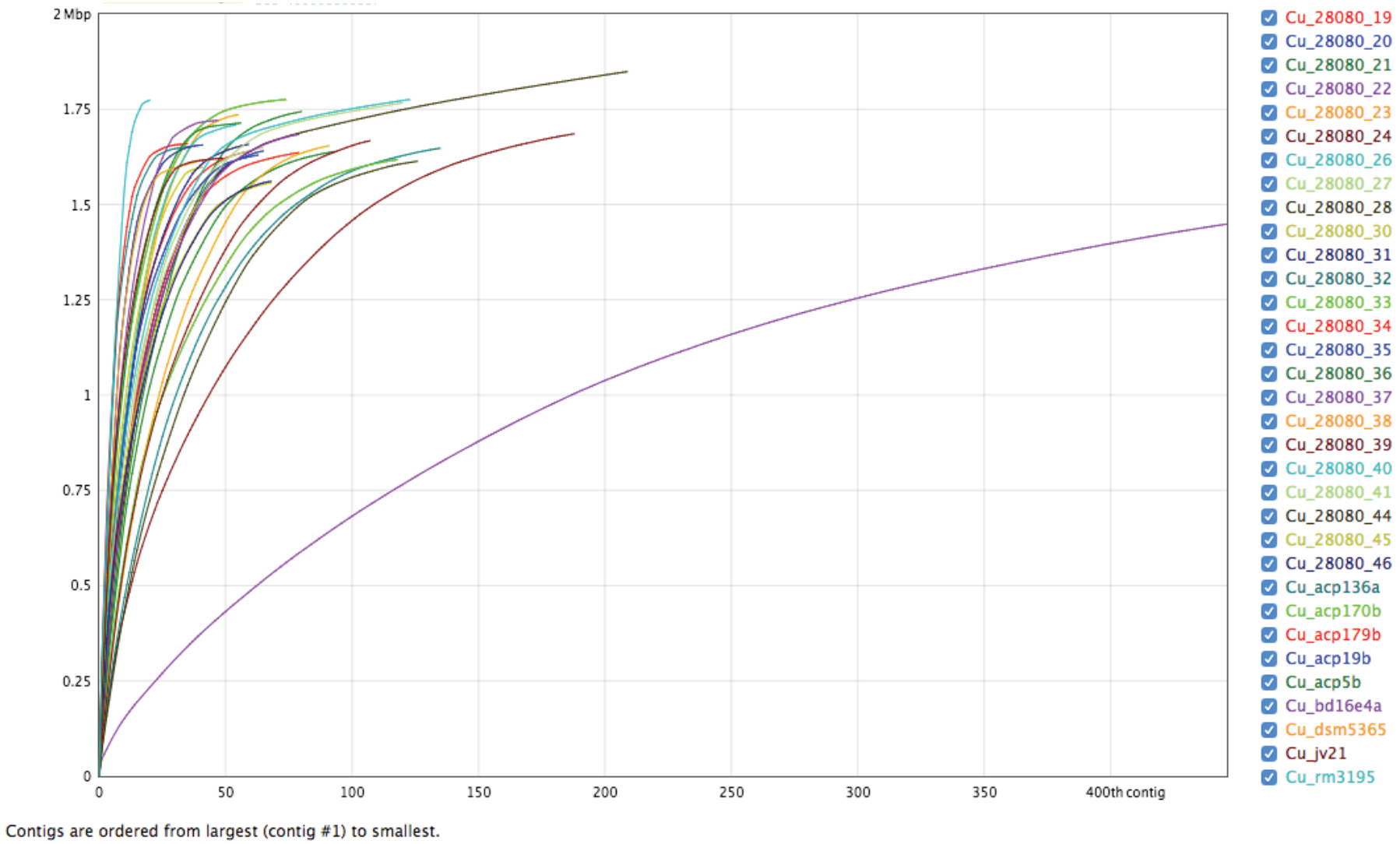
Gene	Description	COG	COG group	Mechanism	*C. jejuni* (n = 42)		*C. upsaliensis* (n = 33)		*C. helveticus* (n = 9)		Reference
					Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	Num. of strains	Mean gene num.	
AnsB	Cytoplasmic asparaginase involved in utilisation of asparagine	COG0252	E	Stress response	42	1	33	1.18	9	1.22	(22)
Lon	Lon protease, heat shock protein	COG0466	O	Stress response	42	1	32	1.03	9	1.11	(22)
CdtA	Cytolethal distending toxin subunit A	annotations	-	Toxin	40	1.05	33	1	9	1	(160)
CdtB	Cytolethal distending toxin subunit B (including homologues)	annotations	-	Toxin	42	1.1	32	1	9	1	(160)
CdtA/C	Cytolethal distending toxin A/C family	annotations	-	Toxin	40	1.05	32	1	9	1	(160)
CgtB/ WlaN	1,3 galactosyltransferases involved in lipopolysaccharide production	COG0463	M	Toxin	42	2.17	31	1.68	8	1.5	(160)

Supplemental Table 7.3. Number of predicted pathogenic proteins (and % proportion from total predictions) in genome compartments of *Campylobacter* species.

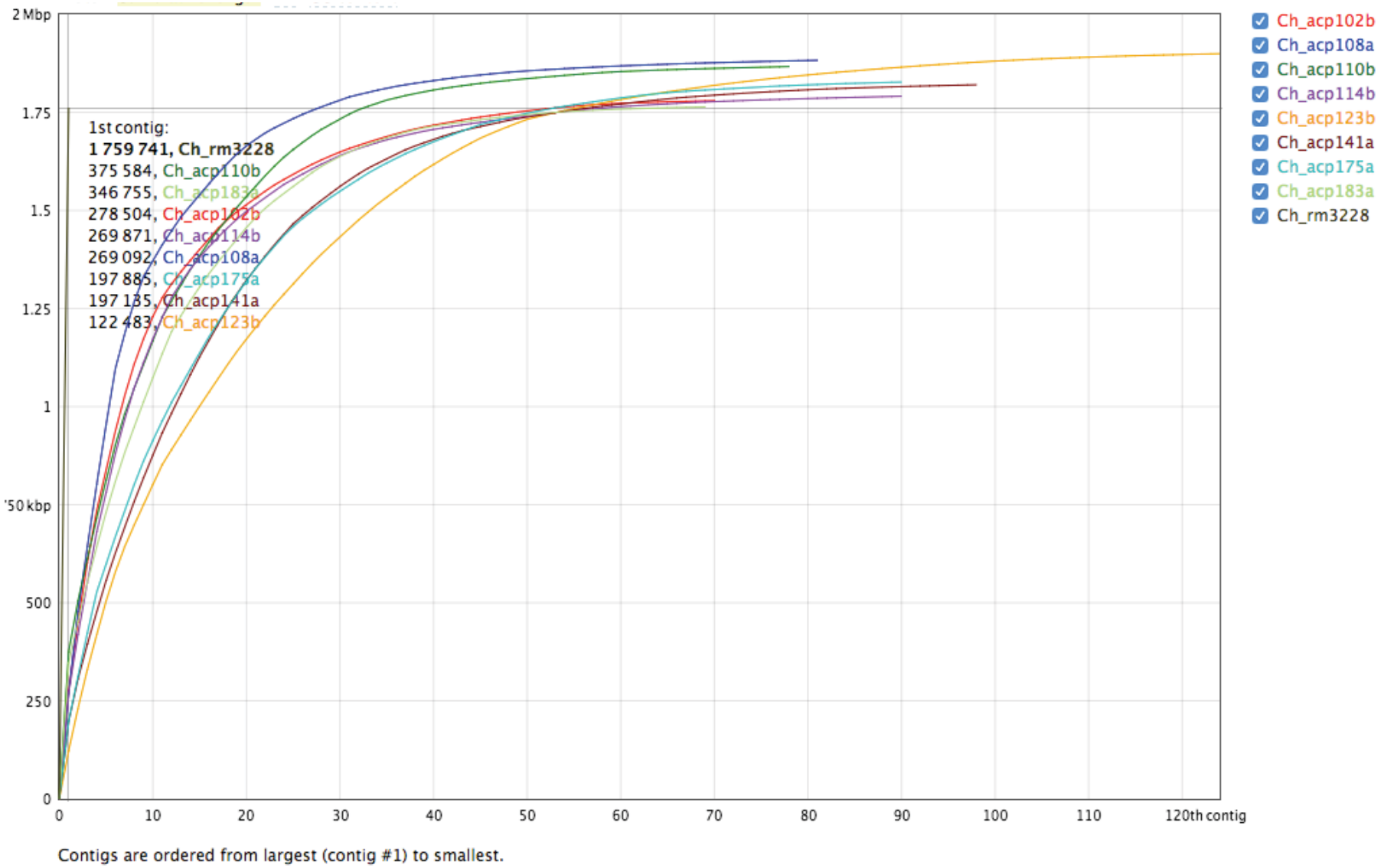
Genome feature	*C. jejuni* (n = 42)	*C. upsaliensis* (n = 33)	*C. helveticus* (n = 9)	*Campylobacter* spp. (n = 84)
A) MP3 prediction software				
Core	9,176 (16.89)	3,853 (14.42)	1,841 (14.0)	10,241 (16.89)
Accessory	2,445 (25.0)	3,069 (14.46)	240 (26.91)	10,425 (15.94)
Singletons	44 (50.57)	58 (37.18)	9 (34.62)	69 (41.57)
Pan-genome	11,665 (18.22)	6,980 (14.51)	2,090 (14.85)	126,192 (16.43)
B) PathogenFinder prediction software				
Core	15,372 (95.41)	4,254 (93.91)	1,907 (91.46)	14,146 (95.57)
Accessory	3,508 (97.36)	3,478 (91.02)	93 (85.32)	14,484 (93.59)
Singleton	22 (100)	10 (100)	6 (100)	20 (100)
Pan-genome	18,902 (95.77)	7,742 (92.60)	2,006 (91.18)	28,650 (94.56)



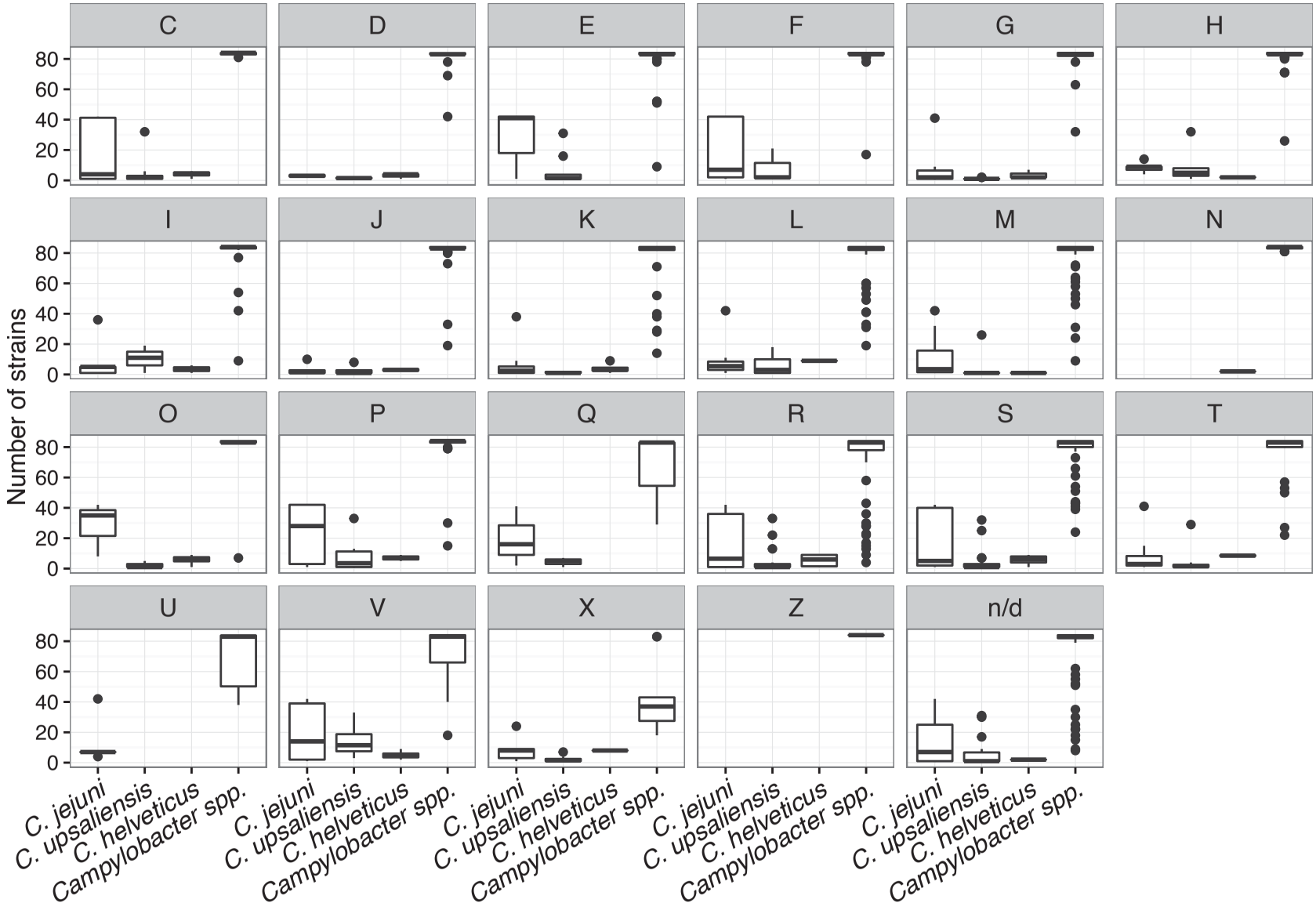
Supplemental Fig. 7.1. Cumulative contig length plots for 42 *C. jejuni* genome assemblies.



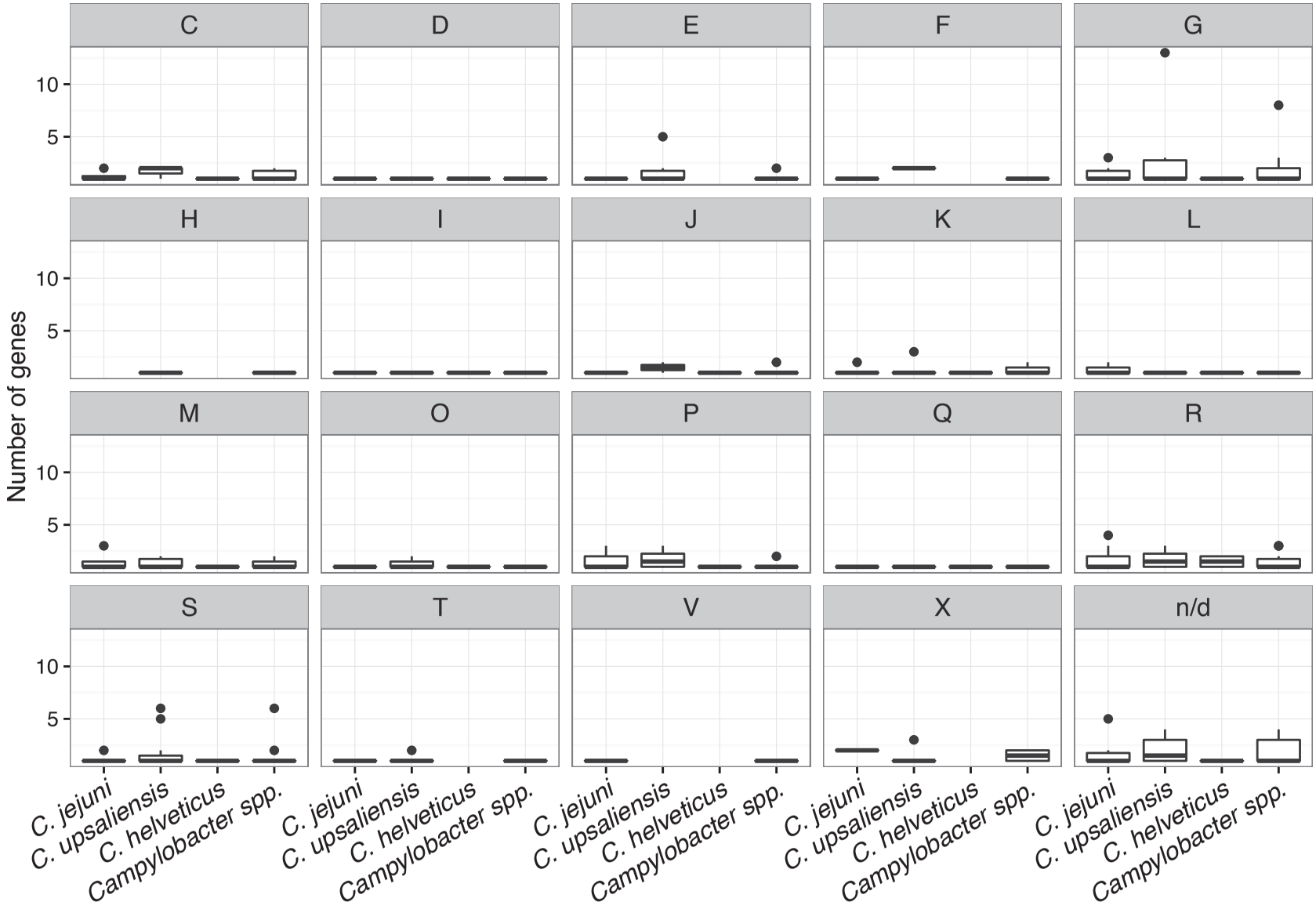
Supplemental Fig. 7.2. Cumulative contig length plots for 33 *C. upsaliensis* genome assemblies.



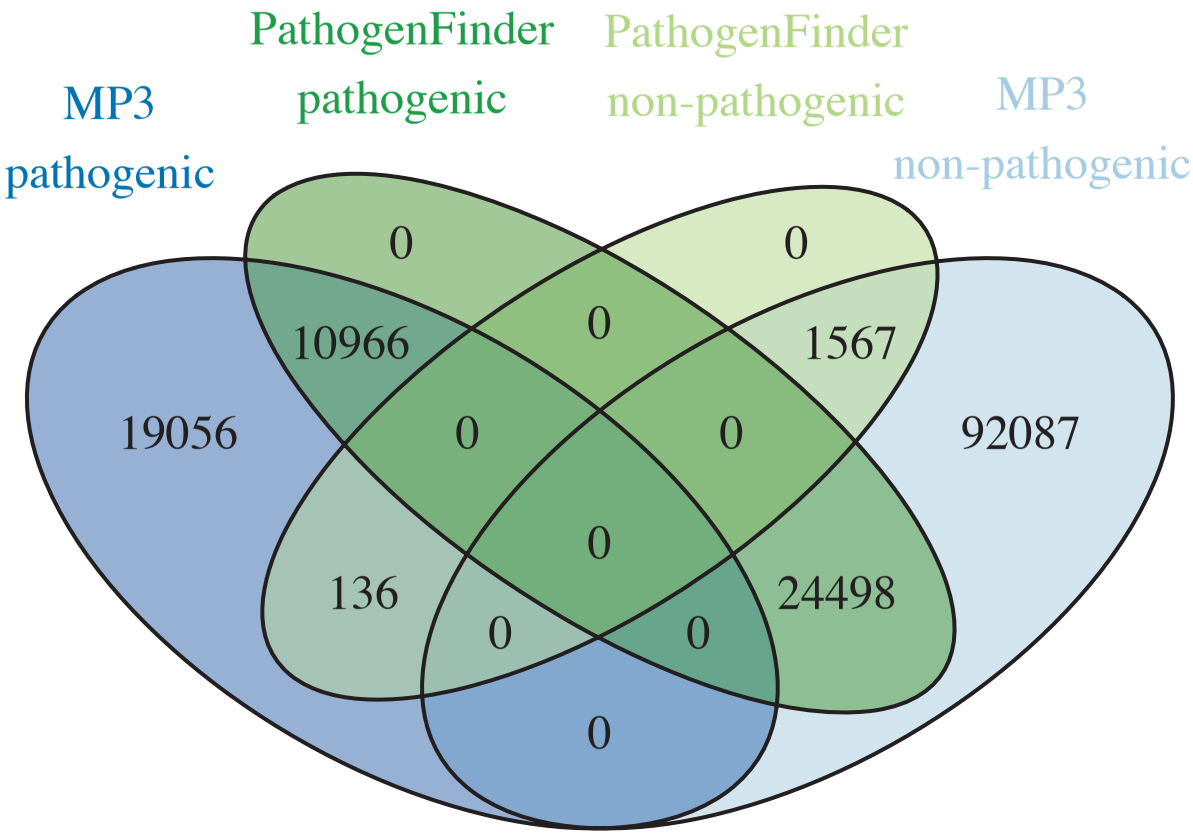
Supplemental Fig. 7.3. Cumulative contig length plots for nine *C. helveticus* genome assemblies.



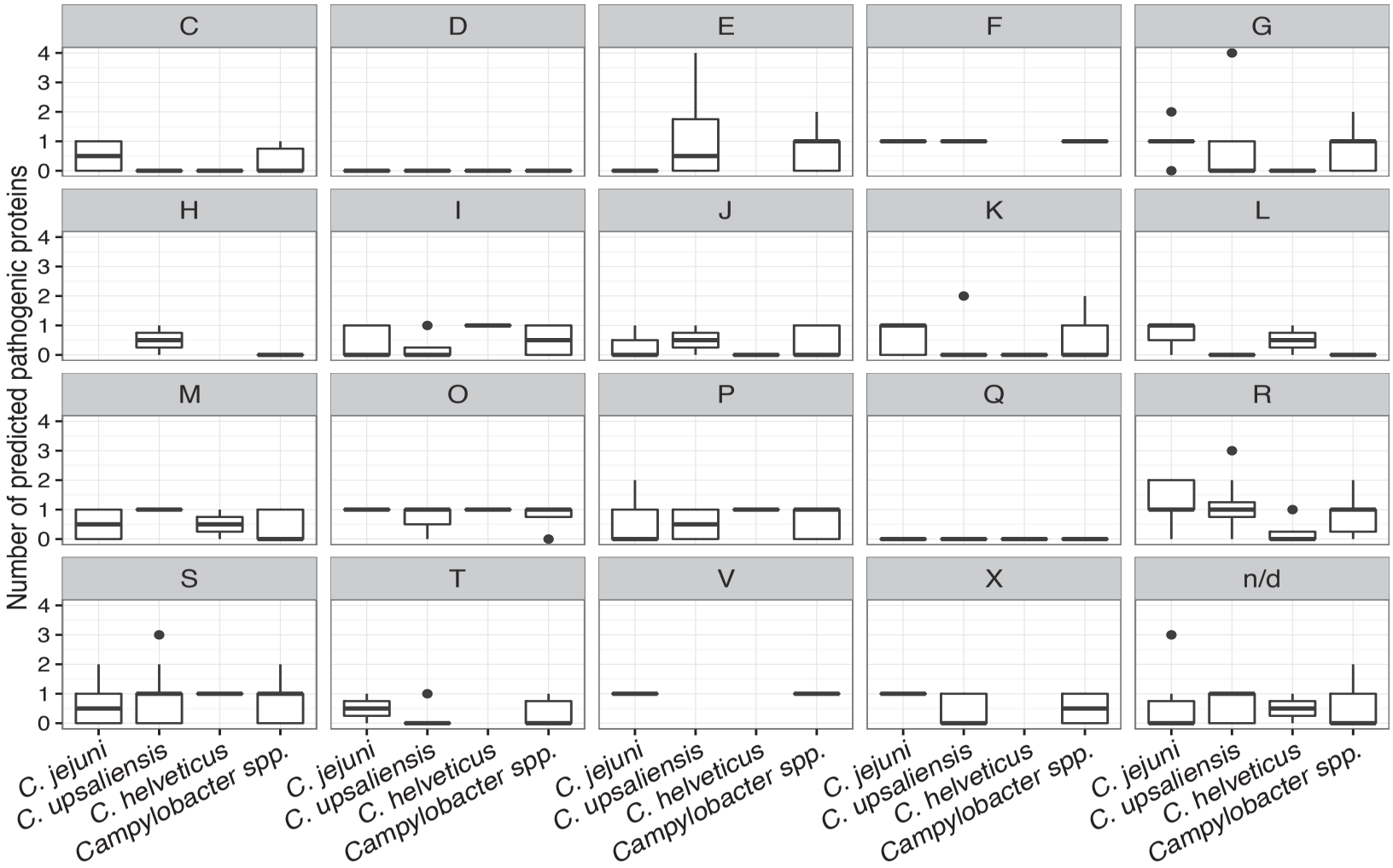
Supplemental Fig. 7.4. Distribution of the number of unique Clusters of Orthologous Groups (COG) by functional groups per strain in 42 *C. jejuni* (257 unique COGs), 33 *C. upsaliensis* (169 unique COGs) and nine *C. helveticus* (49 unique COGs) isolates**.** *Campylobacter* spp. respresents the number per strain of 1,111 COGs shared between the three species.



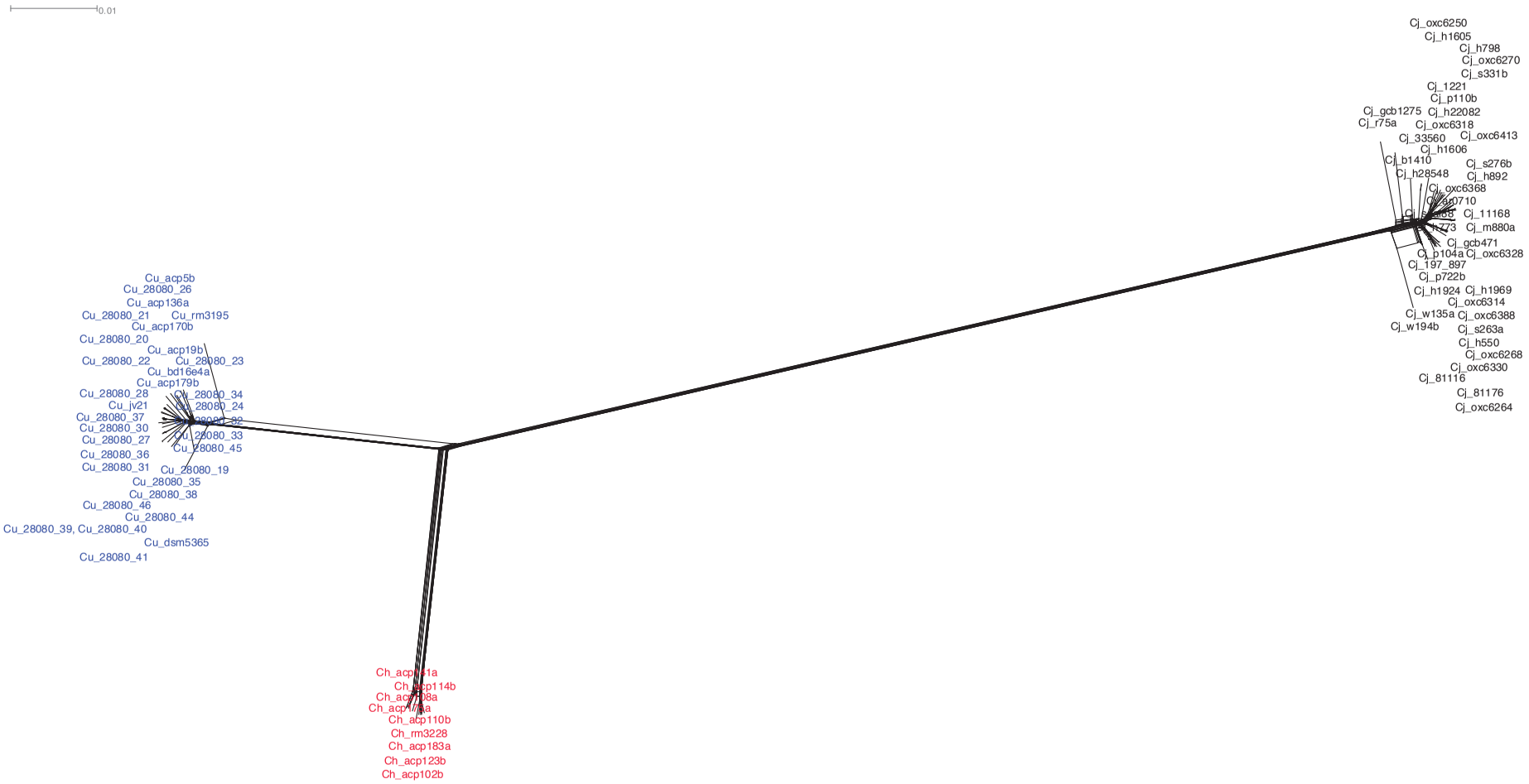
Supplemental Fig. 7.5. Distribution of the number of singleton genes per strain in 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups**.**



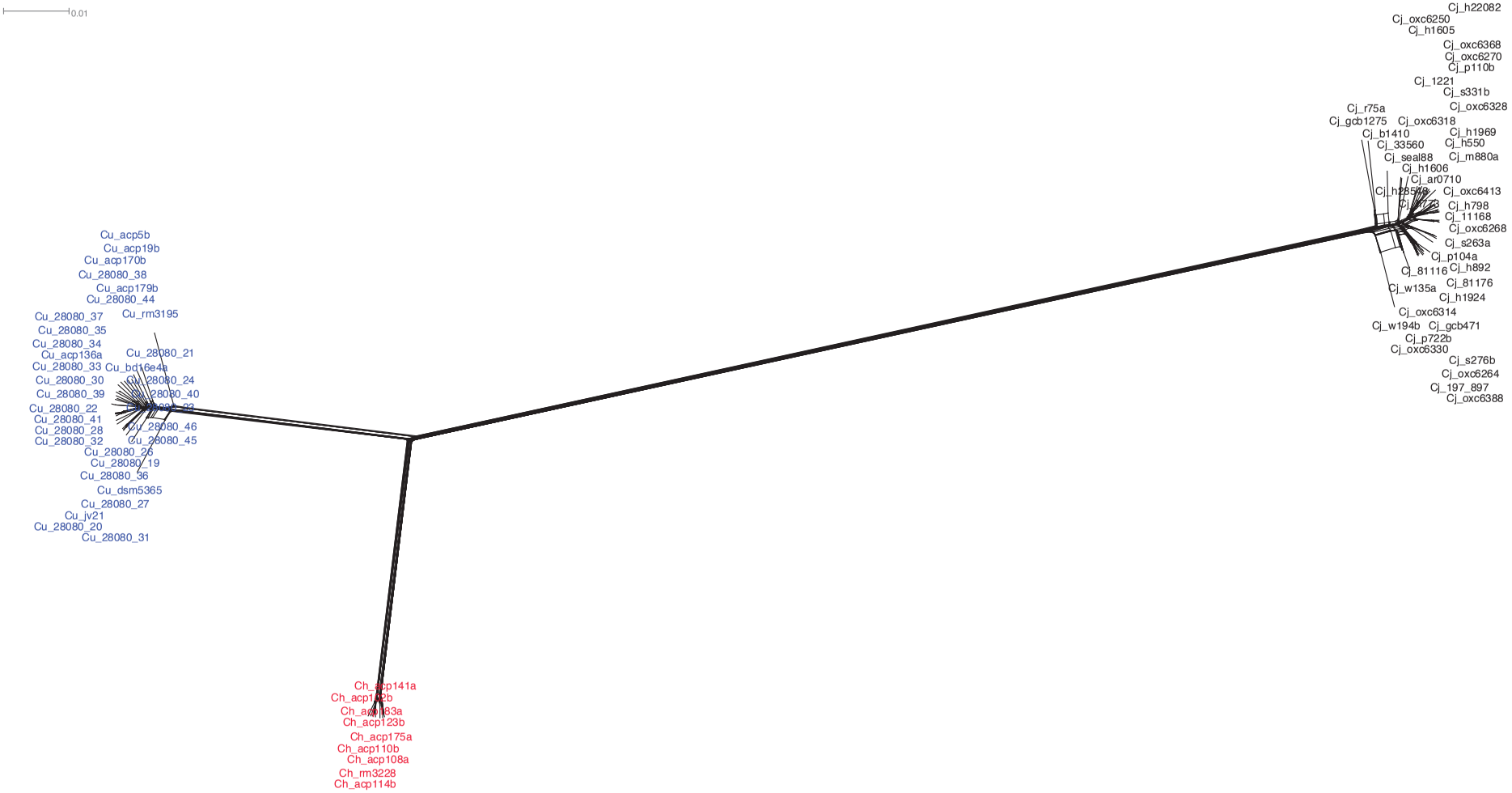
Supplemental Fig. 7.6. Agreement between MP3 and PathogenFinder predictions of pathogenic proteins of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* genomes.



Supplemental Fig. 7.7. Distribution of the number of MP3 predicted pathogenic proteins per strain in singletons of 42 *C. jejuni*, 33 *C. upsaliensis* and nine *C. helveticus* isolates by Clusters of Orthologous Groups (COG) functional groups.



Supplemental Fig. 7.8. NeighborNet visualised in SplitsTree for the amino acid sequences of 182 core genes in 84 genomes of *Campylobacter* species. Only genes of same length were used and had an alignment of 45,949 amino acids in length with 9,840 (21.42%) variable sites. Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus*, respectively.



Supplemental Fig. 7.9. NeighborNet visualised in SplitsTree for the amino acid sequences of 649 core genes in 84 genomes of *Campylobacter* species. The genes included are those that have a length range of 20% in the cluster and had an alignment of 197,053 amino acids in length with 59,486 (30.19%) variable sites. Cj, Cu, and Ch denote *C. jejuni*, *C. upsaliensis* and *C. helveticus*, respectively.