Molecular epidemiological studies of *Campylobacter* isolated from different sources in New Zealand between 2005 and 2015

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Abstract

Campylobacteriosis is one of the most important food-borne diseases worldwide, and a significant health burden in New Zealand. *C. jejuni* is the predominant species worldwide, accounting for approximately 90% of human cases, followed by *C. coli*.

The first study evaluated whether the time elapsing from sampling to culture has an impact on the recovery rate of *Campylobacter*, and explored whether some sequence types are more likely than others to be missed due to delayed culture. The study revealed that, whereas delayed culture may affect the recovery rate of *Campylobacter*, there was no evidence of a bias due to specific sequence types being under detected.

The second study aimed to analyse the differences in the *Campylobacter* viable counts and in population genetic structure between chicken drumsticks and whole carcass meat for retail sale. The results indicate that the *Campylobacter* population genetic structure did not differ between the two types of retail chicken meat. However, the difference in *Campylobacter* viable counts suggest that consumption of different chicken meat products may pose different risks of campylobacteriosis associated with an exposure to different infection doses.

In the third study, we genotyped *C. coli* isolates collected from different sources between 2005 and 2014, to study their population structure and estimate the contribution of each source to the burden of human *C. coli* disease. Modelling indicated ruminants and poultry as the main sources of *C. coli* infection.

The fourth study aimed to genotype *C. jejuni* isolates collected between 2005 and 2015 from different sources, to assess changes in the molecular epidemiology of *C. jejuni* following the food safety interventions implemented by the New Zealand poultry industry in 2007/2008. Modelling indicated that chicken meat from 'Supplier A' was

the main source of *C. jejuni* human infection before the interventions; but after the interventions, ruminants became the main source of infection, followed by chicken meat from Supplier A.

This thesis has made us aware of the aetiology of *C. coli* infections and the change in the attribution of *C. jejuni* infections. These findings should be used in developing further strategies to reduce the total burden of human campylobacteriosis.

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Nomenclature

BA	Blood agar
BB	Bolton Broth
BPW	Buffered peptone water
CC	Clonal complex
cfu	colony forming unit
CrI	Credible interval
DALY	Disability Adjusted Life Years
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Environmental Science and Research Ltd.
GBS	Guillain-Barré syndrome
HL	Heat-labile
HS	Heat-stable
IID	Infectious intestinal disease
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
mCCDA	modified Cefoperazone Charcoal Deoxycholate agar
MCL	Maximum composite likelihood
^{m}EpiLab	Molecular Epidemiology and Public Health laboratory
ML	Maximum likelihood
MLST	Multi locus sequence typing
MU	Massey University
NMDS	Non-metric multidimensional scaling
PCR	Polymerase chain reaction

PERMANOVA	Permutational multivariate analysis of variance
PFGE	Pulsed field gel electrophoresis
PSI	Proportional similarity index
spp.	Species
ST	Sequence type
WHO	World Health Organisation

Thesis structure and format

This thesis is composed of six chapters covering a literature review, four research-based chapters and a final discussion. Raw data are presented in Appendices. Some repetition between chapters was inevitable due to the style of the thesis presentation, especially in the materials and methods sections. These repetitions allow each chapter to be read in isolation.

Chapter one

This chapter is a general overview covering the main concepts and overviewing the influential literature addressed in the thesis. It discusses the molecular epidemiological studies of human campylobacteriosis in New Zealand and other countries and summarises the studies done in this PhD projects.

Chapter two

This chapter compares direct versus delayed culture for *Campylobacter* in human faeces, titled: **"Detection and recovery rate of *Campylobacter* from faecal swabs: Direct vs delayed culture"**

Chapter three

This chapter compares two types of chicken retail meat (whole carcasses versus drumsticks) collected from different suppliers, titled: **"Abundance and multilocus genotypes of *Campylobacter* species isolated from chicken drumsticks and whole carcasses obtained from different suppliers in the retail chain".**

Chapter four

This chapter titled **"Molecular epidemiology of *Campylobacter coli* isolated from different sources in New Zealand between 2005 and 2014"** formed the basis of a paper published in Applied and Environmental Microbiology:

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Chapter five

This chapter compares the source attribution of *C. jejuni*-associated campylobacteriosis cases before versus after intervention, titled: **"Changes in the molecular epidemiology of *Campylobacter jejuni* following food safety interventions by the poultry industry".**

Chapter six

This chapter summarises and discusses the significance of the results of the previous studies.

Appendices: The raw data and supplementary materials of each chapter are presented in this study.

Bibliography: The literature cited is presented in the format required by Applied and Environmental Microbiology journal.

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Chapter 1

General overview

1.1 Campylobacter

The genus *Campylobacter* was first proposed in 1963 (1) and included just two species, *Campylobacter fetus* and '*Campylobacter bubulus*' (today known as *Campylobacter sputorum*). The genus currently includes 28 species and 11 sub-species (2), where *Campylobacter hepaticus* sp. nov. (3) and *Campylobacter geochelonis* sp. nov. (4) were identified in 2016. *C. jejuni* is the predominant species isolated from cases of human campylobacteriosis worldwide, followed by *C. coli* (5-7). Other species, such as *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter fetus* have also been occasionally isolated from campylobacteriosis cases in humans (7).

Campylobacter spp. are Gram-negative, non-spore forming rods (8) that are 0.2 to 0.5 μm in diameter and 1 to 8 μm long, with the shape of a comma, an 'S', a helix, or spiral (9). The cells have a darting motility conferred by a unipolar or bipolar flagella (10, 11). *Campylobacter* spp. are microaerophilic with optimal growth in atmospheres of 5-10% O_2 and 3-5% CO_2 with some species like *C. gracilis*, *C. hyointestinalis* and *C. showae* able to grow under anaerobic conditions (10), and *C. ureolyticus* being incapable of growing in a microaerobic atmosphere unless hydrogen is supplied (12). *Campylobacter* spp. that hydrolyse hippurate are *Campylobacter avium* sp. nov. and *C. jejuni*, although the *C. jejuni* subspecies *doylei* varies in its ability for hippurate hydrolysis (13, 14). Accordingly, the hippurate hydrolysis test (15) became widely used to identify *C. jejuni* and differentiate it from *C. coli*, a species phenotypically and genotypically very similar to *C. jejuni* (16).

Thermophilic campylobacters do not grow at temperatures below 30°C and their optimal growth temperature is 42°C (17). Bolton Broth (*Campylobacter* enrichment

broth - Bolton formula) can be used for the recovery of campylobacters from wide range of sample types and it is recommended in protocols produced by the U.S. Food and Drug Administration (18). Several *Campylobacter* selective agars can be used for the culturing of *Campylobacter* isolates. In the present project, the modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland, New Zealand) that include charcoal as an oxygen quencher, were used. High temperatures (>20°C), low temperatures (<0°C), and fluctuating temperatures should be avoided, and it is recommended to store clinical samples at approximately 4°C if there is a delay between sample collection and processing (19). Amies medium (charcoal based) is recommended for the transport of swab samples because this medium protects the swab content from drying and the toxic effects of oxygen (19). Once in the laboratory, the samples should be processed as soon as possible, preferably no longer than 3 days from arrival, and samples should be kept refrigerated at 4°C in case of delayed processing (19, 20).

Campylobacter spp. can be detected in a broad range of hosts, mainly in the intestinal tract, and to less extent in other organs such as spleen, liver (21) and the oral flora (22, 23). It can be isolated from humans and animal hosts such as poultry, ruminants, dogs, cats, wild birds and pigs, and from environmental sources like water and sand (24-26). *Campylobacter* spp. are able to survive, but not grow, outside the host for a long period of time (27), entering a 'viable but nonculturable' state (A state of very low metabolic activity and do not divide) (28). Having the ability to survive for a long period of time (> 1 week) in the environment, *Campylobacter* spp. are often found in rivers, streams and lakes (29), which allows them to be transmitted non-selectively between different host species through indirect routes (30).

1.2 Campylobacteriosis

Campylobacteriosis is one of the most important food-borne diseases worldwide, and the incidence of reported cases varies between countries (31). It has a significant impact on the economy and health care systems around the world due to its high incidence. In the 1990s, the direct cost of campylobacteriosis in New Zealand was estimated at $NZ 77 million annually (32), and a loss of more than four million working days per year (33). The high costs associated with this infection prompted the New Zealand government to initiate source attribution studies in order to better understand the transmission of *Campylobacter* bacteria to humans (34-36).

The symptoms of campylobacteriosis are generally observed two to five days after the infection (incubation period range: 1-10 days) (6). *Campylobacter jejuni* is highly infectious, and as few as 800 colony-forming units are sufficient to establish an infection in humans (37). However, the dose-response relation differs among different *C. jejuni* isolates (38). Common symptoms of campylobacteriosis include diarrhoea (often bloody stools), abdominal pain, fever, headache, nausea and vomiting, which can last from three to six days (6). Mortality rate is usually low and limited to very young or elderly patients, or to patients suffering from serious immunosuppression (6). Several complications have also been reported at various rates, including bacteraemia, hepatitis, pancreatitis and miscarriage. Post-infection complications may include neurological disorders such as Guillain-Barré syndrome (GBS) and Miller Fisher Syndrome (39-41), occurring in approximately 0.1% of cases (42). These complications are serious, resulting in progressive muscle weakness or paralysis and, in a small proportion of cases, death. Campylobacteriosis is also associated with Reiter's syndrome, a reactive arthritis estimated to occur in 1% of cases (43).

Children <4 years old, adults (20 - 40 years old) and elderly (>75 years old) seem to be more susceptible to *Campylobacter* infection than individuals of other ages (44, 45). However, Stafford et al. (1996) explained that the higher rate of campylobacteriosis observed in young children might be due to a biased reporting, with parents of young children more likely to seek medical care for their children (46). On the other hand, the higher risk of campylobacteriosis among the 20 to 29 years-old may be due to greater travel activity and less experience in safe food handling. Generally, campylobacteriosis is a self-limiting illness and no pharmacological treatment is required, except for fluid replacement and rehydration (6). However, antimicrobial treatment is recommended in cases of bacteraemia, if the infection is severe, the patient is immunocompromised, severe or bloody diarrhoea develops, or if the duration is prolonged (47, 48).

1.3 Reported incidence rate of campylobacteriosis cases around the world

In 2010, thirty one foodborne hazards caused ~600 million illnesses globally and *Campylobacter* spp. accounted for ~96 million of those cases (49). The Disability Adjusted Life Years (DALYs) caused by the 31 foodborne hazards was 33 million in which *Campylobacter* spp. accounted for ~2.1 million of the DALYs (49). DALY is a measure of the overall disease burden, expressing the number of years lost due to illness, disability or early death.

1.3.1 North and Central America

In the United States, based on 10 years outbreak data (1998-2008), the number of campylobacteriosis cases was estimated to be ~845,000, resulting in ~8,500 hospitalisations and 76 deaths annually (50). In 2014, the incidence of campylobacteriosis was 13.45 cases per 100,000 population (51), and the annual cost was estimated to be 1.7 billion USD (52).

In Quebec, Canada, the estimated campylobacteriosis annual incidence rate between 1996 and 2006 was of 35.2 cases per 100,000 population (a total of ~28,500 cases) (53), whereas in the Waterloo region of Ontario, the reports from 1990 to 2004 indicated an incidence of 49.69 cases per 100,000 population (54).

In Guatemala, campylobacteriosis is common among children, with an incidence rate ranging between 185.5 and 1,288 per 100,000 children (55). In Barbados, one study estimated an incidence of 5.4 cases per 100,000 population in 2000 (56).

According to the WHO 2015 report (49), the 2010 median rates for foodborne DALYs for the North American sub-region ranged between 35 and 315 DALYs per 100,000 population, and *Campylobacter* spp. was responsible for 9 to 15 DALYs per 100,000 population (Appendix 2 of the WHO report).

1.3.2 South America

Usually, there are no national surveillance programs for campylobacteriosis in developing countries, and the incidence rates of campylobacteriosis in South America are not known. The data in this region indicate that *Campylobacter* spp. contribute to the aetiology of gastroenteritis; however, the contribution of campylobacteriosis relative to that of other pathogens, is unclear (57).

1.3.3 Europe

An estimate of the incidence of campylobacteriosis among 27 European Union state members ranged between 30 to 13,500 cases per 100,000 population in 2009 (9.2 million cases) including the underreporting factor estimates, with Finland and Sweden reporting the lowest incidence, and Bulgaria the highest (58). In the United Kingdom, *Campylobacter* was responsible for approximately 572,000 cases in 2009, with a rate of 9.3 cases per 1,000 person-years, and 80,000 general practitioner consultations (59). In

Germany, the annual incidence ranged between 53.4 to 81.4 cases per 100,000 population between 2005 and 2011 (60), with ~70,000 reported cases in 2011 (61). In Poland, it has been hypothesised that campylobacteriosis may be underdiagnosed and underreported (57), since the estimated incidence from reported cases in 2012 was only 1.12 cases per 100,000 population (62). In contrast, in 2011 in the Netherlands, the incidence was estimated to be 51 cases per 100,000 population (63), with an estimated €21 million cost of illness per year (64). The median rates for foodborne DALYs for the European sub-regions in 2010 ranged between 41 and 52 DALYs per 100,000 population, and *Campylobacter* spp. accounted for 8 to 10 DALYs per 100,000 population (49).

1.3.4 Asia and Middle East

Epidemiological data about campylobacteriosis in Asia and the Middle East region are limited (57). In China, a study predicted that 1.6% of the urban and 0.37% of the rural population may be affected every year (65), based on the detection rates of *Campylobacter* in raw chicken and the consumption of chicken products. Miyagi Prefecture, Japan, had an incidence rate of ~1,500 cases per 100,000 population in 2005-2006 (66). However, that unusually high rate might have been due to outbreaks identified during the time frame analysed, and/or the methodology used to estimate the incidence rate. Further active surveillance of food safety is needed in central regions of Asia (57). The 2010 median rates for foodborne DALYs for the South-East Asian sub-region ranged between 685 and 711 DALYs per 100,000 population, and *Campylobacter* spp. accounted for 33 to 37 DALYs per 100,000 population (49).

Data from the Middle East indicate that gastroenteritis due to *Campylobacter* spp. is becoming a major problem. For example, a study done in Israel showed an increase of campylobacteriosis rate, from 31 cases per 100, 000 population in 1999 to 91 cases per

100,000 population in 2010 (67). During that period, the consumption of poultry meat had markedly changed from a consumption of mainly frozen products, to fresh or chilled meat, which might explain the increase in campylobacteriosis rate. In fact, studies showed that the number of *Campylobacter* colony forming unit (cfu) on meat drops after freezing (68, 69). In this Eastern Mediterranean sub-region, the 2010 median rates for foodborne DALYs was 362 DALYs per 100,000 population and *Campylobacter* spp. accounted for 75 DALYs per 100,000 population

1.3.5 Africa

Many developing African countries do not have national surveillance programs for campylobacteriosis. Therefore, incidence data for campylobacteriosis in African cases have not been retrieved. However, according to the WHO 2015 report (49), the 2010 median rates for foodborne DALYs for the African sub-region ranged between 1,179 and 1,276 DALYs per 100,000 population and *Campylobacter* spp. accounted for ~70 DALYs per 100,000 population.

1.3.6 Oceania

Campylobacteriosis is the most common notifiable food-borne infection in Australia, with an incidence rate of 112.3 cases per 100,000 population in 2010 (~17,000 notified cases) which is a 6% increase from 2008 and 2009 (70-72).

In New Zealand, the incidence of campylobacteriosis showed a gradual increase since the disease became notifiable in 1980, and peaked in 2006, with ~390 cases/100,000 population. In 2008, food safety interventions applied at the poultry industry level have led to a dramatic drop in campylobacteriosis incidence, with ~173 cases/100,000 population (Figure 1.1). Despite this 60% decrease, campylobacteriosis remained the most commonly notified enteric disease in New Zealand, and comprised about 45% of

all enteric diseases notifications in 2014 (73) (campylobacteriosis rates between 2003 and 2016 were taken from the New Zealand Public Health Surveillance Reports (Figure 1.1), and are available on https://surv.esr.cri.nz/surveillance/NZPHSR.php).

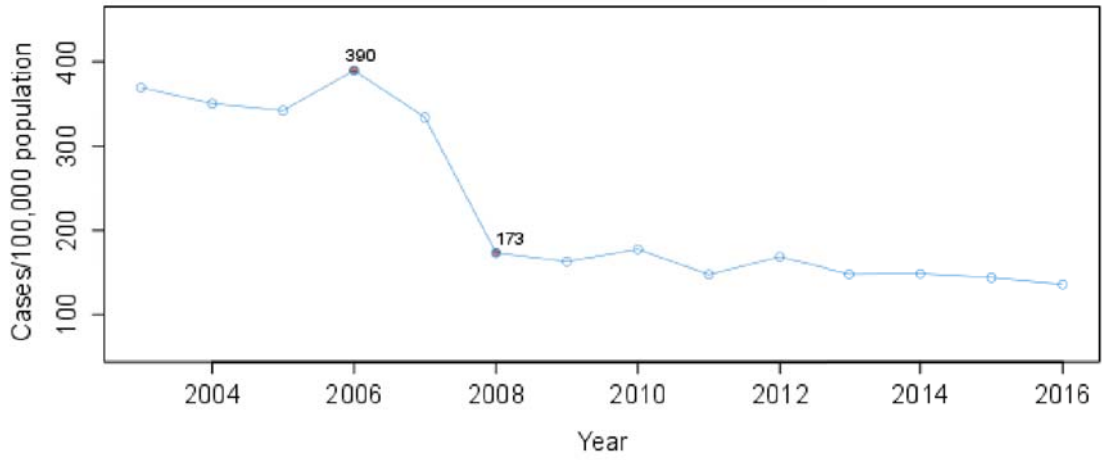

Figure 1.1: Campylobacteriosis rate per 100,000 population between 2003 and 2016 in New Zealand.

1.4 Manawatu sentinel surveillance site

The high rate of the campylobacteriosis in New Zealand has led to the establishment of a sentinel surveillance program in the Manawatu region, from 2005 onward. The region has a population of ~223,000 that live in rural and urban areas, with its main city, Palmerston North, of a population of ~80,000 (74, 75). The purpose of the program was to assess the contribution of the different infection sources to the burden of campylobacteriosis through molecular epidemiological studies. For that purpose, all the human faecal specimens submitted to the regional diagnostic laboratory (MedLab Central, Palmerston North) during the sampling period, which tested positive for *Campylobacter* spp., were delivered for bacterial culture to the Molecular Epidemiology and Public Health laboratory (^{m}EpiLab) of Massey University (MU).

This surveillance program has helped to elucidate the common infection sources in the region. Bolwell et al. (2014) evaluated the representativeness of the Manawatu sentinel

program based on the following criteria: 'representativeness' of the sentinel site, completeness of data collected, and purpose of the surveillance activities (76). The authors concluded that other surveillance sites may be required in order to capture a wider population and to adequately inform control policies. However, the Manawatu sentinel site can be used to capture the broad features of the wider population, enabling to monitor sub-populations in an efficient way (76).

1.5 Typing methods for *Campylobacter* spp.

Several phenotyping and genotyping methods for *Campylobacter* isolates are available, and each method has advantages and disadvantages.

1.5.1 Phenotyping

The phenotype is a set of observable characteristics of an individual that result from the interaction of its genotype with the environment. Therefore, phenotypes reflect the nature of the organism (77, 78). Phenotyping methods include biotyping, serotyping and phage typing (79). These methods have the limitation of a lack of, or difficult standardization and portability, frequent cross-reactivity between strains and the presence of non-typeable strains (80). Moreover, phenotypic methods have relatively low discriminatory power (81).

Biotyping

Biotyping is a phenotypic typing method that assesses an organism's ability to utilise biochemical substrates and grow under differing environmental conditions (82). A biotyping scheme for *Campylobacter* spp. was first developed utilising 12 biochemical tests (83). It is a simple method to implement and interpret, but the discriminatory power is relatively low among *Campylobacter* strains, so biotyping is usually combined with other typing methods for better results (84).

Serotyping

Two serotyping schemes have been widely used for the classification of *C. jejuni*: i) the Penner scheme (85), based on the recognition of soluble heat-stable (HS) antigens; and ii) the Lior scheme (86), which exploits the variation in heat-labile (HL) antigens (87). The combined use of these schemes provides high level of discrimination for outbreak investigations and epidemiological source tracking (88, 89). However, the use of these tests is time consuming, laborious and costly, mainly due to the requirement for a large panel of antisera.

Phage typing

Viruses that infect bacteria, or bacteriophages, do so by attaching to surface receptors. Phage typing relies on the host–specific nature of such viral infection. Phage typing has been widely used for the typing of many bacterial species, such as *Staphylococcus aureus* (90) and *Salmonella enterica* (91). In general, *Campylobacter* phage typing has a relatively poor discriminatory power, which could be improved by adding new phages targeting phase-variable structures (92).

1.5.2 Genotyping

Genotyping methods exploit the variability of DNA sequence to discriminate between different bacterial isolates, and these methods have replaced phenotypic methods for species identification and subtyping of many microorganisms, including *Campylobacter*, due to their higher discriminatory power and reproducibility (93).

Campylobacter species identification

In the present project, *Campylobacter* identification to the species level was accomplished by the polymerase chain reaction (PCR) using primers targeting different species. PCR is a fast and inexpensive technique used to "amplify" small segments of

DNA necessary for molecular and genetic analyses. The primers *map*A-F (5'-CTTGGCTTGAAATTTGCTTG-3') and *map*A-R (5'-GCTTGGTGCGGATTGTAAA-3') were used for the *C. jejuni* species-specific PCR for the detection of the *map*A gene (94), has a sensitivity and specificity of 100% and 92% respectively (95). The primers *ceu*E-F (5'-AATTGAAAATTGCTCCAACTATG-3') and *ceu*E-R (5'-TGATTTTATTATTTGTAGCAGCG-3') were used for the *C. coli* species-specific PCR, for the detection of the *ceu*E gene (96), and has a 100% sensitivity and specificity. A multiplex PCR was run for the identification of both *C. jejuni* and *C. coli* species using the primers CJF (5'-ACTTCTTTATTGCTTGCTGC-3') and CJR (5'-GCCACAACAAGTAAAGAAGC-3') to detect the *hip*O gene (97) associated with *C. jejuni* (100% sensitivity and specificity), and the primers *ceu*E-F and *ceu*E-R for the detection of the *ceu*E gene associated with *C. coli*.

Molecular subtyping

Molecular subtyping, that is, genetic characterization of bacterial isolates below the species level, has helped in the investigations of infectious disease outbreaks and also in long-term epidemiological studies which aimed to identify risk factors for disease and strains of zoonotic pathogens. The two most commonly used genotyping methods used for *Campylobacter* spp. subtyping are: Pulsed Field Gel Electrophoresis (PFGE) and Multilocus Sequence Typing (MLST).

Pulsed Field Gel Electrophoresis

PFGE is a technique to fractionate high-molecular weight DNA (size from 10 kb to 10 Mb) by electrophoresis in agarose gel with an electric field that alternates in two directions (98). The process of the PFGE technique is described on the following link http://www.cdc.gov/pulsenet/pathogens/protocol-images.html#pfge. PFGE is used for

Campylobacter spp. to assess polymorphisms throughout the genome (79). Even though PFGE has high resolution (99) and very good discriminatory power (100), it is unsuitable for typing large numbers of isolates because it is time consuming, laborious and needs special equipment (99).

Multilocus sequence typing

MLST was considered the gold standard for epidemiological subtyping of several pathogenic microorganisms (101, 102), due to its efficiency and high reproducibility of the sequence data. Results can be shared online (103); However, MLST is somewhat costlier than other methods due to the cost of the seven PCR and sequencing reactions. The MLST technique has been applied throughout this PhD project for the characterisation of *C. jejuni* and *C. coli*. So far, 29 MLST schemes have been developed for different bacterial species (http://www.mlst.net/databases/). In the *Campylobacter* MLST scheme, fragments of seven defined housekeeping genes of the bacterium are amplified by PCR, and the nucleotide sequence of the amplicons are determined based on the methods outlined by Dingle et al., 2001 (104). As a final step, sequence data are collated and submitted online for allele and sequence type (ST) designation, with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/*Campylobacter*/).

1.6 Source attribution of campylobacteriosis

As previously indicated, *Campylobacter* is a successful organism that can be detected in a broad range of hosts and can survive, but not grow, outside the host for a long period of time. Therefore, source attribution studies are used to determine to what extent certain sources (e.g. chicken, ruminants, etc.), and pathways (e.g. food-borne, person-to-

person, water-borne routes), contribute to the burden of a specific disease (e.g. campylobacteriosis).

Case control is one of the methodologies used for source attributional studies in which campylobacteriosis patients (cases) are compared retrospectively to patients who do not have campylobacteriosis (controls) in order to compare the frequency of exposure to a risk factor present in each group, so that the relationship of particular risk factors with campylobacteriosis is determined. Before 2005, several studies in New Zealand (105-107) have identified consumption of poultry meat as a significant risk factor for campylobacteriosis using the case-control methodology. However, observations from one study (107) showed ambiguous results, with 81% of both cases and controls having consumed poultry meat, which underscores the limitations of case-control studies for infectious diseases risk factor analysis. Domingues et al. (2012) performed a meta-analysis of case-control studies to identify the major risk factors of human campylobacteriosis (108). The authors estimated that the highest risk factor was international travel, followed by consumption of undercooked chicken, environmental exposure to *Campylobacter* and direct contact with farm animals. Consumption of undercooked chicken and unpasteurised dairy products were the main sources of food-borne campylobacteriosis. Another case-control study conducted in the Netherlands (109) considered the consumption of chicken meat as a significant risk factor for campylobacteriosis.

After 2005, campylobacteriosis source attribution based on molecular epidemiological studies were conducted in New Zealand. Mullner et al. (2009) estimated that the consumption of poultry meat was the major source of campylobacteriosis in New Zealand between 2005 and 2008, causing an estimated 58–76% of cases, followed by ruminant sources (~20% to ~30%) (34). Other molecular studies around the world (24,

110, 111) also implicated chicken meat as the major source of food-borne *C. jejuni* infections. However, other potential sources should not be underestimated. For instance, in Finland, in 2003, bovine and poultry sources have been deemed of equal importance as sources of human *C. jejuni* infections (112). Ruminant- derived campylobacteriosis cases are assumed to be due to direct environmental and occupational exposures, rather than food-borne (113). Occupational exposures are the handling and treatment of animals on-farm and the exposure of meat workers to faecal matter during the slaughtering process (114). On the other hand, poultry-derived campylobacteriosis cases are believed to be due to the handling and consumption of meat, rather than occupational exposures (115), and this could be explained by the high level of *Campylobacter* contamination found in chicken samples (116, 117).

Determining the major source for human campylobacteriosis using a robust methodology is crucial to develop and evaluate appropriate control strategies to reduce the burden of disease. That has been achieved in New Zealand, where control strategies aimed at the poultry industry have led to a dramatic reduction in the campylobacteriosis rate (118).

1.7 Methods and analytical tools used in campylobacteriosis molecular epidemiological source attribution studies

1.7.1 The proportional similarity index

The similarity between the frequency distributions of the *Campylobacter* STs among different sources can be estimated by using the proportional similarity index (PSI) or Czekanowski index, that measures the area of intersection between two frequency distributions (119). However, an individual source responsible for a significant proportion of human cases can be masked when using the PSI if the same source also

has a high proportion of low, or non-pathogenic STs resulting in a low index value (120). The PSI was used in chapters 3, 4 and 5 of this thesis.

1.7.2 Minimum spanning trees

Minimum spanning trees are a graphical tools developed to represent the relationships among individuals from a population. In this project minimum spanning tree was implemented using the Bionumerics software (Applied Maths; http://applied-maths.com/bionumerics), that links allele designations within an MLST database to a minimal spanning tree calculated by Prim's algorithm (121). The minimum spanning tree inspects the relationships of closely related genotypes within the CC, and identifies the most likely existing ancestral genotype (founder) of each CC from which the clonal variants might have descended. STs with the largest numbers of single locus variants are given the highest priority as possible founders. In this project, minimum spanning trees were applied in the studies presented in chapters 2, 3, 4 and 5.

1.7.3 The asymmetric island model

This retrospective model of population genetics uses an evolutionary approach based on Wright's island model (122). The asymmetric island model is a Bayesian approach that models the genealogical history of all the isolates based on their allelic profiles, taking into consideration the relatedness of STs (34). In campylobacteriosis, this technique was used to estimate the mutation, recombination, as well as the migration rates from each reservoir to the human 'island' based on their allelic profiles, and probabilistically assign each human case originating from each of the source populations (111). This model was applied in chapters 4 and 5 of this thesis.

1.7.4 Bayesian hierarchical model

The Bayesian hierarchical model, based on the modified Hald model (123), was used to implement a non-parametric source attribution model to attribute human campylobacteriosis cases to sources in a Bayesian framework with source and sequence type effects. This model was applied in chapter 5, along with the asymmetric island model, in order to ascertain whether the source attribution outcome of one model is similar using a second model.

1.8 Poultry meat production chain and *Campylobacter*

Poultry comprises chickens, ducks, turkeys and laying hens. Production of chicken meat is the predominant poultry production branch, accounting for 70% to 80% of the production, worldwide (124). In 2014, the global production of poultry meat reached 95.8 million tonnes, almost twice the production in 2000 (58.5 million tonnes). It has been estimated that chicken meat production will keep growing at a rate of 2.3% per year, to reach 134.5 million tonnes, making it the largest meat sector from 2020 onwards (http://www.thepoultrysite.com/articles/3230/global-poultry-trends-2014-poultry-set-to-become-no1-meat-in-asia/). The global burden of campylobacteriosis has been clearly affected following this increase in the consumption of poultry meat products. The continuous increase will put significant pressure on the poultry industry to control campylobacteriosis associated with the consumption of poultry.

The chicken meat production and processing chain consists of several stages that may play a role in the transmission of *Campylobacter,* from farm to fork: i) primary production at the rearing farms, ii) transport to slaughter, iii) slaughtering process and the processing of chicken meat products, iv) sale of the products via the retail chain, v) handling and consumption of chicken meat products either at home or restaurants (124).

Identified risk factors for contamination of meat with *Campylobacter* at slaughter are: thinning of the flock (portion of birds are removed from poultry house for slaughter and processing while other birds left to grow to normal clearance age), slaughter in the summer season, increasing bird age at slaughter, poor flock health based on mortality, and the higher the number of rearing houses on-farm (125, 126). Transport to slaughtering has a limited effect on the contamination of carcasses, unlike the slaughtering process, where there is a higher risk of contamination during plucking and evisceration (124). After the slaughtering process, different types of chicken meat products (fresh or frozen whole carcasses and various pieces of chicken meat) are sold, but whether consumption of different types of chicken meat products pose different risks of campylobacteriosis, is not well understood.

In April 2008, the New Zealand Food Safety authority (NZFSA) announced the implementation of a *Campylobacter* Risk Management Strategy in collaboration with the poultry industry, to reduce campylobacteriosis burden in New Zealand. Those interventions led to > 50% decrease in campylobacteriosis incidence rate. The key interventions introduced, included monitoring activities that are linked with actions based on the findings, are summarised as follows according to Sears et al. (118):

1. **Interventions during primary production**
    - Development of the Broiler Growing Biosecurity Manuals by industry in order to identify effective on-farm biosecurity procedures.
    - Improved the catching and transporting of birds and the cleaning of transport crates.
    - Monitoring the prevalence of *Campylobacter* spp. in bird caecal samples in each shed.

2. **Interventions during processing**
    - Monitoring the enumeration levels of *Campylobacter* spp. from rinsates of bird carcasses.
    - Establishment of a system to exchange information within the industry and implementation of developments during the primary processing.
    - Implementation of the Code of Practice for primary processing of poultry that includes slaughter and dressing.
    - Establishment of mandatory targets for the contamination levels of *Campylobacter* spp. on poultry carcasses after primary processing.
3. **Interventions at the retail chain**
    - Implementation of leak-proof bags.
    - Implementation of sporadic monitoring of *Campylobacter* spp. contamination in retail poultry.
4. **Interventions on the consumer**
    - Enhanced consumer education to increase public awareness of food safety risk mitigation behaviours.
5. **Other interventions**
    - Improved surveillance and source attribution research into human campylobacteriosis.

1.9 Aims of this PhD project

The main aim of this project was to determine the major infection sources of human campylobacteriosis caused by *C. coli* and *C. jejuni*. Four studies were implemented in order to achieve this aim:

1. The Molecular Epidemiology and Veterinary Public Health laboratory (^{m}EpiLab) of Massey University was in charge of the Manawatu *Campylobacter*

Sentinel study since 2004. The laboratory analysed ELISA-positive human faecal samples submitted by the main human diagnostic laboratory (Medlab Central in Palmerston North) for *Campylobacter* culture. Many of these samples were cultured with a delay of up to 7 days from collection. The first study of this project was designed to assess whether the delay in culture has a negative impact on the recovery of *Campylobacter* bacteria, and in particular whether this delay inhibits the isolation of specific *C. jejuni* STs (Chapter 2). The working hypothesis was that culture methods for *Campylobacter* used at the ^{m}EpiLab may introduce bias to source attribution studies.

2. Most studies in New Zealand analysed *Campylobacter* spp. isolates recovered from whole chicken carcasses and little is known about isolates recovered from the chicken pieces meat. Since chicken pieces meat are widely available in the retail chain in New Zealand, Chapter 3 compared i) the *Campylobacter* viable count and ii) the population genetic structure of *C. jejuni* and *C. coli* between the two types of chicken retail meat (whole carcasses versus drumsticks). One of the reasons for investigating this difference was to see if the choice of meat type might have any influence on the source attribution results. The working hypothesis was that sampling methods for *Campylobacter* used at the ^{m}EpiLab may introduce bias to source attribution studies.
3. This study (Chapter 4) assessed the relative contribution of *C. coli* to campylobacteriosis morbidity and analysed the genetic relatedness of the clinical *C. coli* isolates with that of isolates obtained from different sources over a ten year period in order to infer possible transmission pathways of this important, but under-studied pathogen.

4. The aim of the study presented in Chapter 5 was to compare the sources of *C. jejuni*-associated campylobacteriosis before and after the industry intervention using molecular epidemiology methodology, in order to provide additional information on the effectiveness of the campaign to inform policy makers that can develop new strategies to further reduce human campylobacteriosis incidence.

Chapter 2

Detection and recovery rate of *Campylobacter* from faecal swabs: Direct vs delayed culture

2.1 Abstract

Campylobacteriosis is one of the most important food-borne diseases worldwide and is a significant health burden in New Zealand. *C. jejuni* and *C. coli* are the most important human pathogens of the genus accounting for approximately 95% of human cases of campylobacteriosis. The Molecular Epidemiology and Public Health Laboratory (^{m}EpiLab) receives on weekly basis ELISA-positive *Campylobacter* human faecal samples from the diagnostic laboratory MedLab Central, Palmerston North, and 19% of those samples are negative for *Campylobacter* by culture. Since there is often a delay in receiving the ELISA-positive samples from the ^{m}EpiLab, the objective of this study was to evaluate whether the time elapsing from sampling to culture could have an impact on the recovery rate of *Campylobacter*, so that measures can be taken to expedite culture in order to maximise the number of isolates recovered. Another aim was to explore whether some sequence types are more likely to be missed by culture than others, potentially biasing source attribution analyses. The methods used in this study were: 1, direct processing by culture of human faecal samples (referred to as the 'gold standard'); 2, delayed culturing where samples were preserved and kept in charcoal-Amies swabs at room temperature for up to 7 days before culture. Both direct and delayed cultures were performed using mCCDA plates incubated in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 48 hours. The sensitivity and the specificity of the delayed culture were 76% and 100% respectively, whereas the ELISA method had a sensitivity of 53% and a specificity of 98% compared to the gold standard. However, there was no evidence of source attribution bias due to selection

bias of specific sequence types. In conclusion, the results of this study indicated that delayed culture, as often implemented at the ^{m}EpiLab, may affect the recovery rate of *Campylobacter* in ELISA-positive samples submitted for culture by MedLab Central.

2.2 Introduction

Campylobacter species are a major cause of food-borne disease worldwide (108, 127). *Campylobacter* is a genus of Gram-negative, microaerophilic, oxidase-positive, non-fermentative bacteria associated with bacterial gastroenteritis known as campylobacteriosis, mostly caused by the species *Campylobacter jejuni* and *Campylobacter coli* (128). *Campylobacter jejuni* is highly infectious, and as few as 800 colony-forming units are sufficient to establish an infection in humans (37). *Campylobacter* infections are usually self-limiting and with adequate supportive treatment the patient usually fully recovers (129). However, antimicrobial therapy could be necessary in severe cases accompanied by high fever, severe or bloody diarrhoea, and if the duration of the symptoms is prolonged (47, 48). Infection with *Campylobacter* species may precede Guillain-Barré syndrome (GBS), an autoimmune condition that affects the peripheral nervous system (47, 130). *Campylobacter* is sensitive to environmental conditions such as dehydration, atmospheric oxygen, sunlight and elevated temperature. High temperatures (>20°C), low temperatures (<0°C), and fluctuations in temperature should be avoided and it is recommended that clinical samples are stored at approximately 4°C if there is a delay between sample collection and processing (19). Amies medium (charcoal based) is recommended for the transport of the swab samples because this medium protects the swab content from drying and the toxic effects of oxygen (19). Once in the laboratory, the samples should be processed as soon as possible, preferably no longer than 3 days from arrival, and samples should be kept refrigerated at 4°C in case of delayed processing (19, 20).

In the past decade, as part of a regional source attribution study of campylobacteriosis, all human faecal specimens that were submitted to the main medical laboratory (MedLab Central, Palmerston North) during the sampling period, and tested positive for *Campylobacter* spp. by ELISA (ProSpecT ®, Remel, USA), were delivered for bacterial culture to the Molecular Epidemiology and Public Health laboratory (^{m}EpiLab) using charcoal-Amies transport swabs (Copan, Italy). The charcoal-Amies swabs were kept in MedLab at room temperature and were submitted to the ^{m}EpiLab once a week. Therefore, swabs were analysed from 1 to 7 days delay depending on the day of sampling. Between 2005 and 2014, 19% percent of the ELISA positive C*ampylobacter* samples were negative by culture at the ^{m}EpiLab. A number of possible reasons were postulated, including the occurrence of false positive ELISA-results, viable but unculturable *Campylobacter* or, death of *Campylobacter* bacteria on swabs subject to delay in culture. Therefore, the main aim of this study was to assess whether the delay in culture could have a statistically significant negative impact on the recovery of *Campylobacter* bacteria compared with culturing on the same day or up to 24 h from sampling. In parallel to this study, another diagnostic study was performed to compare different methods for detection of *Campylobacter* species. In this study, all the samples that were negative for *Campylobacter* spp. by the ELISA method and positive by culture were sent for MLST in order to assess the probability of specific sequence types (ST) being overlooked. As source attribution studies make extensive use of molecular sub-typing methods which require the isolation of bacterial strains, failure to recover all STs with equal probability bias source attribution studies.

2.3 Materials and methods

2.3.1 Study population

All faecal samples were collected from Medlab Central in Palmerston North in the MidCentral District Health Board area of New Zealand North Island. The region's population consists of 223,000 people and ranks 6th out of 16 New Zealand regions. Its main city is Palmerston North, which has a population of ~80,000 people (74, 75).

2.3.2 Samples analysed

All human faecal specimens submitted to MedLab Central in Palmerston North that required an enteric bacterial pathogenicity testing were collected and tested by culture for the detection of *Campylobacter* spp. at the ^{m}EpiLab. A total of 702 samples were collected in 3 months (May 2014, December 2014 and January 2015) and processed at ^{m}EpiLab on the same day which will be referred to in the present study as "direct culturing". These same samples preserved in charcoal-Amies transport swabs (Copan, Italy) at room temperature and cultured on a weekly basis, will be referred to in the present study as "delayed culturing". The delay in culturing ranged from 1 day to 7 days.

2.3.3 Bacterial culture and identification

Samples were cultured on modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland) and in a 3 ml Bolton Broth (BB) (Lab M, Bury, England) in a loose-capped bijou and incubated in microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 48 hours (MACS-VA500-microaerobic workstation, Don Whitley Scientific, West Yorkshire). A single colony resembling *Campylobacter* spp. on mCCDA was subcultured to Columbia horse blood Agar (BA) (Fort Richard, Auckland) and incubated microaerobically at 42 °C for two days. If the

mCCDA plate was negative for colonies resembling *Campylobacter* spp., a subculture was made from BB onto another mCCDA plate and the plate was incubated in a microaerobic atmosphere at 42 °C for 48 hours. Pure cultures were frozen for future reference in 15% Glycerol Broth at -80 °C.

Deoxyribonucleic acid (DNA) extraction was done after 24 hours of incubation from freshly grown pure cultures by boiling for ten minutes in 1 ml 2% Chelex™ (Biorad, USA) in sterile Milli-Q water, followed by centrifugation (13000 rpm, 3 mins) to remove cell debris and the Chelex™ which inhibits polymerase chain reaction (PCR). The supernatant, containing the nucleic acids, was transferred to a fresh Eppendorf tube and used for PCR and MLST for species confirmation and sequence type respectively. The isolates of *Campylobacter* were speciated by multiplex PCR to detect the *ceu*E gene associated with *C. coli* and the *hip*O gene associated with *C. jejuni*. Primer pairs used were *ceu*E forward (5'-AATTGAAAATTGCTCCAACTATG-3') and primer *ceu*E reverse (5'-TGATTTTATTATTTGTAGCAGCG-3') (131) and CJF primer (5'-ACTTCTTTATTGCTTGCTGC-3') and CJR primer (5'-GCCACAACAAGTAAAGAAGC-3') (97) were used to target those genes for identification. Amplification was performed in a 20 µl reaction volume containing 0.2 µl of Platinum Taq polymerase (Invitrogen, USA), 2 µl of 10× PCR reaction buffer (Invitrogen, USA), 1 µl dNTP (Bioline, UK) (2mM), 0.6 µl $MgCl_2$, 2 µl of each forward and reverse primers (*hip*O and *ceu*E) (2pmol/µl), 6.2 µl H_2O and 2 µl of DNA. The reactions in the PCR were carried out in a SensoQuest lab Cycler (SensoQuest, Germany) by heating the sample to 96 °C for 2 mins, followed by 40 cycles of 94 °C for 20sec, 55 °C for 20 sec, 72°C for 30 sec, with a final extension of 72 °C for 5 mins. Electrophoresis was used to visualise the PCR products in a 1% agarose gel (Axygen® Biosciences, Spain) in TBE buffer which was then stained with ethidium bromide

(Thermo Fisher Scientific, New Zealand) and exposed to UV light. The presence of a 462 bp product indicated *C. coli* and 323 bp indicated *C. jejuni*.

2.3.4 Sequence typing

Multilocus sequence typing (MLST) for *Campylobacter* isolates was performed after species determination using seven house-keeping genes: *asp*A (aspartase A), *gln*A (glutamine synthase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *unc*A (ATP synthase alpha subunit) based on the methods outlined by Dingle et al., 2001. Each amplification reaction was performed in a 20 µl reaction volume containing 2 µl of the DNA preparation, 5 pmoles of both forward and reverse amplification primers and 14 µl of the PCR Mastermix (200 µl 10× PCR reaction buffer, 100 µl dNTP, 60 µl $MgCl_2$, 20 µl Platinum Taq polymerase and 1020 µl H_2O). A SensoQuest lab Cycler (SensoQuest, Germany) was used to perform the amplification under the following conditions: Initial denaturation for 15 mins at 94 °C followed by 30 cycles of 94 °C denaturation for 30 sec, 50 °C annealing for 30 sec and 72 °C extension for 90 sec. Final extension was for 72 °C for 7 mins. PCR products were precipitated with 25 µl 20% PEGS / 2.5 M NaCl solution, washed with 80% ethanol, dried and taken up in 13 µl H_2O and screened on 1% agarose gels. Sequencing reactions were performed using 2 µl of the PCR product, 3.2 pmoles primer, 1 µl ABI BigDye (Applied Biosystems, USA), 2 µl of 5× buffer and water to a total volume of 10 µl. The following conditions were used to perform the reactions: Initial denaturation for 3 mins at 96 °C followed by 15 cycles of 96 °C for 15 sec, 50 °C for 15 sec and 60 °C extension for 4 mins. Sequence products were precipitated with 3 M Na acetate / 96% ethanol (EtOH) solution, washed with 70% EtOH, dried and taken up in 12 µl H_2O and the sequence read at the Institute of Environmental Science and Research (ESR, Kenepuru, New Zealand) on an ABI 3130XL automated DNA

sequencer using ABI BigDye v3.1 following the manufacturer's instructions. Sequence data were collated and submitted for allele and ST designation with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/). Isolates yielding novel STs or alleles that did not give clear sequences were re-amplified and sequenced using the same protocol. If the sequence products were not readable, they were re-sequenced with the reverse primer and if this failed, the locus was re-amplified and re-sequenced. If this new assay also failed, no ST was assigned to the isolate.

2.3.5 Data analysis

The accuracy of a diagnostic test is called validity, and it is usually assessed by calculating the sensitivity and specificity (132). "Gold standard" is a test considered the best test or method for the detection of a disease. In this study, the 'gold standard' test was assumed to be *Campylobacter* culture without delay as opposed to delayed *Campylobacter* culture. The resulting data can be summarised in a 2x2 table (Table 2.1). The true positive (TP) and true negative results (TN) (Table 2.1 cells a and d) are the results that correctly identify the status of the sample by the new test. False positive (FP) results (Table 2.1cell b) are defined as positive test results that are negative by the gold standard. False negative (FN) results (Table 2.1 cell c) are negative results by the new test, but positive by the gold standard test. Sensitivity and specificity, including the 95% confidence interval (CI) as described by Newcombe 1998 (133), was calculated online (http://vassarstats.net/clin1.html).

Table 2.1: A 2×2 table for diagnostic test performance showing the true positive (TP), false positive (FP), true negative (TN) and false negative (FN).

	Gold standard test positive	Gold standard test negative	
Test positive	TP a	FP b	Total test positives a + b
Test negative	FN c	TN d	Total test negatives c + d
	Total diseased a + c	Total normal b + d	Total population a + b + c + d

Sensitivity measures the likelihood of a test to pick up the presence of a disease among people who have it whereas specificity measures the likelihood of a test to be negative among disease-free people.

$$\text{Sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}}$$

$$\text{Specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}}$$

$$95\%\ \text{confidence interval} = \text{p} \pm 1.96 \times \sqrt{\text{p}(1-\text{p})/\text{n}}$$

2.3.6 Minimum spanning tree

Minimum spanning trees were implemented to visualise allelic differences between STs of isolates that were not detected by the ELISA method using the pairwise Hamming distance matrix. The trees were calculated by Prim's algorithm (121) as implemented in the Bionumerics software (Applied Maths; http://applied-maths.com/bionumerics).

2.3.7 PERMANOVA

In order to formally test for significant population differentiation between *Campylobacter* isolates that were missed by the ELISA method and were detected by direct culture, permutational multivariate analysis of variance (PERMANOVA) (134, 135) was utilised. This analysis was implemented using the PERMANOVA+, an 'add in' to the PRIMER 6 software (136).

2.4 Results

A total of 702 samples were collected and processed by direct culture; the same samples were preserved in charcoal-Amies transport swabs at room temperature and cultured after a maximum delay of 7 days. During May 2014, a total of 191 samples were collected and the *Campylobacter* positivity rate was 4.18% (8/191), whereas in December 2014 and January 2015 the *Campylobacter* positivity rate was 10.17% (52/511). Direct culture of faecal samples detected the highest number of *Campylobacter* (60/702; 8.5%) compared to delayed culture (46/702; 6.55%) and to the ELISA method (46/702; 6.55%). The dominant *Campylobacter* spp. was *C. jejuni*, detected in 59 out of the 60 samples that were *Campylobacter*–positive by direct culture. *C. coli* was the only non-*jejuni Campylobacter* species isolated and was detected by all methods.

Table 2.2 shows comparison of outcomes between "direct vs delay culture", between "direct culture vs ELISA method" and between "delay culture vs ELISA method" of human faecal samples.

Table 2.2: Comparison of outcomes: direct vs delay culture, direct culture vs ELISA method and delay culture vs ELISA method.

		Direct culture (Gold standard)		
		Positive	Negative	Total
Delay culture	Positive	46	0	46
	Negative	14	642	656
	Total	60	642	702
ELISA method	Positive	32	14	46
	Negative	28	628	656
	Total	60	642	702
		Delay culture		
		Positive	Negative	Total
ELISA method	Positive	28	18	46
	Negative	18	638	656
	Total	46	656	702

The accuracy of the ELISA method is summarised in Table 2.2, showing the results of direct culture vs ELISA method.

Sensitivity = TP / (TP+FN) = 32/60 = 0.53 (95% CI: 0.40 - 0.66).

Specificity = TN / (TN+FP) = 628/642 = 0.98 (95% CI: 0.96 – 0.98).

The accuracy of the delay culture is summarised in Table 2.2, showing the results of direct culture vs delay culture.

Sensitivity = TP / (TP+FN) = 46/60 = 0.76 (95% CI: 0.63 - 0.86).

Specificity = TN / (TN+FP) = 642/642 = 1 (95% CI: 0.99 – 1).

The accuracy of the ELISA method compared to the delay culture is summarised in Table 2.2 giving the following results:

Sensitivity = TP / (TP+FN) = 28/46 = 0.60 (95% CI: 0.45 - 0.74).

Specificity = TN / (TN+FP) = 638/656 = 0.97 (95% CI: 0.95 – 0.98).

The fourteen true positive *Campylobacter* isolates by the 'gold standard' that were negative by delay culture had differing number of days delayed ranging from one day delay (5 isolates) to seven days of delay (2 isolates). Among the true positive *Campylobacter* isolates by the ELISA method, 4/32 (12.5%, 95% CI: 3.5 - 28.9%) were negative by delayed culture; the number of days delay in culturing for those four samples were one, five, six and seven days.

Sequence types

Fourteen isolates that were *Campylobacter* positive by direct culture and were not recovered by delayed culture were sequence typed. Four of those 14 isolates were *Campylobacter* positive by the ELISA method at MedLab Central. The allelic profiles, clonal complex (CC) and the ST assignments of those 14 isolates are listed in Table 2.3.

Table 2.3: CC assignment with ST profile recovered from samples that were negative in delayed culture but positive in direct culture.

		Allelic profile							
ST	CC	ASP	GLN	GLT	GLY	PGM	TKT	UNC	ELISA*
21	21	2	1	1	3	2	1	5	negative
42	42	1	2	3	4	5	9	3	positive
45	45	4	7	10	4	1	7	1	negative
45	45	4	7	10	4	1	7	1	positive
61	61	1	4	2	2	6	3	17	negative
257	257	9	2	4	62	4	5	6	negative
354	354	8	10	2	2	11	12	6	negative
474	48	2	4	1	2	2	1	5	negative
583	45	4	7	10	4	42	51	1	positive
704	U/A	2	29	4	27	10	25	57	negative
2026	403	10	1	16	19	10	5	7	negative
3105	U/A	7	276	5	2	135	68	26	positive
3798	403	239	27	16	19	10	5	7	negative
6964	354	7	2	2	2	11	12	6	negative

* *Campylobacter* detection by the ELISA method at MedLab Central
U/A = CC unassigned

Twenty eight isolates from samples that were 'false negative' by the ELISA method at MedLab Central and were positive by direct subculture were sequence typed; eighteen of those 28 isolates were recovered by delayed subculture. The details of CC assignment and allelic profile of the STs of the 'false negative' isolates by the ELISA method are listed in Table 2.4.

Table 2.4: CC assignment with ST profile recovered from samples that were negative by ELISA but positive in direct culture.

		Allelic profile						
ST	CC	ASP	GLN	GLT	GLY	PGM	TKT	UNC
42	42	1	2	3	4	5	9	3
45	45	4	7	10	4	1	7	1
53	21	2	1	21	3	2	1	5
61	61	1	4	2	2	6	3	17
137	45	4	7	10	4	42	7	1
190	21	2	1	5	3	2	3	5
422	21	2	1	5	3	2	5	5
474	48	2	4	1	2	2	1	5
538	45	4	7	10	4	42	25	1
677	677	10	81	50	99	120	76	52
1286	U/A	95	2	94	127	172	144	114
2026	403	10	1	16	19	10	5	7
21*	21	2	1	1	3	2	1	5
257*	257	9	2	4	62	4	5	6
354*	354	8	10	2	2	11	12	6
704*	U/A	2	29	4	27	10	25	57
3798*	403	239	27	16	19	10	5	7
6964*	354	7	2	2	2	11	12	6

* The same ELISA negative isolates as in Table 2.3
U/A = CC unassigned

The minimum spanning tree visualizing the ST clusters of the *C. jejuni* isolates that were missed by the ELISA method and detected by direct culture is shown in Figure 2.1. One *C. coli* isolate (ST-4009) was the only *Campylobacter* species detected other than *C. jejuni* and it was detected by all methods.

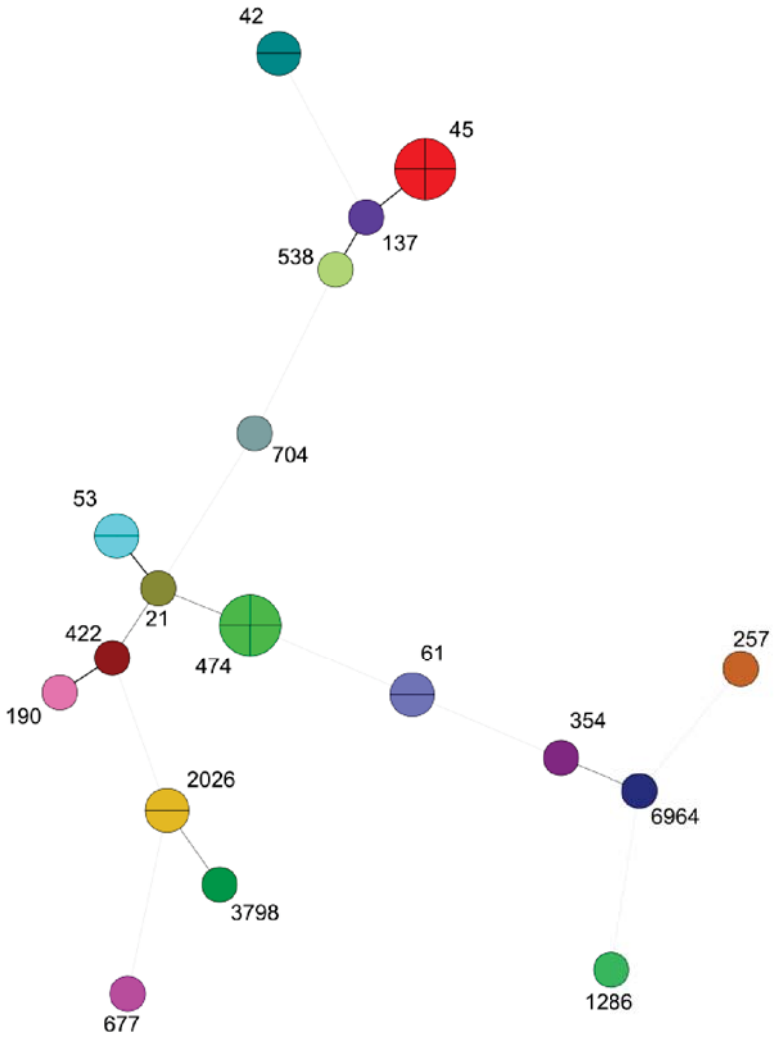

Figure 2.1: A minimum spanning tree of *Campylobacter* STs from clinical cases that were undetected by ELISA method and were positive by direct culture. Each node represents a ST, its size is proportional to the frequency of isolation. The thickness of the connecting lines is proportional to the similarities between STs, with the thickest connector linking single locus variants.

All twenty eight cases that were undetected by the ELISA method were *C. jejuni* and had 18 different STs. ST-474 and ST-45 were the most frequent STs that were not detected (4/28; 14%) followed by ST-2026, ST-42, ST-53 and ST-61 (2/28; 7%); the rest of the 12 STs were equally missed once by the ELISA method.

PERMANOVA outcome results, a pseudo-F statistic was 0.51 (P=0.82), indicated that the two populations (29 ELISA positive and 28 ELISA negative STs) did not differ from each other during the same sampling period. Moreover, the two populations also did not differ when we added 206 ELISA positive STs collected in 2014 to the 28

ELISA negative STs collected in our study period; the pseudo-F statistic was 0.48 (P=0.86). Figure 2.2 and Figure 2.3 show the non-metric multidimensional scaling (NMDS) plot of the two populations during the sampling period and after including all isolate from year 2014 respectively.

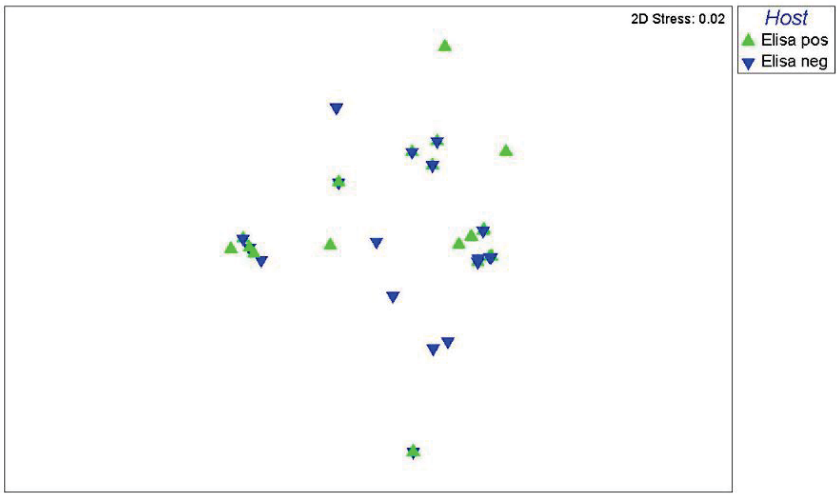

Figure 2.2: NMDS 2D plot of the 57 ELISA positives and ELISA negatives isolates (detected by direct culture) during the same sampling period.

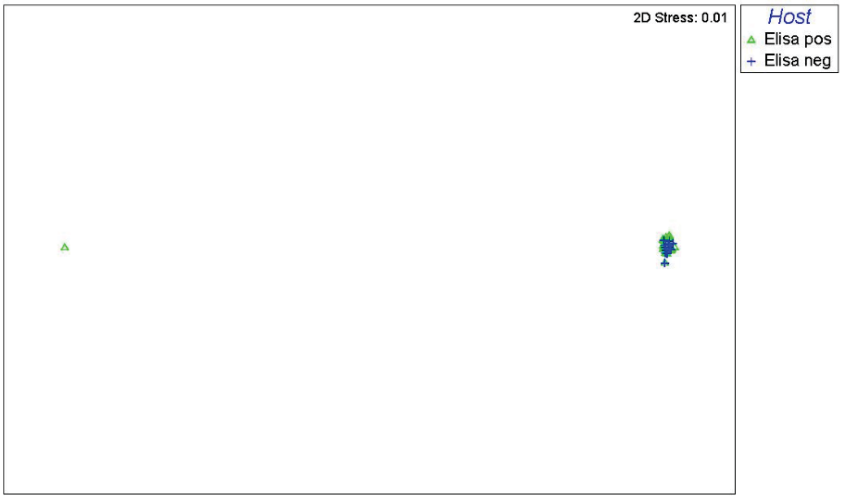

Figure 2.3: NMDS 2D plot of the 206 ELISA positive STs collected in 2014 and 28 ELISA negative STs (detected by direct culture) collected in our study period.

2.5 Discussion

Several studies have shown that the peak of *Campylobacter* infection rates in humans differs between one country and another depending on the season (137, 138). In the present study, the *Campylobacter* positivity rate was higher in the warmer months of the

year (December and January) with a 10.17% rate and lower in the colder months of the year (May) with a 4.18% rate. This difference in the positivity rates was expected and a similar study showed that the campylobacteriosis rate in Palmerston North, New Zealand, is higher in the summer seasons rather than in autumn and winter seasons (139).

In the past decade more than 95% of campylobacteriosis cases detected at MedLab belonged to *C. jejuni* followed by *C. coli*, which is quite similar to *Campylobacter* rates found in different countries where *C. jejuni* is always the dominant strain with around 90% to 95% of the campylobacteriosis cases (117, 127, 140). In this study there were fifty nine *C. jejuni* isolates and only one *C. coli* isolate out of the sixty positive samples which suggests that the ELISA method was not significantly missing non-*jejuni* isolates in the last decade which might have affected the source attribution studies.

Delayed subculture of up to seven days led to 23% of the positive samples not being recovered. The ELISA method had around 23% false positive samples or non-culturable isolates compared to a total of 60 positive isolates that were detected by the gold standard method. Four out of thirty two isolates were negative by delayed culture and positive by both the ELISA method and direct subculture; however it is difficult to know what length of delay day-wise affects recovery because all four samples had different lengths of delay ranging between one and seven days. Therefore we can say that there is no systematic relationship between the days of delay in processing. An explanation of why nineteen percent of the ELISA positive C*ampylobacter* samples being sent from MedLab Central for the past 10 years were not recovered by culture at the ^{m}EpiLab is either because of the delay in subculturing the sample, or it was a 'false positive' sample by the ELISA method, or positive ELISA samples that cannot be cultured because the organism was dead.

Although there were isolates being missed or undetected by the ELISA method or isolates that were not recovered at the ^{m}EpiLab, there were no missed STs that could have affected the source attribution studies. The STs (ST-474 and ST-45) of the 28 culture positive samples that were missed by ELISA were among the most common STs isolated from clinical cases in our lab. Moreover, the PERMANOVA results indicated that the two populations (ELISA positive and ELISA negative STs) did not differ from each other either during the same sampling period or during the whole year of 2014. This suggests that the isolates not detected by ELISA were random and that failure to detect was not associated with any particular ST. Hence this did not lead to bias in source attribution analyses. ST-704 and ST-1286 are rare STs that were previously detected once in our laboratory from a clinical human case and a water source respectively. Of the remaining ten STs that were 'false negative' by the ELISA method, those STs were frequently detected in human cases with a frequency reaching 72 times for ST-190 over a 10 year period. ST-3105, which was detected by the ELISA method and by direct subculturing but was not recovered by delayed subculture, was previously detected 14 times by our lab and only from poultry sources. Therefore, although those STs were missed they would not have changed or affected the ongoing source attribution studies because they are very rare STs that were detected less than 5 times in a 10 year period. The reason for the lack of detection of those STs is most probably due to random sampling rather than due to the ELISA method itself. This is because the common STs have been regularly detected before by the same method and the rare STs were not detected in higher amounts by 'Gold Standard' method. This hence supports the conclusion that it was a statistical sampling issue rather than a problem with the ELISA test sensitivity that resulted in these STs not being detected in human cases. In conclusion, because the ^{m}EpiLab is receiving the positive *Campylobacter* ELISA

samples once a week, the delay in processing those samples is affecting the recovery rate. In addition, the ELISA method used at MedLab Central is not detecting all the campylobacteriosis cases. It is acknowledged that there is a compromise between collecting as many samples as possible to capture all strain variants in the population versus what can be reasonably processed in the laboratory. However, the results indicate that the protocol employed does not introduce any major bias to source attribution analyses.

2.6 Acknowledgements

The work was funded by the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) postgraduate application fund supporting student research. I would like to thank Rukhshana Akhter and Lynn Rogers from the ^{m}EpiLab team for their laboratory work contribution and Dr Philip Carter from ESR for help with sequence typing. Last but not least, I want to thank my colleague Krunoslav Bojanic for enabling the cooperation with MedLab Central, collecting the samples and also laboratory work.

Chapter 3

Abundance and multilocus genotypes of *Campylobacter* species isolated from chicken drumsticks and whole carcasses obtained from different suppliers in the retail chain

3.1 Abstract

In the past decade, the major attributed source for campylobacteriosis infection in New Zealand was the consumption of poultry meat. Given that New Zealanders consume different types of chicken meat which undergo different processing before entering the retail chain, the aim of this study was to analyse the differences in the *Campylobacter* viable count and the population genetic structure between chicken drumsticks and whole carcass meat for retail sale over a one year period. *Campylobacter* isolates were identified by polymerase chain reaction and typed by multilocus sequence typing (MLST). *C. jejuni* was the dominant species among whole carcasses (63.5%; 40/63) and drumsticks samples (73.8%; 48/65) followed by *C. coli* 27% (17/63) and 23.1% (15/65) respectively. MLST revealed 21 different sequence types (STs) among whole carcass samples, two of which were novel STs (ST-8066 and ST-8067). No new STs were detected among the 20 STs isolated from drumsticks samples. No difference between the population genetic structure of *C. jejuni* and *C. coli* isolated from the two types of retail meat was observed based on statistical analyses. However, the *Campylobacter* viable counts differed significantly between the two types of retail meat, where whole carcasses showed higher viable counts than drumsticks. Additionally, there was a significant difference in the viable counts between the different suppliers in both types of retail meat. In conclusion, the *Campylobacter* population genetic structure did not differ between the two types of chicken retail meat; therefore, source attributional studies based on MLST are unlikely to be affected by the selection of those two

different types of retail meat during sampling. However, the difference in *Campylobacter* viable counts suggest consumption of different chicken meat products may pose different risks of campylobacteriosis associated with an exposure to different infection doses.

3.2 Introduction

Campylobacteriosis is a major health problem worldwide, mostly caused by the species *Campylobacter jejuni* and *Campylobacter coli* (141). It has been a notifiable disease in New Zealand since 1980 (142) and accounted for 45% of all notifiable bacterial gastroenteritis cases in 2014 (73). In New Zealand, the major risk factor for campylobacteriosis has been attributed to poultry consumption (143) causing an estimated 58% to 76% of cases (34). A study conducted in New Zealand (2003 and 2004) showed higher *Campylobacter* contamination in retail poultry meat compared to other meat products (144), which was also similar to findings from studies done in Belgium (145) and greater Washington, D.C. area, USA (146). New Zealanders are at high risk of *Campylobacter* infection due to their high consumption of retail poultry meat (147). A total of 136,728 tonnes of poultry meat were consumed in New Zealand in 2009 which was 35.79% of the total meat consumption (http://pianz.org.nz/industry-information/industry-statistics/meat-consumption/meat-consumption-percentages).

According to the New Zealand National Nutrition Survey (NNS) in 1997 and the Children's National Survey (CNS) in 2002, adult New Zealanders consumed mostly the breast poultry portion (28% of servings), followed by drumsticks (11.4%) from the total poultry portion types, whereas children's highest consumption was drumsticks (25.9%) followed by breast (19.9% of servings) (148). Most studies on *Campylobacter* spp. in New Zealand analysed bacterial isolates recovered from the whole chicken carcasses, and little is known about *Campylobacter* spp. isolates recovered from chicken pieces,

which are widely available in the retail chain in New Zealand. Furthermore, there is limited published comparative data concerning the prevalence, species and subtypes of *Campylobacter* spp. among different types of chicken meat products sold at retail chains globally. The manipulations and cutting performed during the jointing of chicken carcasses into chicken pieces have the potential to cross-contaminate the different products sold. For example, a study showed that 51.1% of 350 chicken products (thighs, breasts, tenders, gizzards or livers) were contaminated if the chicken products were derived from *Campylobacter*-positive flocks; in contrast, only 7.2% of 250 chicken products derived from *Campylobacter*-negative flocks were contaminated (149). Therefore, the aim of this study was to analyse the differences in the total *Campylobacter* viable count between whole carcasses and chicken drumsticks for retail sale in New Zealand over a one year period. An additional aim was to compare the population genetic structure of *C. jejuni* and *C. coli* between these two retail products using multilocus sequence typing (MLST).

3.3 Materials and methods

3.3.1 Sampling for *Campylobacter* isolation

Two different types of retail chicken meat products were sampled from several suppliers: whole carcasses and drumsticks. Six samples of fresh drumsticks and six of whole carcasses of approximately similar average weight were sampled every month at random from different supermarkets in Palmerston North during the period between October 2013 and September 2014. Sampled products were labeled with different brands, which in this paper are referred as "Supplier", which were arbitrarily assigned as Supplier A, Supplier B, and Supplier 'Others' (5 different suppliers combined). Suppliers A and B were the dominant brands in the retail chain at the time of the sampling. Over the same period, all human specimens that were submitted to MedLab

Central, Palmerston North and tested positive for *Campylobacter* spp. by ELISA (ProSpecT R, Remel, USA) were sent to the Molecular Epidemiology and Veterinary Public Health Laboratory (^{m}EpiLab) using charcoal transport swabs (Copan, Italy). Duplicate *Campylobacter* isolates with similar sequence types (STs) from the same sample were excluded.

3.3.2 *Campylobacter* isolation and identification

Human faecal swabs were cultured on modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland, New Zealand) and in a 3 ml Bolton Broth (BB) (Lab M, Bury, England) in a loose-capped Bijou and incubated in microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 48 hours (MACS-VA500-microaerophilic workstation, Don Whitley Scientific, West Yorkshire). A single colony resembling *Campylobacter* spp. on mCCDA was subcultured to Columbia Horse Blood Agar (BA) (Fort Richard, Auckland, New Zealand) and incubated microaerobilically at 42 °C for two days. If the mCCDA plate was negative for colonies resembling *Campylobacter* spp., a subculture was made from BB onto another mCCDA plate and the plate was incubated in a microaerobic atmosphere at 42 °C for 48 hours. Pure cultures were frozen in 15% glycerol broth (Oxoid, UK) at -80 °C.

Chicken parts were washed and massaged in 200 ml of sterile buffered peptone water (BPW) (Difco, USA). The wash was centrifuged at 16,000×*g* at 4°C for 30 mins (Sorvall LYNX 4000 centrifuge) and the pellet resuspended in 5 ml BPW. The resuspended pellet was added to 90 ml of Bolton Broth (Lab M, Bury, UK) and incubated at 42°C microaerobically (85% N_2, 10% CO_2, and 5% O_2) for 48 hours (MACS-VA500-microaerobic workstation, Don Whitley Scientific, West Yorkshire, UK), after which, it was subcultured onto modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) (Fort Richard, Auckland, New Zealand) and incubated

microaerobically at 42 °C for 48 hours. A single colony morphologically resembling *Campylobacter* spp. on mCCDA was subcultured to Columbia horse blood agar (BA) (Fort Richard, Auckland, New Zealand) and incubated microaerobically at 42 °C for 48 hours. Pure cultures were frozen in 15% glycerol broth (Oxoid, UK) at -80 °C.

DNA extraction was done after 24 hours of incubation from freshly grown pure cultures, by incubation in a heating block at 100 °C for ten minutes in 1 ml of 2% Chelex™ (Biorad, USA) in sterile Milli-Q water, followed by centrifugation at 12,470×*g*, for 3 mins to remove cell debris and Chelex™ which may inhibit PCR. The supernatant containing the nucleic acids was transferred to a fresh Eppendorf tube and used for PCR and MLST for species and sequence type determination. Isolates were confirmed as *Campylobacter* spp. by a *Campylobacter* genus-specific PCR using primers C412-F (5'-GGATGACACTTTTCGGAGC-3') and C1288-R (5'-CATTGTAGCACGTGTGTC-3') (150). Agarose gel electrophoresis was used to verify the presence of amplicons (150). *Campylobacter* isolates were identified to species level by multiplex PCR to detect the *ceu*E gene associated with *C. coli* or the *hip*O gene associated with *C. jejuni,* using the following primers: *ceu*E forward (5'-AATTGAAAATTGCTCCAACTATG-3') and primer *ceu*E reverse (5'-TGATTTTATTATTTGTAGCAGCG-3'). (131), and CJF primer (5'-ACTTCTTTATTGCTTGCTGC-3') and CJR primer (5'-GCCACAACAAGTAAAGAAGC-3') (97). Amplifications were performed in a 20 µl reaction volume containing 0.2 µl of DNA polymerase (Platinum Taq polymerase; Invitrogen, USA), 2 µl of 10× PCR reaction buffer (Invitrogen, USA), 1 µl dNTP (Bioline, UK) (2mM), 0.6 µl $MgCl_2$, 2 µl of each forward and reverse primers (*hip*O and *ceu*E) (2pmol/µl), 6.2 µl H_2O and 2 µl of template DNA. The PCR were carried out in thermocycler (SensoQuest Lab Cycler; SensoQuest, Germany) by heating the sample

to 96 °C for 2 mins, followed by 40 cycles at 94 °C for 20 sec, 55 °C for 20 sec, 72°C for 30 sec, with a final extension of 72 °C for 5 mins. Electrophoresis in 1% agarose gel (Axygen® Biosciences, Spain) and staining with ethidium bromide (Thermo Fisher Scientific, New Zealand) was used to visualise PCR amplicons. The presence of a 462 bp product indicated *C. coli* and a 323 bp product indicated *C. jejuni*.

3.3.3 Sequence typing

MLST for *Campylobacter* isolates was performed after speciation using seven house-keeping genes: *asp*A (aspartase A), *gln*A (glutamine synthase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *unc*A (ATP synthase alpha subunit) based on the methods outlined by Dingle et al., 2001 (104). Each amplification reaction was performed in a 20 µl reaction volume containing 2 µl of the DNA preparation, 5 pmoles of both forward and reverse amplification primers and 14 µl of the PCR Mastermix (200 µl 10× PCR reaction buffer, 100 µl dNTP, 60 µl $MgCl_2$, 20 µl Platinum Taq polymerase and 1020 µl H_2O). A SensoQuest lab Cycler (SensoQuest, Germany) was used to perform the amplification under the following conditions: Initial denaturation for 15 mins at 94 °C followed by 30 cycles of 94 °C denaturation for 30 sec, 50 °C annealing for 30 sec and 72 °C extension for 90 sec. Final extension was for 72 °C for 7 mins. PCR products were precipitated with 25 µl 20% PEGS / 2.5 M NaCl solution, washed with 80% ethanol, dried and taken up in 13 µl H_2O and screened on 1% agarose gels. Sequencing reactions were performed using 2 µl of the PCR product, 3.2 pmoles primer, 1 µl ABI BigDye (Applied Biosystems, USA), 2 µl of x5 buffer and water to a total volume of 10 µl. Following conditions were used to perform the reactions: Initial denaturation for 3 mins at 96 °C followed by 15 cycles of 96 °C for 15 sec, 50 °C for 15 sec and 60 °C extension for 4 mins. Sequence products were precipitated with 3 M Na acetate / 96%

EtOH solution, washed with 70% EtOH, dried and taken up in 12 µl H2O and the sequence read at the Institute of Environmental Science and Research (ESR, Kenepuru, New Zealand) on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 following the manufacturer's instructions. Sequence data were collated and submitted for allele and ST designation with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/). Isolates yielding novel STs or alleles that did not give clear sequences were re-amplified and sequenced using the same protocol. If the sequence products are not readable, they were re-sequenced with the reverse primer and if this failed, the locus was re-amplified and re-sequenced. New MLSTs were submitted to the online database.

3.3.4 Genetic relatedness

Genetic relatedness assessment between *Campylobacter* STs utilised permutational multivariate analysis of variance (PERMANOVA) (134, 135). This analysis was implemented using the PERMANOVA+, an 'add in' to the PRIMER 6 software (136). PERMANOVA works with any distance measure that is appropriate to the data and uses permutations to make distribution free. Finally, the genetic diversities of *Campylobacter* spp. from the different sources were compared by combining the results of the Simpson's and Shannon's diversity indices and their 95% bootstrap credible intervals (CrI) (calculated using PAST software version 2.17c) (151) and rarefaction analysis (performed using the package 'vegan' in R, version 3.1.3) (152). Shannon index is sensitive to both evenness and richness of the species, whereas Simpson index gives more weight to the dominant species, where rare species will not affect the diversity. Rarefaction analysis allows to compare species richness between samples of different sizes.

3.3.5 Minimum Spanning Tree

Minimum spanning trees were implemented to visualise allelic differences between STs of isolates from the different sources using the pairwise Hamming distance matrix. The method inspects the relationship of closely related genotypes within CC and identifies the most likely existing ancestral genotype. The trees were calculated by Prim's algorithm (121) as implemented in the Bionumerics software (Applied Maths; http://applied-maths.com/bionumerics).

3.3.6 Proportional Similarity Index

The proportional similarity index (PSI) estimated the similarity between the frequency distribution of human *Campylobacter* STs and those of the different sources and their bootstrap CrIs, as previously described (120). The PSI measures the area of intersection between two frequency distributions (119) and ranges between 0 and 1, where 0 indicates no similarity and 1 indicates identical frequency distributions. The PSI is calculated by the following equation: $PSI = 1 - 0.5 \sum i \mid pi - qi \mid = \sum i \min (pi,qi)$, where pi and qi are the proportion of strains that belong to type i out of all strains typed from sources P and Q (153). Calculations were performed using R, version 3.1.3.

3.3.7 Colony counts on chicken samples

Enumeration of *Campylobacter* colonies from whole carcasses and drumsticks samples were done using the spiral plater (Wasp, Don Whitley, England) and manual spread plating. During the process of chicken washing discussed above, duplicate mCCDA plates were inoculated with 1 ml (manual spread plate) and 50 µl (spiral plater) aliquots of wash and 100 µl (spiral plater) aliquots of resuspended wash pellet. The mCCDA plates were incubated microaerobically at 42 °C for 48 hours and the *Campylobacter*-like colonies were counted using a plate reader (aCOLyte, Synbiosis, England), or

manually. All counts were calculated to reflect the number of bacteria in 1 ml of the 200 ml chicken wash.

3.3.8 Analysis of *Campylobacter* colony counts

We aimed at assessing whether there were statistically significant differences in the *Campylobacter* viable counts among the two chicken retail meat types, after adjusting for the weight of the sample. A zero-inflated Poisson model was used for the analysis, as the variance of the viable counts was higher than the mean due to the presence of many samples that had zero counts, which led to over-dispersion. The R Studio version 3.1.3 software ("pscl" package) was used to apply the following equation:

$$Yi \sim \begin{cases} Poisson\ (Vi\ \lambda i), Zi = 0 \\ 0, Zi = 1 \end{cases}$$

Where Yi is the number of colonies counted from replicate i

$Zi \sim Bernoulli\ (Pi)$

$Pi =$ Probability of a zero

$Vi =$ Volume of rinse that is plated

$\lambda i =$ average colony count per milliliter (ml)

$$log\lambda i = Type\ of\ sample\ i + Supplier\ i + Type\ of\ sample\ i{:}\ Supplier\ i + Weight\ of\ sample\ i$$

$$logit(Pi) = Type\ of\ sample\ i + Supplier\ i + Type\ of\ sample\ i{:}\ Supplier\ i + Weight\ of\ sample\ i$$

This equation will allow both the probability of a zero count (i.e. probability of a negative) and the expected colony count, given that it is positive, to differ for each type and allowing for these type effects to differ between each supplier, after adjusting for the sample weight.

3.4 Results

Two hundred and sixty-five human clinical faecal samples and 144 chicken samples were collected between October 2013 and September 2014 and used in this study (Table 3.1). There were 72 drumsticks samples with an average weight of 1478 g and 72 whole carcasses with an average weight of 1586 g. The dominant *Campylobacter* species found in human clinical faecal samples was *C. jejuni*, present in 189/195 (97%), followed by *C. coli*, found in 6/195 (3%) samples. *C. jejuni* was also the dominant species followed by *C. coli* among the two types of retail chicken meat. Among whole carcass isolates, *C. jejuni* accounted for 40/63 (63.5%) of the isolates, followed by *C. coli* in 17/63 (27%), and unidentified *Campylobacter spp.* in 6/63 (9.5%). Among the drumstick isolates, *C. jejuni* accounted for 48/65 (73.8%) of the isolates, followed by *C. coli* (15/65; 23.1%) and unidentified *Campylobacter spp.* (2/65; 3.1%).

Table 3.1: Overview of samples collected between October 2013 and September 2014.

Source	Samples analysed	*Campylobacter* culture-positive samples	Isolates confirmed *C. jejuni*	Isolates confirmed *C. coli*	Isolates confirmed *Campylobacter* spp.	Number of isolates typed [a]
Human clinical faecal samples	265	195	189	6	0	192
Whole carcasses [b]	72	60	47	17	6	64
Drumstick[c]	72	63	48	15	2	63
Total	409	318	277	38	8	319

[a] Only *C. jejuni* and *C. coli* isolates were typed by MLST.
[b] Three samples had mixed *Campylobacter* spp. (*C. jejuni* and *C. coli*) and eight samples had mixed *C. jejuni* STs.
[c] Two samples had mixed *Campylobacter* spp. (*C. jejuni* and *C. coli*).

3.4.1 Multilocus sequence typing

Sixty-four different STs were observed among the 319 *Campylobacter* isolates from faeces and chicken samples (Table 3.2). Eight new STs (ST-7769, ST-7796, ST-8065, ST-8066, ST-8067, ST-8069, ST-8070 and ST-8076) that had not been reported in the PubMLST database were detected in this study. ST-7769, ST-7796, ST-8065, ST-8069, ST-8070 and ST-8076 were isolated from clinical cases and ST-8066 and ST-8067 were isolated from whole chicken carcasses.

Table 3.2: Frequency of ST isolated from human cases and chicken samples.

	Source Type			
ST	Human	Whole carcasses	Drumsticks	Total
45	21	11	11	43
50	17	2	3	22
583	10	6	5	21
48	6	4	7	17
354	3	6	8	17
42	12	0	0	12
2345	9	1	2	12
61	11	0	0	11
53	9	0	0	9
257	6	1	2	9
6964	2	3	3	8
474	5	0	2	7
190	6	0	0	6
520	5	1	0	6
677	6	0	0	6
2026	6	0	0	6
21	4	0	1	5
508	5	0	0	5
3105	0	4	1	5
422	4	0	0	4
436	4	0	0	4
538	4	0	0	4
38	3	0	0	3
535	0	3	0	3
51	2	0	0	2
3676	3	0	0	3
2343	1	0	1	2
2350	1	1	0	2
137	1	0	0	1
356	0	1	0	1
403	1	0	0	1
696	0	0	1	1
704	1	0	0	1
991	1	0	0	1
1517	1	0	0	1

Continued on next page

	Source Type			
ST	Human	Whole carcasses	Drumsticks	Total
1919	1	0	0	1
2076	1	0	0	1
2212	1	0	0	1
2535	1	0	0	1
3538	1	0	0	1
3711	1	0	0	1
3717	1	0	0	1
3792	0	1	0	1
3798	1	0	0	1
4338	1	0	0	1
4492	1	0	0	1
4500	0	0	1	1
7769	1	0	0	1
7796	1	0	0	1
8065	1	0	0	1
8066	0	1	0	1
8067	0	1	0	1
8069	1	0	0	1
8070	1	0	0	1
8076	1	0	0	1
1581*	0	9	4	13
1590*	1	2	3	6
2256*	0	1	4	5
825*	2	0	2	4
854*	1	2	1	4
3230*	0	3	0	3
1016*	1	0	0	1
3072*	1	0	0	1
4009*	0	0	1	1
Total isolates	192	64	63	319

* *C. coli* ST isolates

Table 3.3 provides the frequency of the different STs isolated from different retail chicken meat suppliers. Two of the most common STs found in humans in New Zealand

(ST-45 and ST-50) were only isolated from Suppliers B and 'Others'. Three clinical isolates belonged to ST-354, which was only isolated from Suppliers A and 'Others'.

Table 3.3: Frequency of STs isolated from different suppliers of retail chicken meat products

	Suppliers			
ST	A	B	'Others'	Total
45	0	9	13	22
354	10	0	4	14
583	4	1	6	11
48	0	8	3	11
6964	1	1	4	6
3105	4	1	0	5
50	0	4	1	5
257	3	0	0	3
2345	3	0	0	3
535	3	0	0	3
474	0	0	2	2
4500	1	0	0	1
3792	0	1	0	1
8066	0	0	1	1
696	0	0	1	1
2350	0	1	0	1
520	0	0	1	1
8067	0	0	1	1
2343	0	0	1	1
356	1	0	0	1
21	0	0	1	1
1581*	3	8	2	13
1590*	0	2	3	5
2256*	0	0	5	5
854*	0	1	2	3
3230*	0	0	3	3
825*	0	0	2	2
4009*	1	0	0	1
Total	34	37	56	127

* *C. coli* ST isolates.

3.4.2 Genetic relatedness

The genetic diversities of *Campylobacter* spp. from the different sources were compared using the Simpson's and Shannon's diversity indices (Table 3.4) and rarefaction analysis (Figure 3.1), with their 95% bootstrap credible intervals (CrI). Human *Campylobacter* spp. showed the greatest number of STs (n=52), followed by whole carcasses (n=21), and drumsticks samples (n=20). Some human STs were only found in drumsticks samples (ST-2343, ST-825, ST-474 and ST-21) whilst others were only found in whole carcass samples (ST-2350 and ST-520). The rarefaction curves indicated a greater ST richness of human *Campylobacter* spp. compared with the other sources, which was consistent with the values of the Simpson's and Shannon's diversity indices.

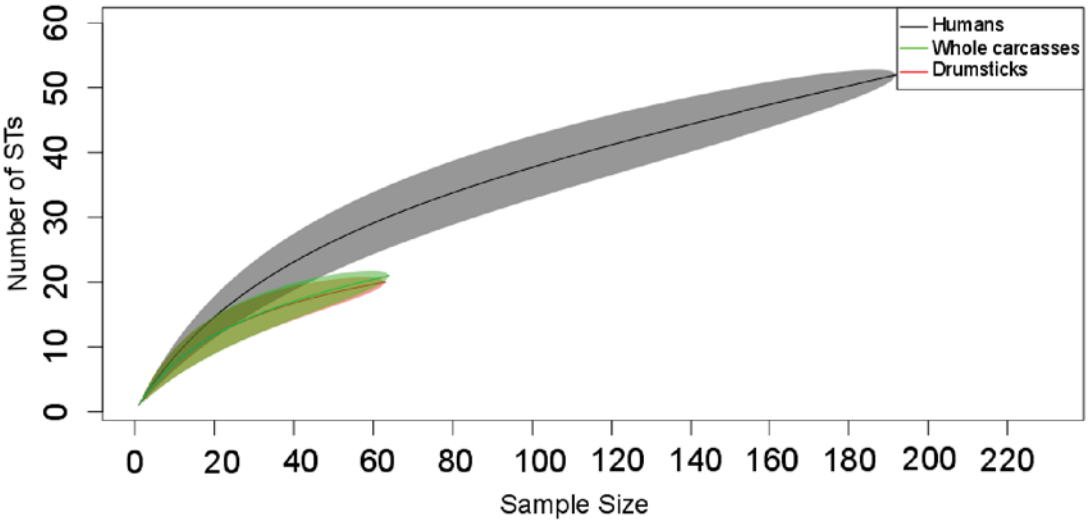

Figure 3.1: Rarefaction curves of the observed STs in human cases, whole carcasses and drumsticks samples. The shaded areas represent the 95% CrI. Note: The upper boundary of the 95% CrI in both types of chicken samples does not reach the point estimate of the human curve at maximum sample size.

Table 3.4: Simpson's and Shannon's diversity indices of the different sources (95% CrI are in brackets)

Source	Humans	Whole carcasses	Drumsticks
Simpson's	0.95 (0.93-0.95)	0.91 (0.91-0.95)	0.91 (0.91-0.95)
Shannon's	3.46 (3.21-3.50)	2.70 (2.84-3.30)	2.69 (2.84-3.31)

Campylobacter spp. among supplier 'Others' showed the greatest number of STs (n=19), followed by suppliers A and B (n=11 each). Rarefaction curves of the different suppliers are represented in Figure 3.2.

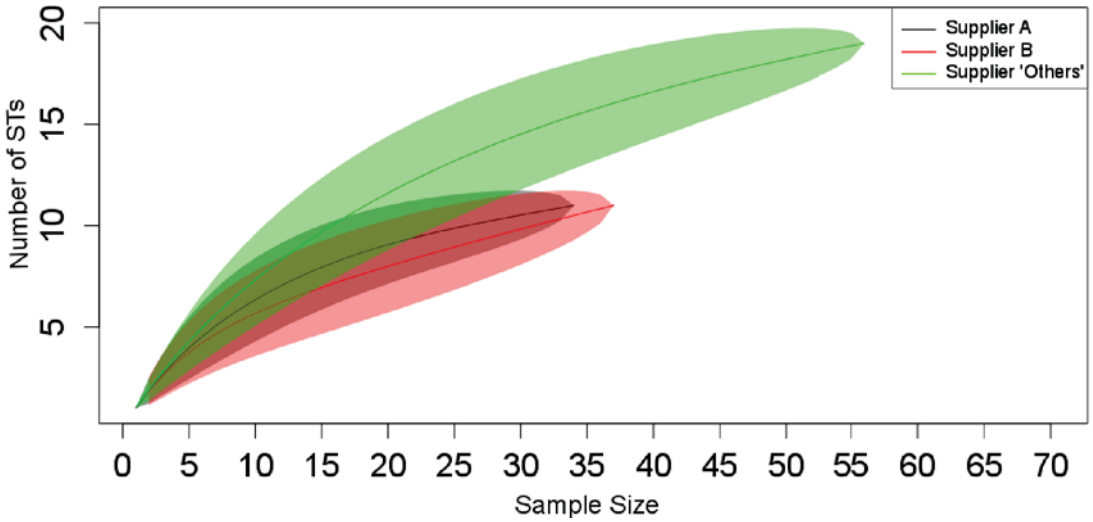

Figure 3.2: Rarefaction curves of the observed STs in different chicken meat suppliers. The shaded areas represent the 95% CrI. Note: The upper boundary of the 95% CrI in Suppliers A and B does not reach the point estimate of Supplier 'Others' curve at maximum sample size.

PERMANOVA was used to test for patterns of population differentiation, to compare the mean pairwise Hamming distances of *Campylobacter* spp. from clinical cases, whole carcasses and drumsticks. The pseudo-F statistic was 5.33 (P = 0.01), indicating that at least one of the populations differed from the other two. The non-metric multidimensional scaling (NMDS) 2D plot (Figure 3.3) did not reveal a conclusive difference between the populations.

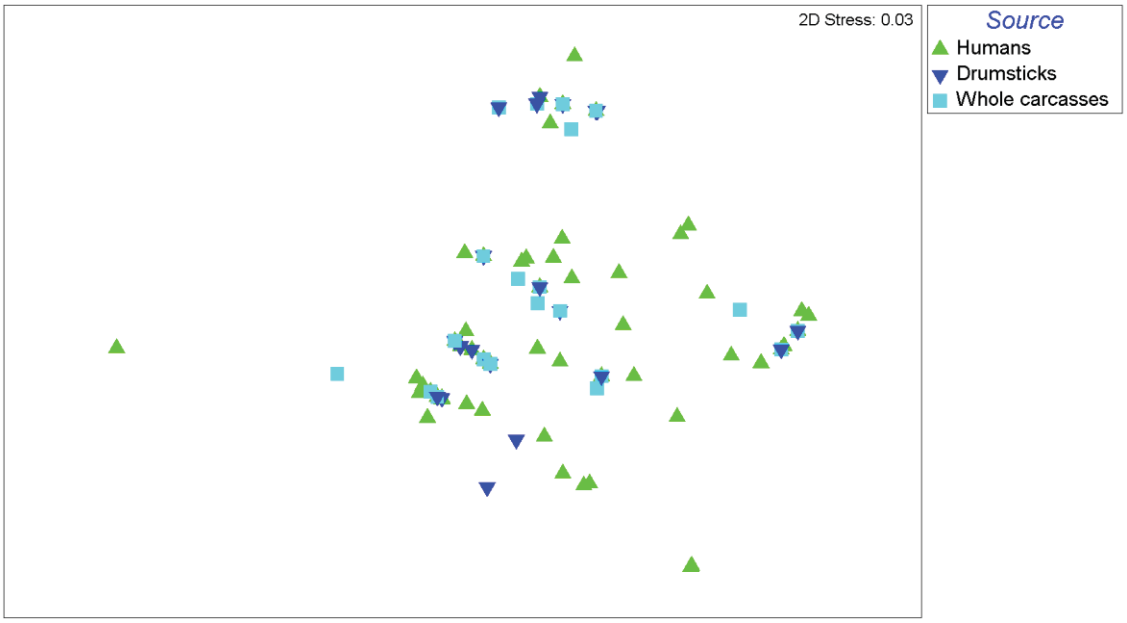

Figure 3.3: NMDS 2D plot of STs derived from different sources, humans, whole chicken carcasses and chicken drumsticks.

Another PERMANOVA was done to compare the mean pairwise Hamming distances of *Campylobacter* spp. from whole carcasses and drumsticks. The pseudo-F statistic was 0.72 (P = 0.60), which implies that the two populations did not show evidence of differentiation. The NMDS 2D plot (Figure 3.4) also suggested that there is no difference between drumsticks and whole carcasses. Therefore, the significant difference observed in the first PERMANOVA was likely to be attributable to a difference between the human sample and the chicken meat samples.

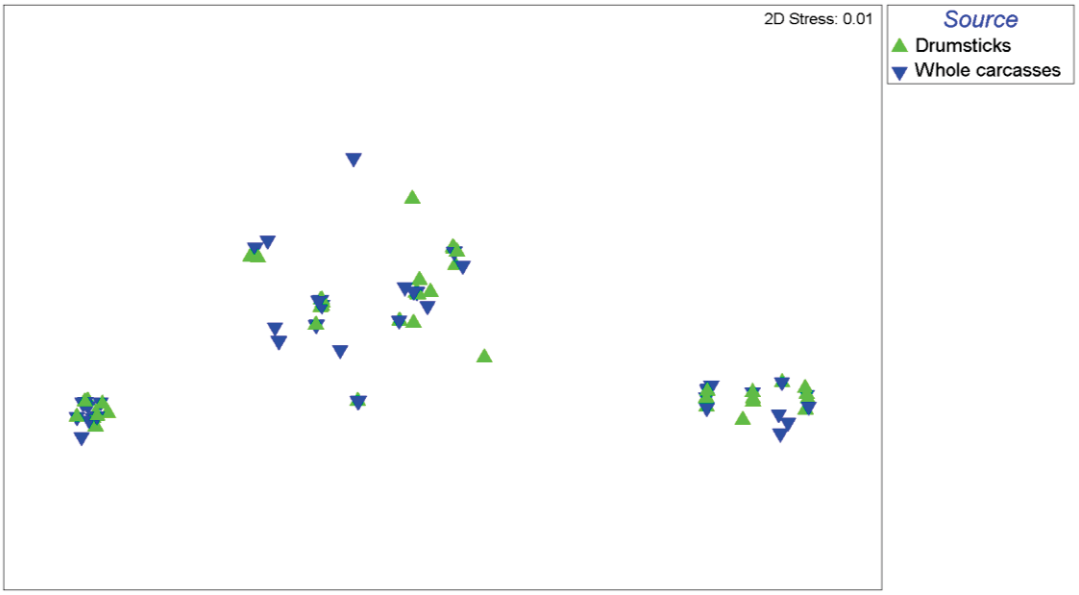

Figure 3.4: NMDS 2D plot of STs derived from whole chicken carcasses and chicken drumsticks.

A third PERMANOVA was used to test for significant population differentiation among different suppliers of the chicken samples. The pseudo-F statistic was 4.73 (P = 0.001), indicating that at least one of the populations differed from the other two. The non-metric multidimensional scaling (NMDS) 2D plot (Figure 3.5) did not reveal a conclusive difference between the populations.

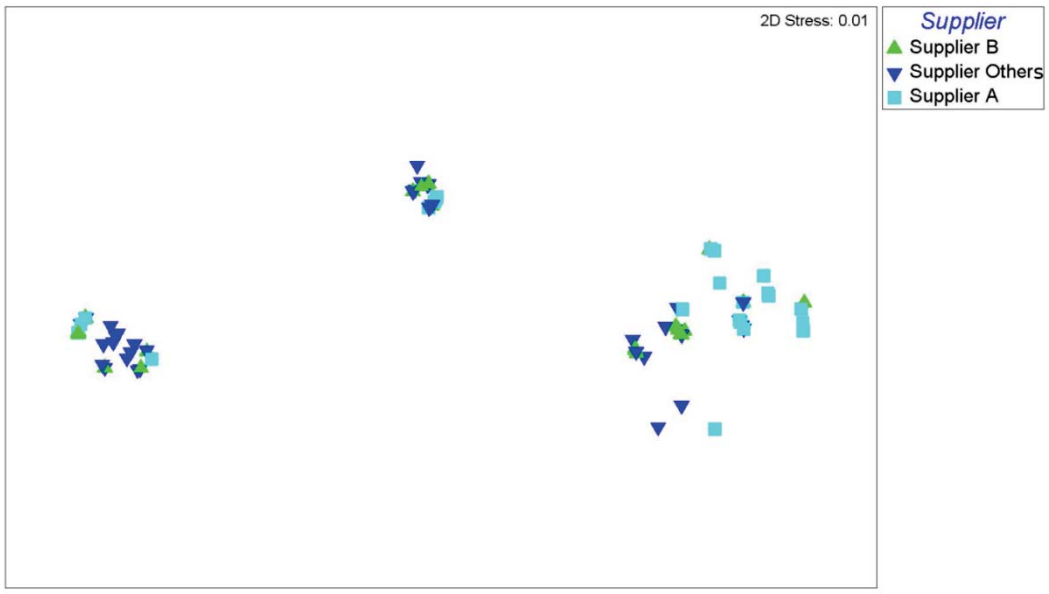

Figure 3.5: NMDS 2D plot of STs derived from chicken meat Supplier A, Supplier B and Supplier 'Others'.

Another PERMANOVA was done to compare the mean pairwise Hamming distances of *Campylobacter* spp. from Supplier B and Supplier 'Others'. The pseudo-F statistic was 1.60 (P = 0.15), which implies that the two populations did not show evidence of differentiation. Therefore, the significant difference observed in the PERMANOVA analysis among different suppliers was likely attributed to a difference between Supplier A samples and those samples from Supplier B and Supplier 'Others'.

3.4.3 Minimum Spanning Tree

The minimum spanning tree visualising ST clusters of the 319 *Campylobacter* STs isolated from different sources is shown in Figure 3.6.

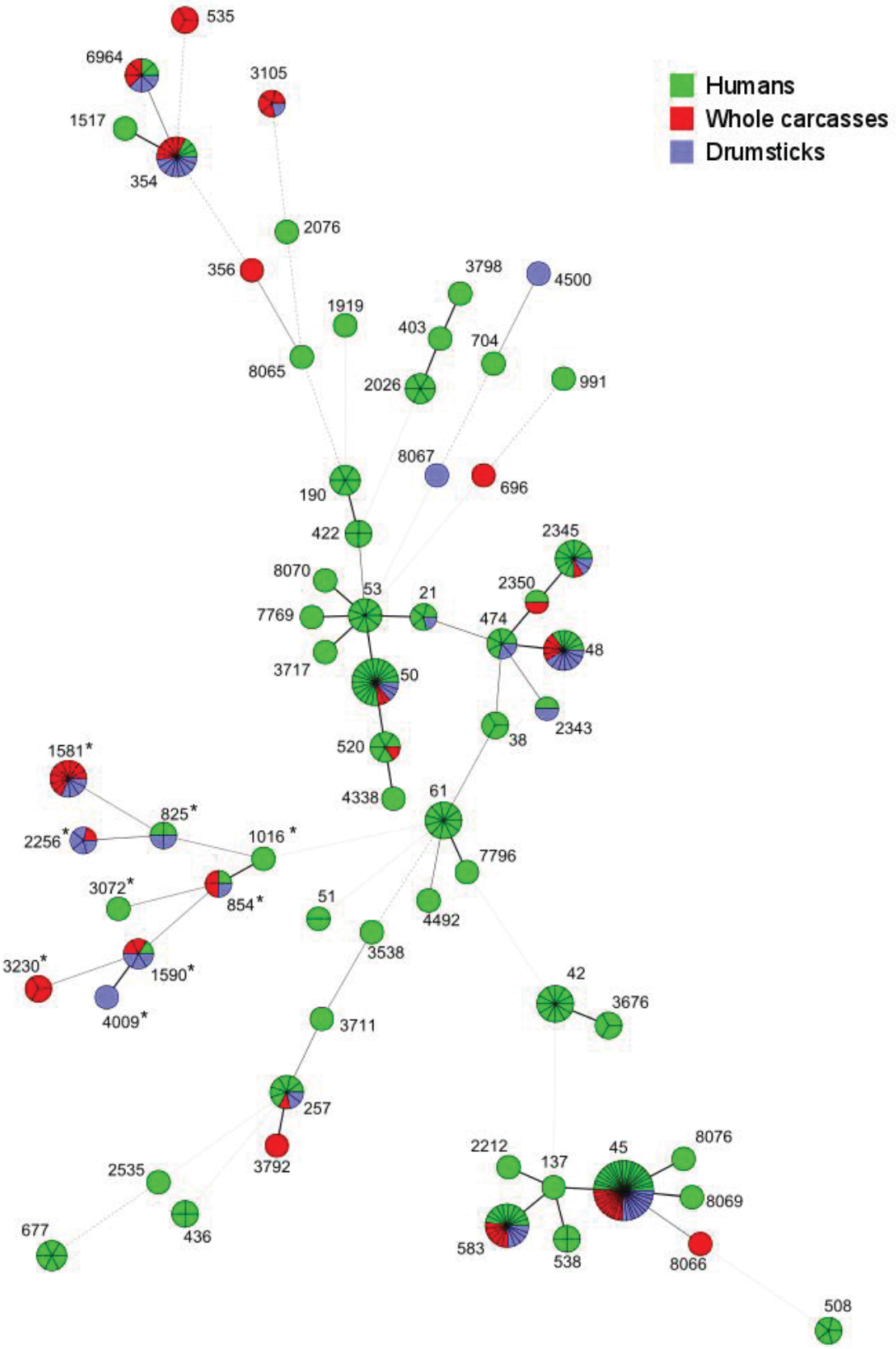

Figure 3.6: A minimum spanning tree of *Campylobacter* STs from human cases (n=192), whole carcasses (n=64) and drumsticks samples (n=63). *C. coli* STs are indicated with an asterix (the remaining are *C. jejuni)*. Each node represents a ST, its size is proportional to the frequency of isolation and the colours represent the different source type. The thickness of the connecting lines is proportional to the similarities between STs.

3.4.4 Proportional similarity index

The proportional similarity indices (PSI) and 95% CrI are reported in Table 3.5. The PSI between drumsticks and clinical isolates (PSI= 0.39, 95% CrI 0.27 – 0.44) was similar to that between whole carcasses and human isolates (PSI= 0.31, 95% CrI 0.21 – 0.37), and the 95% CrI overlapped. Among suppliers, the highest PSI was observed between Supplier 'Others' and humans isolates, with a significant difference compared to Supplier A since the 95% CrI did not overlap. The PSI comparing whole carcasses with drumsticks was 0.65 (95% CrI 0.43 – 0.67).

Table 3.5: The PSI for each source compared to the human genotypes distribution including 95% bootstrap CrI. The higher the value the stronger the similarity between the genotypes detected in human cases and the source.

Source	PSI	Lower Limit 95% CrI	Upper Limit 95% CrI
Type of sample			
Drumsticks	0.39	0.27	0.44
Whole carcasses	0.31	0.21	0.37
Suppliers			
Supplier A	0.15	0.08	0.19
Supplier B	0.28	0.17	0.33
Supplier 'others'	0.32	0.22	0.37

3.4.5 *Campylobacter* count data analysis

The prevalence of *Campylobacter* spp. positive samples throughout the duration of sampling in retail chicken samples was high (85.4%). Supplier A had the highest percentage of *Campylobacter* positive samples (94.1%), followed by Supplier B (86.3%), and Supplier 'Others' (80.3%). Counts of *Campylobacter* spp. on positive whole carcasses ranged from 50 to 260,000 colony forming units (cfu) per 'carcass', whereas the counts on positive chicken drumsticks ranged from 50 to 928,000 cfu per 'carcass'. Table 3.6 shows a comparison between the CFU counts on the positive

chicken meat among the different types of product and suppliers, based on the geometric means. Boxplot comparisons are represented in appendices 7.1.1, 7.1.2 and 7.1.3.

Table 3.6: Prevalence and count level of *Campylobacter* spp. on different types of samples and suppliers.

	No. (%) of positive *Campylobacter* samples	No. (%) of samples with detectable count	*Campylobacter* level (log CFU/sample) on positive samples *		
			Mean	Standard deviation	Median
Type of sample					
Drumsticks	63/72 (87.5%)	46/72 (63.8%)	6.22	1.85	5.99
Whole carcasses	60/72 (83.3%)	49/72 (68.0%)	7.65	1.97	7.69
Suppliers					
Supplier A	32/34 (94.1%)	25/34 (73.5%)	6.50	1.90	5.99
Supplier B	38/44 (86.3%)	27/44 (61.3%)	7.55	2.14	7.60
Supplier 'others'	53/66 (80.3%)	43/66 (65.1%)	6.95	1.98	6.62
Type within Supplier					
Drumsticks within Supplier A	16/17 (94.1%)	11/17 (64.7%)	6.26	1.35	5.99
Whole carcasses within Supplier A	16/17 (94.1%)	14/17 (82.3%)	6.65	2.18	6.19
Drumsticks within Supplier B	14/15 (93.3%)	8/15 (53.3%)	6.50	2.63	5.99
Whole carcasses within Supplier B	24/29 (82.7%)	19/29 (65.5%)	7.87	1.87	7.90
Drumsticks within Supplier 'Others'	33/40 (82.5%)	27/40 (67.5%)	6.12	1.73	5.84
Whole carcasses within Supplier 'Others'	20/26 (76.9%)	16/26 (61.5%)	8.08	1.73	8.29

* The geometric mean was used versus the arithmetic mean because the geometric mean is more suitable as the count data are closer to being symmetric on the log scale.

The use of median and mean log-transformed counts of *Campylobacter* spp. did not take into consideration the weight of the samples. Therefore, in order to know if there was a significant difference between *Campylobacter* counts among different types of chicken samples and different suppliers, the zero-inflated Poisson model was used for the statistical analysis taking into account the weight of the samples. According to the *Campylobacter* count model (Table 3.7), *Campylobacter* counts are higher in whole carcasses than drumstick samples within the same supplier. Moreover, the coefficients also showed that Supplier B had the highest *Campylobacter* counts ($p = 2\times10^{-16}$), followed by Supplier 'Others' ($p=0.03$). The weight of the sample was significantly related to *Campylobacter* counts, where higher weights were associated with higher *Campylobacter* counts ($p = 2\times10^{-16}$). It should be noted that 2 samples of chicken drumsticks from Supplier A that weighed 2.3kg and 2.5kg, respectively, and had very low (no more than 1) *Campylobacter* counts, and were removed from the model as the model was found to be highly sensitive to their inclusion (these were the only samples greater than 2kg).

The probability of a sample having a *Campylobacter*-zero-count after adjusting for the effect of sample weight is reported in Table 3.7. Whole carcass samples showed a lower probability of a zero-count than drumsticks ($p = 0.07$). The probability of zero-count was smaller for samples originating from Supplier 'Others' than the Suppliers A and B ($p = 0.01$). The probability of a sample being positive was estimated to increase 4.35 times (95% confidence interval: 2.14 - 8.82) for every 1kg increase in sample weight ($p <0.001$).

Table 3.7: Zero-inflated Poisson model results.

Model comparing *Campylobacter* colony counts between retail chicken meat products and suppliers, after adjusting for the weight of the sample				
	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)*	-2.71	0.15	-17.92	2×10^{-16}
Type Whole	2.02	0.10	19.96	2×10^{-16}
Source Supplier B	2.14	0.10	19.98	2×10^{-16}
Source Supplier 'Other'	0.79	0.10	7.43	0.03
Weight	1.91	0.06	31.38	2×10^{-16}
Type Whole: Source Supplier B	-0.50	0.10	-4.61	0.41
Type Whole: Source Supplier 'Others'	-0.41	0.11	-3.65	0.21
Zero-inflation model comparing the probability of a zero *Campylobacter* colony count between retail chicken meat products and suppliers after adjusting for the weight of the sample				
	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	2.60	0.63	4.08	1×10^{-5}
Type Whole	-0.68	0.35	-1.93	0.07
Source Supplier B	-0.49	0.40	-1.22	0.19
Source Supplier 'Others'	-0.77	0.31	-2.45	0.01
Weight	-1.28	0.33	-3.82	4×10^{-5}
Type Whole: Source Supplier B	0.33	0.47	0.70	0.52
Type Whole: Source Supplier 'Others'	0.97	0.43	2.22	0.02

*Comparison group was drumsticks from Supplier A to whole carcasses from Supplier B and Supplier 'Other'

3.5 Discussion

Chicken meat is sold in different portions which undergo different processing methods. There are little published comparative data on the prevalence, species and subtypes of *Campylobacter* spp. contaminating different types of chicken meat products sold at retail chains. Hence, in this study we analyzed the abundance and population genetic structure of *C. jejuni* and *C. coli* in two types of retail chicken meat products among different suppliers, over a one year period.

The proportions of *Campylobacter*-positive samples were very similar among drumsticks and whole carcasses. However, among the samples positive for *Campylobacter*, average counts were higher in whole carcasses than drumsticks, even after adjusting for the weight of the sample. Although whole carcasses had higher *Campylobacter* average counts than drumsticks, the highest *Campylobacter* count were observed among drumsticks (928,000 cfu/ sample) whereas in whole carcasses the highest count was 260,000 cfu/ sample. That might be due to the process of jointing whole carcasses, which involves contact with the abdominal cavity. Sample weight was directly correlated with the *Campylobacter* colony count in chicken samples. Heavier samples have a bigger surface area, hence, the tendency for higher *Campylobacter* spp. counts. Samples with high *Campylobacter* counts have implications on the human health and most important implication is that they have the potential to increase the risk of contaminating the kitchen environment leading to campylobacteriosis. A limitation of this study was the sampling of only one type of chicken retail meat product (drumsticks). Based on the results of this study, it would be interesting to further investigate the other types of chicken retail meat products. Another limitation might be the removal of the 2 samples of chicken drumsticks from Supplier A but the data suggest that, if anything, supplier A had the smallest *Campylobacter* count difference between whole carcasses and drumsticks, so the model result is counter-intuitive and is likely due to the large weights of those 2 observations relative to the rest. The proportion of the different *Campylobacter* species was very similar between drumsticks and whole carcasses samples. The dominant species in both types of chicken meat was *C. jejuni,* followed by *C. coli,* in agreement with the results of previous studies in New Zealand and abroad (117, 144, 154). One hundred and twenty seven *C. jejuni* isolates obtained from two different types of chicken samples, and 192 human clinical isolates

were subtyped by MLST. PERMANOVA results indicated a significant population differentiation of *Campylobacter* among the different suppliers, but not between the types of chicken meat. Rarefaction analysis indicated that human clinical isolates had higher ST diversity compared to the chicken meat isolates. This is consistent with the presence of other infection sources, such as ruminants and environmental sources (34) that were not analyzed in this study. However, the ST diversities were similar among the two types of chicken meat. The difference in the population genetic structure of *Campylobacter* spp. among the chicken suppliers indicated that some STs were associated with certain suppliers. Indeed, the ST-45 and ST-50 that were the dominant STs among the human clinical isolates were only isolated from Supplier B and Supplier 'Others'. This suggests that both ubiquitous and chicken supplier-specific STs contribute to human campylobacteriosis in the Manawatu region which is consistent with a previous study (117). The greater ST richness detected among Supplier 'Others', compared to Supplier A and B, is presumably because 'Others' represent many more diverse sources (5 different suppliers).

The PSI was calculated to assess the similarity between the *C. jejuni* ST frequency distributions of human clinical isolates, whole carcasses and drumsticks. Although the highest PSI was observed between STs from human clinical isolates and drumsticks, the CrI of the two PSIs overlapped widely, indicating the differences were not likely to be significant. However, there was a significant difference between the PSIs of human clinical isolates and Supplier 'Others' isolates, and between human clinical isolates and Supplier A isolates. This provides evidence that relatively more human campylobacteriosis cases may be attributable to consumption of meat from Supplier 'Others' than Supplier A. This result is consistent with the results of previous studies that also found differences in the attributable impact of different chicken meat suppliers

in New Zealand, although at that time most cases of campylobacteriosis were attributed to Supplier A (34).

In conclusion, we found significant differences between the *Campylobacter* viable counts of the two retail chicken meat products and between the PSIs of the different suppliers. The difference in *Campylobacter* viable counts suggest consumption of different chicken meat products may pose different risks of campylobacteriosis associated with an exposure to different infection doses. Further exploration of the relationship between Supplier 'Others' and human cases would require a larger sample size, representative of the five different suppliers that comprise this category. Moreover, given the differences between suppliers, it would be interesting to assess whether this is attributable to differences in the application of *Campylobacter* management strategies that were developed to reduce the burden of human campylobacteriosis in New Zealand (155).

3.6 Acknowledgements

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Chapter 4

Molecular epidemiology of *Campylobacter coli* isolated from different sources in New Zealand between 2005 and 2014

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4.1 Abstract

Campylobacteriosis is one of the most important food-borne diseases worldwide and a significant health burden in New Zealand. *C. jejuni* is the predominant species worldwide, accounting for approximately 90% of human cases, followed by *C. coli*. Most studies in New Zealand have focused on *C. jejuni*; hence, the impact of *C. coli* strains on human health is not well understood. The aim of this study was to genotype *C. coli* isolates collected in the Manawatu region of New Zealand from clinical cases, fresh poultry meat, ruminant faeces, and environmental water sources, between 2005 and 2014, to study their population structure and estimate the contribution of each source to the burden of human disease. *Campylobacter* isolates were identified by polymerase chain reaction and typed by multilocus sequence typing. *C. coli* accounted for 2.9% (n=47/1,601) of *Campylobacter* isolated from human clinical cases, 9.6% (n=108/1123) of poultry, 13.4%, (n=49/364) of ruminant and 6.4% (n=11/171) of water isolates. Molecular subtyping revealed 27 different sequence types, of which 18 belonged to clonal complex ST-828. ST-1581 was the most prevalent *C. coli* sequence type isolated from both human cases (n=12/47) and poultry (n=44/110). When classified using cladistics, all sequence types belonged to clade 1 except ST-7774, which belonged to clade 2. ST-854, ST-1590 and ST-4009 were isolated only from human cases and fresh poultry, while ST-3232 was isolated only from human cases and

ruminant sources. Modeling indicated ruminants and poultry as the main sources of *C. coli* human infection.

4.2 Introduction

The genus *Campylobacter* currently includes 26 species and 11 sub-species (2). *Campylobacter jejuni* is the predominant species isolated from cases of campylobacteriosis worldwide, followed by *C. coli* (5-7). Campylobacteriosis is one of the most important food-borne diseases worldwide and the incidence of reported cases varies by country (31). For instance, New Zealand had its highest incidence in 2006 with more than 380 cases per 100,000 population (156) whereas the USA in 2014 had an incidence of 13.45 cases per 100,000 (51) where it was considered the third most important bacterial food-borne disease (157) and in Germany the rate in 2010 was 80 cases per 100,000 population where it was considered the leading cause of bacterial gastroenteritis (138). The 2014 rate of campylobacteriosis in New Zealand was 150.3 cases per 100,000 population and, despite the 60% decrease in the rate of campylobacteriosis compared to 2006, campylobacteriosis remains the most commonly notified disease in New Zealand and comprised about 45% of all enteric diseases notifications in 2014 (73).

The distribution and prevalence of *C. jejuni* and *C. coli* strains differs amongst sources; for example it varies between environmental water, food and different animal species (140) and between countries. In England and Wales, *Campylobacter* spp. are the most commonly reported bacterial pathogens isolated from infectious intestinal disease (IID), with ~56,000 laboratory-confirmed cases in 2001. This number of diagnosed cases is an underestimate of the actual number of cases in the population; according to one estimate there could have been ~420,000 cases in England alone (158). There were approximately 572,000 cases of human campylobacteriosis in United Kingdom in 2009

(59) and 845,000 cases occurred annually in the USA (157) of which *C. coli* strains contributed to 9% of the cases (~84,000) (159) and approximately 7% in England and Wales (141). Although the proportion of *C. coli* to *C. jejuni* is small, the high incidence in the population means the case numbers may still be large (140). For example, since there were approximately 500,000 *Campylobacter* IID cases, if 10% were attributed to *C. coli* strains it means that there were 50,000 cases due to *C. coli* strains which is a significant number. Based on recent estimates, infections due to *C. coli* strains have an annual cost of £ 50 million and $ 800 million in UK and USA respectively and despite that, most research including that in New Zealand focused on *C. jejuni* because it is the predominant species affecting humans (160, 161).

Data from the England and Wales sentinel surveillance scheme suggested that different strategies might be needed to control *C. jejuni* and *C. coli* strains (140) because the risk factors for transmission of *C. coli* to humans might be different from the *C. jejuni* strains (141). For instance, in a case-control study in the Netherlands (109) the main risk factor for *C. jejuni* enteritis was consumption of chicken meat, whereas *C. coli* infections had different risk factors; swimming in open water or sea and consumption of game and tripe. Molecular subtyping by means of multilocus sequence typing (MLST) plays an important role in measuring the contribution of different infection sources to the burden of human disease caused by *C. jejuni*, and the same MLST scheme can be applied to *C. coli* (162). MLST was applied to *C. coli* isolates from humans and several other animal sources and water in studies in Scotland (163), Denmark (164), and Switzerland (24). Sheppard et al. (165, 166) carried out phylogenetic analyses of *C. coli* from Scotland using concatenated sequences obtained by MLST, and showed that *C. coli* could be divided into three distinct clades. All the *C. coli* isolated from human clinical cases belonged to clade 1 which was dominated by strains isolated from farm

animal sources, whereas clades 2 and 3 were mainly composed of environmental water strains. Hence, unlike the case control study in the Netherlands (where environmental water sources appeared to be a major source of *C. coli* infection), this study indicated a major role of animal sources in the transmission of *C. coli* to humans in Scotland.

Most cases of human campylobacteriosis in New Zealand are attributable to *C. jejuni*; however the relative contribution of *C. coli* and the sources of these infections had not been previously examined. Therefore, the objectives of this study were firstly to assess the relative contribution of *C. coli* to campylobacteriosis morbidity, by identifying to the species-level 1,601 *Campylobacter* spp. clinical isolates collected between 2005 and 2014 from human cases in one New Zealand region. Secondly, the aim was to analyse the genetic relatedness of the clinical *C. coli* isolates with that of isolates obtained from poultry meat, farmed ruminants, and environmental water sources in the same study area and time frame using MLST, in order to infer possible transmission pathways of this important pathogen.

4.3 Materials and methods

4.3.1 Study population

The study used *Campylobacter* spp. isolated from human faeces from Medlab Central in Palmerston North in the MidCentral District Health Board area of New Zealand North Island between March 2005 and December 2014. The region had a population of ~223,000 and its main city, Palmerston North, had a population of ~80,000 (74, 75).

4.3.2 *Campylobacter* isolates

All the human faecal specimens that were submitted to the main medical laboratory (MedLab Central, Palmerston North) during the sampling period, and tested positive for *Campylobacter* spp. by ELISA (ProSpecT ®, Remel, USA), were delivered for bacterial

culture to the Molecular Epidemiology and Public Health laboratory (^{m}EpiLab) of Massey University (MU). The faeces were delivered weekly on Amies charcoal transport swabs (Copan, Brescia, Italy). Over the same period (2005-2014) fresh whole poultry carcasses (chicken and turkey samples) from different poultry suppliers were sampled each month from supermarkets in Palmerston North. Eleven *C. coli* isolates from environmental water (recreational waterways and pre-treatment drinking water; nine from Manawatu-Tararua area and 2 (ST- 3302 and ST-7774) from the North Island of New Zealand between 2006 and 2014) and 49 *C. coli* isolates from ruminants faeces (cattle and sheep faeces from 9 different farms in the Manawatu-Tararua area between 2006 and 2011) were collected as part of the same, long term monitoring program. These were kept frozen in our laboratory for future reference, and were also included in this study.

Isolates from pig sources were not included in the study due to the low prevalence of *Campylobacter* from this source (more than 650 pig samples were collected from which only one *C. coli* was identified).

4.3.3 Bacterial culture and identification

Human faecal swabs were cultured on modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland, New Zealand) and in a 3 ml Bolton Broth (BB) (Lab M, Bury, England) in a loose-capped bijou and incubated in microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 48 hours (MACS-VA500-microaerobic workstation, Don Whitley Scientific, West Yorkshire). A single colony resembling *Campylobacter* spp. on mCCDA was subcultured to Columbia horse blood Agar (BA) (Fort Richard, Auckland, New Zealand) and incubated microaerobically at 42 °C for two days. If the mCCDA plate was negative for colonies resembling *Campylobacter* spp., a subculture was made from BB onto another mCCDA

plate and the plate was incubated in a microaerobic atmosphere at 42 °C for 48 hours. Pure cultures were frozen in 15% glycerol broth (Oxoid, UK) at -80 °C.

Chickens were washed and massaged in 200 ml of sterile Buffered Peptone Water (BPW) (Difco, USA). The chicken wash was centrifuged (16,000×*g*, 4°C, 30 mins, Sorvall LYNX 4000 centrifuge) and the resultant pellet resuspended in 5 ml of BPW. The resuspended pellet was added to 90 ml of BB and incubated microaerobically at 42 °C for two days after which it was subcultured onto mCCDA and incubated microaerobically at 42 °C for 48 hours. Single colonies resembling *Campylobacter* spp. on mCCDA were subcultured to BA and incubated microaerobically at 42 °C for two days. Pure cultures were frozen in 15% glycerol broth at -80 °C.

Water samples were filtered through a sterile 0.45 µm filter (Millipore, USA) and the filter was transferred to 20 ml BB and incubated at 42°C microaerobically for two days, then it was subcultured onto mCCDA and incubated for two days. A single colony resembling *Campylobacter* spp. on mCCDA was subcultured onto BA and incubated microaerobically at 42°C for two days.

DNA was extracted by boiling a freshly grown culture of frozen isolates for 10 minutes in 1 ml 2% Chelex™ (Biorad, USA) in sterile Milli-Q water, followed by centrifugation (12,470×g, 3 mins) to remove cell debris and the Chelex™. The supernatant was transferred to a fresh eppendorf tube and used for PCR and MLST for species confirmation. Isolates were firstly identified through the use of the *map*A gene for the detection of *C. jejuni* (94), employing the following primers: *map*A-F (5'-CTTGGCTTGAAATTTGCTTG-3') and *map*A-R (5'-GCTTGGTGCGGATTGTAAA-3'). All the non-*jejuni* isolates were tested by a *Campylobacter* genus-specific PCR using the following primers: C412-F (5'-GGATGACACTTTTCGGAGC-3') and C1288-R (5'-CATTGTAGCACGTGTGTC-3') (150). Subsequently, all the non-*jejuni*

Campylobacter spp. were subject to a new *C. coli* species-specific PCR for detection of the *ceu*E gene (96). Primers were *ceu*E-F (5'-AATTGAAAATTGCTCCAACTATG-3'); *ceu*E-R (5'-TGATTTTATTATTTGTAGCAGCG-3'). Amplification was performed in a 20 µl reaction volume containing 0.2 µl of Platinum Taq polymerase (Invitrogen, USA), 2 µl of 10× PCR reaction buffer (Invitrogen, USA), 1 µl dNTP (Bioline, UK) (2mM), 0.6 µl MgCl2, 2 µl of each forward and reverse primer (2pmol/µl), 6.2 µl H2O and 2 µl of DNA. The reactions were carried out in a thermocycler (SensoQuest, Germany) with denaturation at 96°C for 2 mins, followed by 35 cycles of 96°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final extension of 72°C for 2 mins. Electrophoresis in 1% agarose gel and staining with ethidium bromide was used to visualise PCR products. Presence of a ~462 bp product indicated *C. coli* (167).

4.3.4 Sequence typing

MLST for *C. coli* isolates was performed after speciation using seven house-keeping genes: *asp*A (aspartase A), *gln*A (glutamine synthase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *unc*A (ATP synthase alpha subunit) based on the methods outlined by Dingle et al., 2001 (104). Each amplification reaction was performed in a 20 µl reaction volume containing 2 µl of the DNA preparation, 5 pmoles of both forward and reverse amplification primers and 14 µl of the PCR Mastermix (200 µl 10× PCR reaction buffer, 100 µl dNTP, 60 µl $MgCl_2$, 20 µl Platinum Taq polymerase and 1020 µl H_2O). A SensoQuest lab Cycler (SensoQuest, Germany) was used to perform the amplification under the following conditions: Initial denaturation for 15 mins at 94 °C followed by 30 cycles of 94 °C denaturation for 30 sec, 50 °C annealing for 30 sec and 72 °C extension for 90 sec. Final extension was for 72 °C for 7 mins. PCR products were precipitated

with 25 µl 20% PEGS / 2.5 M NaCl solution, washed with 80% ethanol, dried and taken up in 13 µl H_2O and screened on 1% agarose gels. Sequencing reactions were performed using 2 µl of the PCR product, 3.2 pmoles primer, 1 µl ABI BigDye (Applied Biosystems, USA), 2 µl of x5 buffer and water to a total volume of 10 µl. Following conditions were used to perform the reactions: Initial denaturation for 3 mins at 96 °C followed by 15 cycles of 96 °C for 15 sec, 50 °C for 15 sec and 60 °C extension for 4 mins. Sequence products were precipitated with 3 M Na acetate / 96% EtOH solution, washed with 70% EtOH, dried and taken up in 12 µl H2O and the sequence read at the Institute of Environmental Science and Research (ESR) on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1 following the manufacturer's instructions. Sequence data were collated and submitted for allele and ST designation with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/). Isolates yielding novel STs or alleles that did not give clear sequences were re-amplified and sequenced using the same protocol. If the sequence products are not readable, they were re-sequenced with the reverse primer and if this failed, the locus was re-amplified and re-sequenced. New MLSTs were submitted to the online database.

4.3.5 Minimum Spanning Tree

Minimum spanning trees were implemented to visualise allelic differences between STs of isolates from the different sources using the pairwise Hamming distance matrix. The trees were calculated by Prim's algorithm (121) as implemented in the Bionumerics software (Applied Maths; http://applied-maths.com/bionumerics).

4.3.6 Molecular phylogenetic analysis

A maximum likelihood (ML) method based on the Kimura 2-parameter model was used to infer the evolutionary history (168) using the concatenated DNA sequences composed of 3309 nucleotide positions across the seven loci. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (169) to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. Previous phylogenetic work done in Scotland identified 3 *C. coli* clades of closely related STs associated with different sources (170); we examined the position of our STs in the same clades. Therefore, the ML method was implemented using 36 nucleotide sequences representing the different STs found in this study (n=27) with the addition of 9 STs taken at random (4 STs from clade 2: ST-3304, ST-3122, ST-3180, ST-3182; 5 STs from clade 3: ST-2485, ST-2681, ST-3109, ST-3123, ST-3124) from two clades described in Scotland and not present in our study. Clade 1 had 10 shared STs with the Scottish study so no additional STs were sampled from this clade. These analyses were conducted using MEGA6 software (171).

4.3.7 Genetic relatedness

A different approach to assess genetic relatedness between *C. coli* utilised permutational multivariate analysis of variance (PERMANOVA) (134, 135). This analysis was implemented using the PERMANOVA+, an 'add in' to the PRIMER 6 software (136). Finally, the genetic diversities of *C. coli* from the different sources were compared using the Simpson's and Shannon's diversity indices and their 95% bootstrap credible intervals (CrI) (calculated using PAST software version 2.17c) (151) and rarefaction analysis (performed using the package 'vegan' in R, version 3.1.3) (152).

4.3.8 *C. coli* source attribution

Although human-human spread may be implicated in some outbreaks (172), we assume human to human spread as a cause of sporadic cases is negligible in common with other source attribution studies and therefore do not consider anthroponotic spread (117, 170). The relative contribution of different *C. coli* sources to the human disease burden was estimated using a number of tests. The similarity between the frequency distribution of human *C. coli* STs and those of the different sources was estimated using proportional similarity indices (PSI) and their bootstrap CrIs, as previously described (120). The PSI measures the area of intersection between two frequency distributions (119) and ranges between 0 and 1, where 0 indicates no similarity and 1 indicates identical frequency distributions. The PSI is calculated by the following equation: $PSI = 1 - 0.5 \sum i \mid pi - qi \mid = \sum i \min (pi,qi)$, where pi and qi are the proportion of strains that belong to type i out of all strains typed from sources P and Q (153). Calculations were performed using R, version 3.1.3.

The asymmetric island model was used to probabilistically assign each human isolate to one of the source populations (poultry, ruminants or environmental water) (111).

4.4 Results

4.4.1 Proportion of *C. coli* in ELISA-positive faecal specimens

A total of 2,009 ELISA-positive human faecal specimens were submitted by the medical laboratory for culture to ^{m}EpiLab between March 2005 and December 2014 and 1,601 (~80%) were *Campylobacter*-positive by culture. The dominant species was *C. jejuni*, accounting for 1552/1,601 (~97%) isolates, followed by *C. coli,* accounting for 47/1,601 isolates (~2.9%). Two isolates (<0.2%) were not identified as either *C. jejuni* or *C. coli,* and were not further analysed. The annual number of *C. coli* was 9 (2005), 6

(2006), 2 (2007), 6 (2008), none (2009), 4 (2010), 2 (2011), 6 (2012), 7 (2013), and 5 (2014). A total of 1123 *Campylobacter* isolates were isolated from poultry (chicken, n= 1,074; turkey, n= 49) meat samples. *C. jejuni* accounted for 980/1123 (87.3%) and *C. coli* for 108/1123 (9.6%) of the isolates. The *Campylobacter* species remained unidentified in 29/1123 (<3%) isolates. The proportion of *C. coli* was significantly higher in the poultry meat samples than in human faeces (chi-square, P<0. 01).

4.4.2 Sequence Types

Two hundred and fifteen *C. coli* isolates from all sources were subtyped by MLST (47 from human faeces, 49 from ruminant faeces (32 sheep faeces and 17 cattle faeces), 108 from poultry meat and 11 from environmental water). There were 27 different *C. coli* STs. Eighteen STs (66%) belonged to the ST-828 CC, which was the predominant CC in our data and accounted for 54% (116/215) of the isolates. The remaining STs could not be classified into any known CC. Three previously unidentified STs (ST-7767, ST-7774 and ST-7776) were detected. ST-7767 and ST-7776 were isolated from poultry and ST-7774 from environmental water. The dominant sequence type (ST), ST-1581, which was identified in 60/215 (28%) of the isolates (12 from human faeces, 44 from poultry meat, 4 from ruminant faeces and zero from water), is not currently assigned to any CC. The second most prevalent ST was ST-3072 (25/215; ~11.6%), and third were ST-3222 and ST-2397 (with 21 isolates each). These were not predominant STs in other countries (24, 163, 164, 170). Appendix 7.2.1 provides a detailed table of the CC assignment and the allelic profile of the 27 different *C. coli* STs.

4.4.3 Minimum Spanning Tree

The minimum spanning tree visualizing the ST clusters is shown in Figure 4.1.

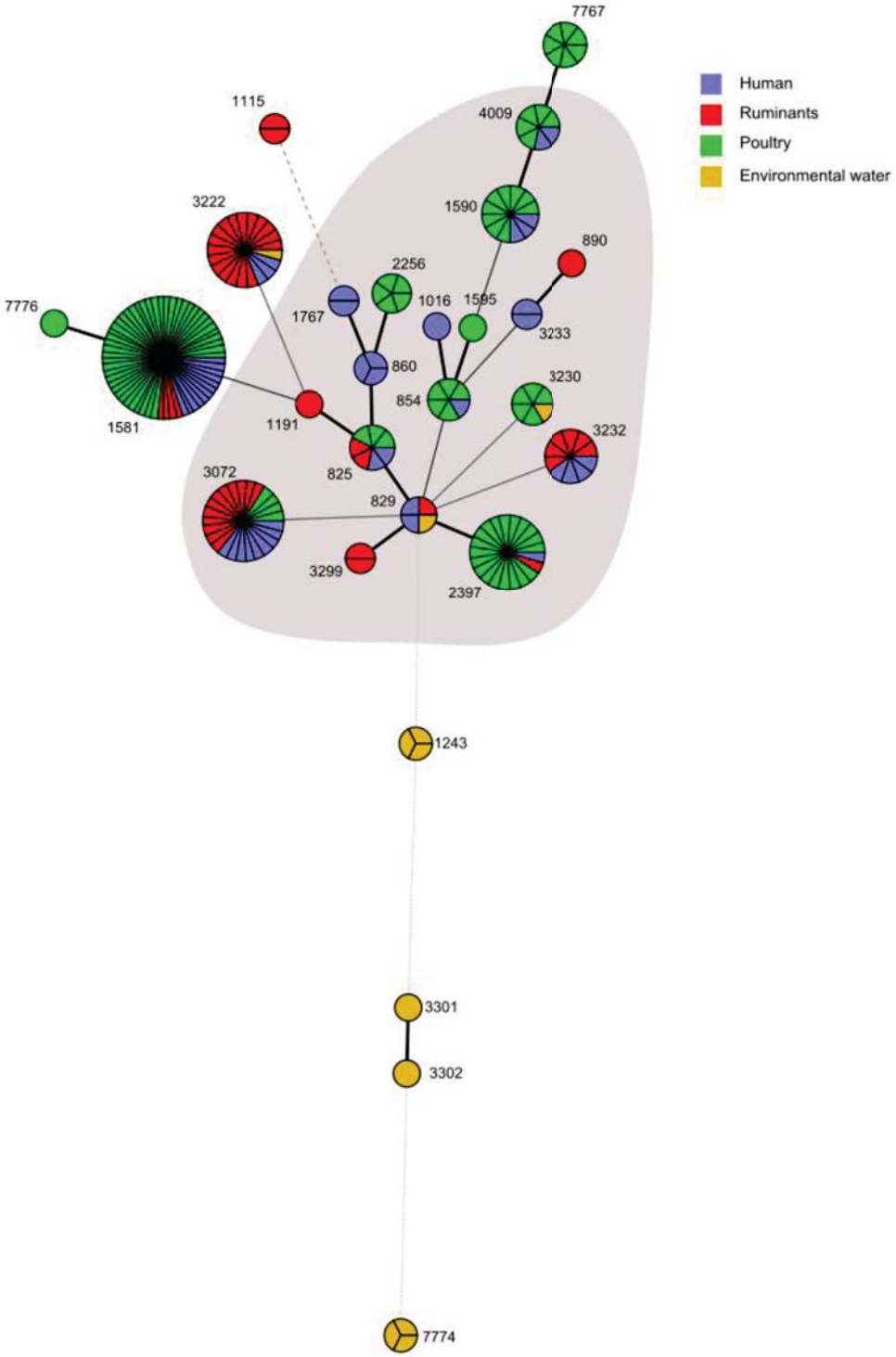

Figure 4.1: A minimum spanning tree of *C. coli* STs from 4 different sources. Each node represents a ST, its size is proportional to the frequency of isolation and the colours represent the different source type. The thickness of the connecting lines is proportional to the similarities between STs, with the thickest connector linking single locus variants. The shaded area represents members of the ST-828 CC.

Except for the environmental water isolates, the diagram (Figure 4.1) showed no distinctive partition of *C. coli* STs between the four sources, with most of the highly

abundant STs represented in at least three sources. The diagram also showed a large cluster of closely related STs. However, four environmental water *C. coli* STs (ST-7774, ST-3302, ST-3301 and ST-1243) appeared dissociated from this cluster, and two of these STs (ST-3301 and ST-3302) were single-locus variants. Moreover, some STs that caused clinical infections were only isolated from poultry sources like ST-854, ST-1590 and ST-4009; on the other hand ST-3232 that caused human campylobacteriosis cases was only isolated from ruminants sources.

4.4.4 Molecular phylogenetic analysis

Ten out of 27 STs found in this study were previously reported by Sheppard et al. in Scotland (170) and belonged to clade 1. Hence, in order to compare the phylogenetic relatedness of *C. coli* from New Zealand and Scotland we generated an ML diagram with the addition of 9 Scottish STs belonging to clades 2 and 3 but not found in our study (Figure 4.2). Interestingly, 26/27 of the New Zealand STs clustered within the Scottish clade 1, only one ST of an environmental water *C. coli* clustered in clade 2 (ST-7774), and no STs clustered in clade 3 (Figure 4.2). Appendix 7.2.2 provides a detailed table about the frequency of STs with their CC and clade designation isolated from different sources.

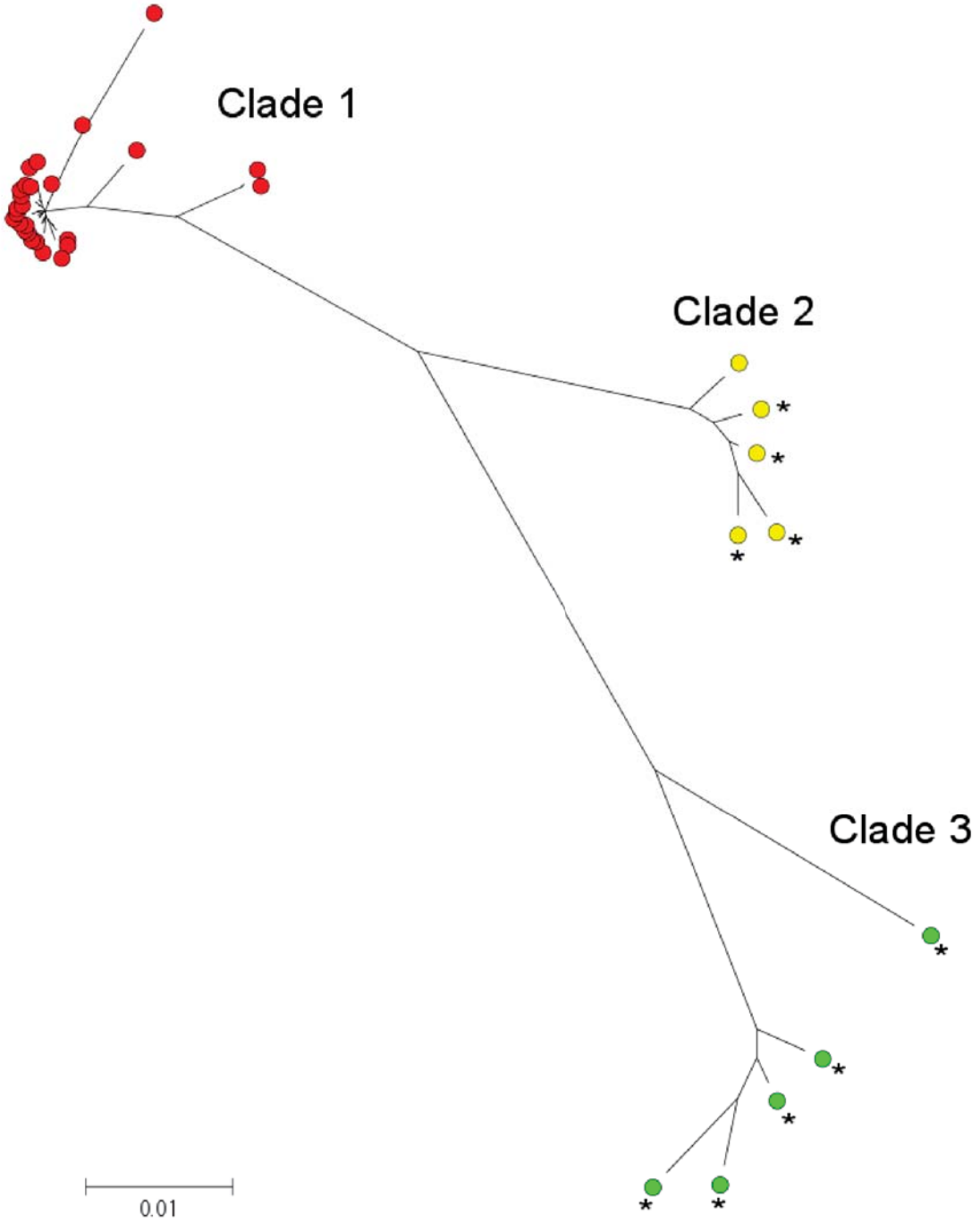

Figure 4.2: Molecular phylogenetic analysis by maximum likelihood method. The 27 STs found in this study and nine STs from clades 2 and 3 from Sheppard et al. (2010) were used. Clade 1 is indicated in red, clade 2 in yellow and clade 3 in green. Sheppard et al. (2010) *C. coli* STs are indicated with an asterix. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar represents a genetic distance of 0.01 (i.e. 1% of the nucleotides differ).

4.4.5 Permutational Multivariate Analysis of Variance

PERMANOVA was used to formally test for significant population differentiation, comparing mean pairwise Hamming distances of *C. coli* isolated from different sources.

The pseudo-F statistic was 8.92 (P=0.001), indicating that at least one of the populations differed from the other three. The degree of freedom (df), sum of squares (SS), mean squares (MS), the pseudo-F statistic and its associated P value are shown in Table 4.1. A non-metric multidimensional scaling (NMDS) plot was used to assess which populations differed (Figure 4.3).

Table 4.1: PERMANOVA outcome of the hosts (Humans, poultry, ruminants, environmental water)

Source	df	SS	MS	Pseudo-F	P value	Unique permutations
Hosts	3	221.09	73.695	8.9269	0.001	97.96
Residual	211	1741.9	8.2554			
Total	214	1963				

Together, NMDS and the minimum spanning tree results indicated that most *C. coli* isolated from water sources were differentiated from the strains circulating in the other sources, although this was not obvious from the results of the maximum likelihood phylogenetic analysis.

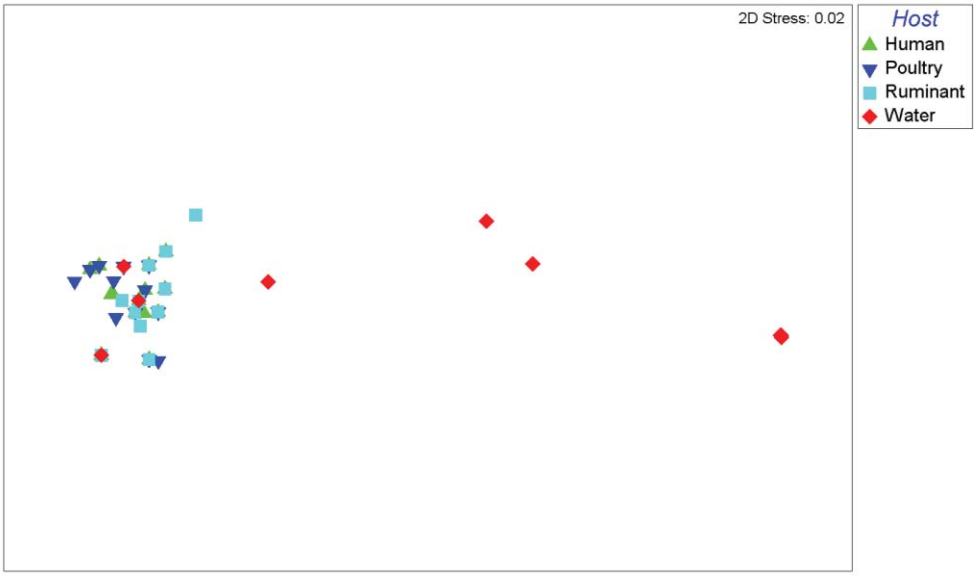

Figure 4.3: NMDS 2D plot of STs derived from different sources, humans, poultry, ruminants and environmental water.

4.4.6 Source attribution for human infections

The PSI and their 95% CrI are reported in Table 4.2. The PSI between ruminant and human *C. coli* (PSI = 0.50, 95% CrI 0.31 – 0.59) was similar to that observed between poultry and human isolates (PSI= 0.46, 95% CrI 0.30 – 0.56), and the 95% CrI overlapped. However, in relation to human *C. coli*, the ruminant and poultry PSI were significantly greater than PSI obtained with environmental water *C. coli* (PSI= 0.10, 95% CrI 0 – 0.17). The PSI changes slightly if the ruminant source was split into sheep and cattle but the differences were still not significant (Appendix 7.2.3).

The asymmetric island model results were consistent with the PSIs (Table 4.2), where ruminants and poultry were identified by both as the main sources for human infection. The model attributed 55% (95% CrI 31 – 79%) and 38% (95% CrI 16 – 58%) of the infections to ruminants and poultry sources, respectively, but the 95% CrIs of the estimated contributions of these sources overlapped widely, whereas the environmental water source accounted for only 7% (95% CrI 0 – 24%) of infections, and the CrI only overlapped slightly with that of the poultry source. As a whole, these results attributed the majority of the infections to ruminant and poultry sources, whereas exposure to environmental water sources appeared to contribute less to the burden of disease. Appendix 7.2.3 provides a numerical indices table measuring diversities, PSI and asymmetric island model in *C. coli* if the ruminant source was split into sheep and cattle.

Table 4.2: Numerical indices measuring diversities, PSI and asymmetric island model in *C. coli* (95% CrI are in brackets).

	Source			
Index	Human	Ruminant	Poultry	Environmental water
Proportional similarity index	NA	0.50 (0.31-0.59)	0.46 (0.30-0.56)	0.10 (0.00-0.17)
Asymmetric island model output	NA	0.55 (0.31-0.79)	0.38 (0.16-0.58)	0.07 (0.00-0.24)
Simpson's	0.87 (0.79-0.89)	0.79 (0.69-0.84)	0.78 (0.70-0.83)	0.81 (0.56-0.83)
Shannon's	2.32 (1.89-2.41)	1.88 (1.49-2.03)	1.93 (1.66-2.06)	1.80 (0.99-1.85)

4.4.7 Diversity index and rarefaction curve

Simpson's and Shannon's indices of diversity are reported in Table 4.2 and rarefaction curves are shown in Figure 4.4. Human *C. coli* showed the greatest number of STs (n=14) followed by poultry (n=12), ruminant (n=11) and environmental water (n=7). Some human STs were only found in poultry (ST-854, ST-1590 and ST-4009) and one (ST-3232) was only isolated from ruminants. The diversity indices of human *C. coli* STs were the greatest among all the sources (Table 4.2) with 95% CrI overlapping. Consistent with the diversity indices, rarefaction curves indicated a greater ST richness of human *C. coli* compared with the other sources except the environmental water source, the curve of which overlapped the human curve. When the ruminant, poultry and environmental water data were combined in one rarefaction curve, this curve did not differ from the human rarefaction curve (not shown).

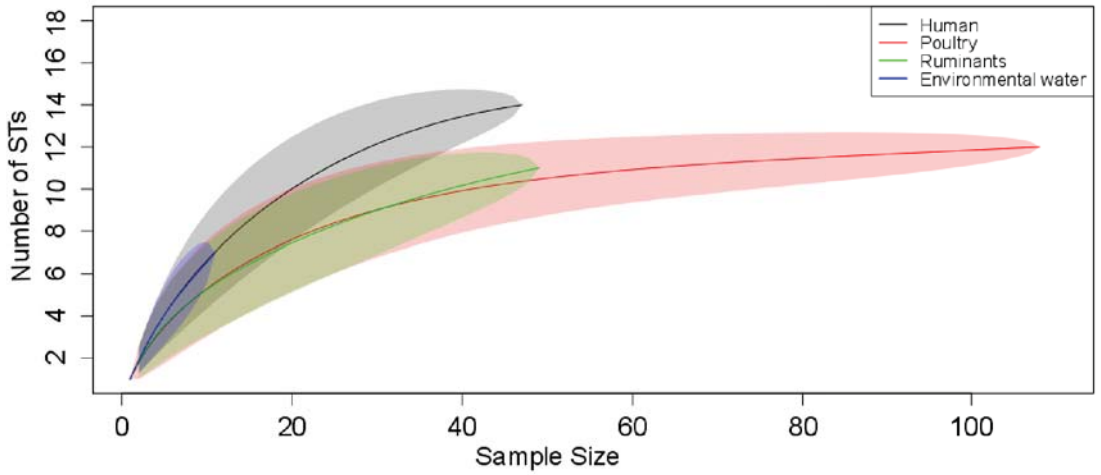

Figure 4.4: Rarefaction curves of the human, poultry, ruminants, and environmental water *C. coli* STs. The shaded areas represent the 95% CrI. Note: poultry and ruminants upper boundary of the 95% CrI does not reach the point estimate of the human curve at maximum sample size. The environmental water curve overlaps the human curve.

4.5 Discussion

In this chapter we report the first molecular source attribution study of *C. coli* campylobacteriosis in New Zealand, and one of the few such studies globally. The study analysed 215 *C. coli* isolates gathered from different sources between 2005 and 2014. Our study did not include pork meat as only one *C. coli* was isolated. Although in some studies *C. coli* has been found at a relatively high prevalence in pig faeces (173, 174), a low *C. coli* isolation rate from pork meat has also been observed in a study in the Greater Washington, D.C., in the United States of America (146). A limitation of this study was an inability to assess variability statistically between the years, due to the modest number of *C. coli* identified each year. However, aggregation of the samples into 3 year intervals did not provide any evidence of temporal changes in dominant STs (data not shown).

C. coli accounted for ~3% of the isolates, a relatively low proportion compared to the situation in other countries where *C. coli* has been identified in 10% of the cases (110, 138, 141, 175). In the last six years the average annual number of campylobacteriosis cases in New Zealand was ~7,000, or 155 cases per 100,000 population

(https://surv.esr.cri.nz/surveillance/surveillance.php). By extrapolation to the whole country without accounting for possible regional differences (76), there would be an average of ~200 *C. coli* infections per year. Estimation of the true incidence of *C. coli* infections in the population is hindered by significant underreporting. For example, in New Zealand it has been estimated that for every reported case of acute gastrointestinal illness (from all causes) there could be 222 unreported cases. However, the majority of these cases could be due to viral infections (176). One estimate in the UK suggested the number of cases of campylobacteriosis in the population could be sevenfold the number of notifications (59). If we extrapolate the UK estimate to New Zealand, there could be ~1,400 (200×7) *C. coli* infections per year, equivalent to an incidence rate of ~31 cases per 100,000 population. This rate is high compared with an estimate of 8.5 cases per 100,000 population in the USA, even though the relative contribution of *C. coli* to the campylobacteriosis burden is approximately three times lower in New Zealand compared to the USA (159). This is due to the relatively high incidence rate of campylobacteriosis (due to any species) in New Zealand.

A comparison between *C. coli* populations cycling in New Zealand and elsewhere revealed a number of key features: STs belonging to CC ST-828 were predominant in our study (54% of the isolates), and interestingly, also predominant in Scotland (94% of the isolates) (110) and Switzerland (71%) (24). Ten out of 27 STs identified in the New Zealand study have also been reported in Scotland. What determines the apparent global distribution and predominance of STs belonging to CC ST-828 is not currently well understood, and may require more samples and the application of higher resolution genotyping using whole genome sequencing to resolve. Three novel STs (ST-7767, ST-7774 and ST-7776) were identified in our study, but none of these were detected in humans. Rarefaction analysis and diversity indices were in broad agreement and

indicated that human clinical isolates had the highest ST diversity (Figure 4.4; Table 4.2). However, when the rarefaction curve for human isolates was compared to all other sources combined, the rarefaction curves were very similar, providing evidence that the increased diversity in clinical isolates could be due to multiple sources contributing to *C. coli*-associated campylobacteriosis.

Most human isolates (32/47) belonged to CC ST-828 (Appendix 7.2.2). However, the most prevalent ST in humans and poultry meat (ST-1581) did not belong to any known CC and appeared sporadically but with regularity in humans in the past ten years. Another ST that appeared with regularity in humans was ST-3072. The remaining 12 STs found in humans were either detected sporadically in a single year, or disappeared and reappeared after several years. For example, ST-3232 was detected in 2005 and disappeared and reappeared in 2010. The predominance of the ST-1581 in humans has not been reported elsewhere. ST-1581 was detected in Denmark from a clinical case, but it was not the dominant strain (164). In a study in Switzerland, none of the 616 *C. coli* isolates collected between 2002 and 2012 from poultry, pigs and humans belonged to the ST-1581. In that study the dominant human ST was ST-827, which belongs to CC ST-828 but was not detected in our study (24). Another study in England did not detect the ST-1581 in the 175 human and water *C. coli* isolates examined (177).

There were 10 STs shared between our study and one study conducted in Scotland (170). These STs belonged to clade 1 as defined by Sheppard et al. (2010) (170) and in the ML tree they clustered with 16 other STs that were not reported in Scotland (Figure 4.2). Conversely, a previously unreported ST (ST-7774) isolated from environmental water clustered with Scottish STs belonging to clade 2, and none clustered with the Scottish clade 3. The finding of human and animal *C. coli* sources harboring STs from clade 1 is consistent with the findings in Scotland. However, in Scotland all the STs

found in environmental water sources clustered in clades 2 and 3 rather than clade 1 (170), whereas in our study we did not find any clade 3 ST in environmental water. Further, the *C. coli* STs isolated from water samples collected in northwest England also belonged to clades 2 and 3 (177). It is possible that increasing the sample size from environmental water sources will result in the identification of clade 2 and clade 3 STs in New Zealand.

In spite of the fact that the ML analysis did not well differentiate the environmental water *C. coli* from isolates obtained from other sources, the PERMANOVA results indicated a significant differentiation of these isolates from *C. coli* cycling in humans, poultry and ruminants. The PSI was calculated to evaluate the similarity between ST frequency distributions and the highest PSI was observed between STs from humans and ruminant sources, but the CrI overlapped widely with the CrI of the PSI between human and poultry *C. coli*. The asymmetric island model results supported the PSI and indicated that ruminant and poultry sources were the largest contributor to human infections, again with overlapping 95% CrIs (Table 4.2). In contrast, up to 76% of *C. jejuni* infections were attributed to poultry sources in New Zealand and ruminants were the second source accounting for up to 20% of the cases (34). In Scotland, the major source of *C. coli* campylobacteriosis, based on the asymmetric island model was attributed to poultry (57%), and 41% was attributed to the ruminant source (170).

Although it is possible that a mutation could occur during culture, and this could result in a change in an allele and the ST, such events are likely to occur at very low frequencies due to the low intrinsic rate of mutation in *Campylobacter* housekeeping genes (178). In fact, only one new ST occurred as a singleton in our study (ST 7776), and the inclusion and exclusion of this isolate in the analyses did not affect the conclusions.

In summary, our results indicate ~3% of human campylobacteriosis cases in the study area could be attributed to infection with *C. coli*. This relative contribution of *C. coli* appears to be smaller than the contribution estimated in other countries. However, calculations suggest that the incidence rate of *C. coli* infection remained higher than in other countries due to the higher incidence of campylobacteriosis in general. Future studies should assess whether these results can be generalised to the whole country given that specific demographic differences between the Manawatu and other New Zealand regions may affect source attribution studies (76). The population genetic structure of *C. coli* in the study area is reminiscent of the structure described in the UK, with the predominance of CC ST-828 and the presence of many shared STs. As in Scotland, our source attribution analysis identified ruminants and poultry as the main infection sources, as well as a smaller contribution from surface water sources. Unlike the situation with *C. jejuni*, our results suggest the ruminant sources might have a greater relative contribution to *C. coli* infection burden than poultry. These results highlight the need to consider each *Campylobacter* species separately when designing public health interventions.

4.6 Acknowledgements

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Chapter 5

Changes in the molecular epidemiology of *Campylobacter jejuni* following food safety interventions by the New Zealand poultry industry

5.1 Abstract

Campylobacteriosis is one of the most important food-borne diseases in New Zealand and worldwide. *C. jejuni* is the species predominantly associated with campylobacteriosis, accounting for approximately 90% of human cases, followed by *C. coli*. In 2006, New Zealand had the highest annual campylobacteriosis notification rate among the Organisation for Economic Co-operation and Development countries, which led to the implementation of food safety interventions in 2008 by the poultry industry resulting in an ~60% decrease in the campylobacteriosis notification rate over a period of 18 months. The aim of this study was to genotype *C. jejuni* isolates collected between 2005 and 2015 in the Manawatu region of New Zealand from human clinical cases, fresh chicken meat, ruminant faeces, environmental water and wild bird sources, to examine the changes in the molecular epidemiology of *C. jejuni* following these interventions. *Campylobacter* isolates were identified by polymerase chain reaction and typed by multilocus sequence typing. *C. jejuni* accounted for 96% (n=1780/1855) of *Campylobacter* spp. isolated from human clinical cases over both periods. Before the intervention, ST-474 was the dominant ST, accounting for 17.2% of the 112 STs; however, after the intervention ST-474 reduced to 6.6% of the 191 STs detected. In both periods, ST-45, ST-50 and ST-48 were isolated in similar proportions and accounted for ~12%, ~8% and ~7% respectively. Before the interventions, modeling indicated that chicken meat, specifically from Supplier A, was the main source of *C. jejuni* human infection; however, after the intervention period ruminants became the main source of *C. jejuni* human infection followed by chicken meat from Supplier A. These results

provide a unique insight into the changes in the source attribution and molecular epidemiology of human cases, following an intervention in one sector of the food industry.

5.2 Introduction

Campylobacteriosis has a significant burden on the economies and health care systems around the world due to its high incidence. Previous estimates identified the cost to the New Zealand economy of approximately $NZ 77 million annually (32), and a loss of more than five million working days each year (33). Among the twenty eight different *Campylobacter* species and 11 sub-species (2), *Campylobacter jejuni* is the predominant species isolated from cases of human campylobacteriosis worldwide (5, 7). The results of the study presented in Chapter 4 indicate this species accounts for more than 95% of cases in the Manawatu region of New Zealand (179). Hence, most research interests focused on *C. jejuni,* not only due to its high prevalence in acute cases of bacterial diarrhoea, but also for the severe sequelae associated with *C. jejuni* infections, such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (39-41).

The most studied potential sources of human *Campylobacter* infection are the contaminated chicken and red meat, environmental water, milk, and contact with pets and farm animals (180, 181). Source attribution studies are a way of determining to what extent certain sources (e.g. chickens, ruminants, etc.), and infectious pathways (e.g. food-borne, person-to-person, etc.), contribute to a campylobacteriosis. In order to assess the contribution of different infection sources to the burden of human disease caused by *C. jejuni*, molecular subtyping by methods such as multilocus sequence typing (MLST) have been applied (162). Several studies (24, 109-111) showed that the consumption of chicken meat was the main risk factor for *C. jejuni* infections, and this result is supported by the high level of *Campylobacter* contamination usually found in

chicken samples (116, 117). On the other hand, other potential sources cannot be underestimated. For instance, findings in Finland implied that bovine and poultry sources are equally important reservoirs for human *C. jejuni* infections (112).

In 2006, New Zealand had the highest campylobacteriosis notification rate in the world, with >380 cases per 100,000 population (156). Mullner et al., (2009) showed evidence that poultry was the leading cause of human campylobacteriosis in New Zealand, causing an estimated 58–76% of cases, followed by ruminants (~20% to ~30%) (34). This high rate triggered the New Zealand Food Safety authority to announce the implementation of *Campylobacter* Risk Management Strategy in collaboration with the poultry industry starting in April 2008 (155). The implementation eventually led to a 60% decrease in the campylobacteriosis notification rate (73). Previous work in the Manawatu region, the sentinel site for campylobacteriosis in New Zealand (76), had shown that the decline in human cases post-intervention was predominately associated with a decline in poultry-associated cases, and a relative increase in the contribution from other sources (182). However only two and a half years of data post-intervention were available from that study (1st January 2008 to July 2010).

The main objective of this study was to compare the source attribution of campylobacteriosis cases caused by *C. jejuni* isolates at two different time periods: the period before the food safety interventions by the poultry industry (2005-2007) and, an extended period after the interventions (2008-2015). Using data from this 11 year time period, the genetic relatedness of the clinical *C. jejuni* isolates was compared with isolates obtained from chicken meat, farmed ruminants, environmental water and wild bird sources in the Manawatu region of New Zealand. In addition, data on the enumeration of *Campylobacter* on chicken carcasses over this time period in the region were compared. This study provides important information for policy makers to enable

appropriate targeting of interventions to further reduce the burden of campylobacteriosis, which is still the most commonly notified gastrointestinal infection, accounting for ~45% of all notified enteric infections (73).

5.3 Materials and methods

5.3.1 Collection of human clinical *Campylobacter* isolates and identification of *C. jejuni* from humans and other sources

The study used *Campylobacter* spp. isolated from human faeces in the Manawatu region (76) of New Zealand between February 2005 and December 2015. The data were subdivided into before and after intervention with 31 December 2007 as a cut-off date. Even though the official implementation date started in April 2008, the poultry companies had already started to trial interventions a few months before that date which resulted in lower carcass contamination (182). The region had a population of ~223,000, and its main city, Palmerston North, had a population of ~80,000 (74, 75). All the human faecal specimens submitted to the main medical laboratory (MedLab Central, Palmerston North) during the sampling period which tested positive for *Campylobacter* spp. by ELISA (ProSpecT ®, Remel, USA), were delivered for bacterial culture to the Molecular Epidemiology and Public Health laboratory (^{m}EpiLab) of Massey University (MU). The faeces were delivered weekly on Amies charcoal transport swabs (Copan, Brescia, Italy). Swabs were first streaked on modified Cefoperazone Charcoal Deoxycholate agar (mCCDA) plates (Fort Richard, Auckland, New Zealand) and then immersed in 3 ml Bolton Broth (BB) (Lab M, Bury, UK) in a loose-capped bijou bottle and incubated at 42°C microaerobically (85% N_2, 10% CO_2, and 5% O_2) for 48 hours (MACS-VA500-microaerobic workstation, Don Whitley Scientific, West Yorkshire, UK). A single colony resembling *Campylobacter* spp. on mCCDA was subcultured onto Columbia horse blood agar (BA) (Fort Richard, Auckland, New Zealand), and

incubated microaerobically at 42°C for 48 hours. If the mCCDA plate was negative for colonies resembling *Campylobacter* spp., a subculture was made from BB onto another mCCDA plate and the plate was incubated in a microaerobic atmosphere as above. Pure cultures were frozen in 15% glycerol broth (Oxoid, UK) at -80°C for future reference.

Campylobacter identification to species level was done by molecular methods. Briefly, DNA was extracted by boiling a freshly grown culture of frozen isolates for 10 minutes in 1 ml 2% Chelex™ (Biorad, USA) in sterile Milli-Q water, followed by centrifugation (12,470×*g*, 3 mins) to remove cell debris and Chelex™. The supernatant was transferred to a fresh tube and used for polymerase chain reaction (PCR) for species confirmation and multilocus sequence typing. Between 2005 and 2014, isolates were firstly identified through the use of the *map*A gene for the detection of *C. jejuni* (94), employing the following primers: *map*A-F (5'-CTTGGCTTGAAATTTGCTTG-3') and *map*A-R (5'-GCTTGGTGCGGATTGTAAA-3'). Amplification was performed in a 20 µl reaction volume containing 0.2 µl of *Taq* polymerase (Platinum Taq, Invitrogen, USA), 2 µl of 10× PCR reaction buffer (Invitrogen, USA), 1 µl dNTP (Bioline, UK) (2mM), 0.6 µl $MgCl_2$, 2 µl of each forward and reverse primer (2pmol/µl), 6.2 µl H_2O and 2 µl of DNA. The reactions were carried out in a thermocycler (SensoQuest, Germany) with denaturation at 95°C for 2 mins, followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec, 72°C for 30 sec, and a final extension of 72°C for 2 mins. Electrophoresis in 1% agarose gel and staining with ethidium bromide was used to visualise PCR products. Presence of a ~589 bp product indicated *C. jejuni* (167). Between August 2014 and December 2015, *Campylobacter* were identified by multiplex PCR to detect the *ceu*E gene associated with *C. coli* and the *hip*O gene associated with *C. jejuni* (97).

5.3.2 Collection of chicken meat samples

Over the same sampling period (2005-2015), fresh chicken carcasses from different commercial chicken suppliers (Supplier A, Supplier B and five small suppliers that are collectively designated 'Others') were sampled each month from supermarkets in Palmerston North. The bags containing the whole carcasses were first checked if they were leaking any fluid material by squeezing the bag to detect the air tightness of it. Carcasses were washed and massaged in 200 ml of sterile Buffered Peptone Water (BPW) (Difco, USA). The liquid from the wash was centrifuged (16,000×*g*, 4°C, 30 mins) and the pellet resuspended in 5 ml of BPW. This suspension was added to 90 ml of BB and incubated microaerobically as above, after which it was subcultured onto mCCDA and incubated for two days. Two colonies resembling *Campylobacter* spp. on mCCDA were subcultured to separate BA and incubated microaerobically at 42°C for two days and pure cultures were frozen at -80°C. Identification of *C. jejuni* proceeded as per the human clinical isolates. Isolates for other poultry samples (turkey and ducks) were not included in this study for comparison purposes because they were not collected in both period intervals.

5.3.3 *Campylobacter* colony counts on chicken samples

Enumeration of *Campylobacter* colonies from chicken samples was done using a spiral plater (Wasp, Don Whitley, England) and by means of manual spread plating. During the chicken washing described earlier, duplicate mCCDA plates were inoculated with 1,000 µl (manual spread plate) and 50 µl (spiral plater) aliquots of chicken wash and 100 µl (spiral plater) aliquots of resuspended chicken wash pellet. The mCCDA plates were incubated microaerobically at 42 °C for 48 hours and then the colonies resembling *Campylobacter* were counted using a plate reader (aCOLyte, Synbiosis, England), or

manually. All counts were calculated to reflect the number of bacteria in 1 ml of the 200 ml chicken wash.

5.3.4 Analysis of *Campylobacter* colony counts

The research question of interest was, "Is there statistically significant differences in the *Campylobacter* viable counts between the two periods (before and after intervention)?" The zero-inflated Poisson model was used for the analysis and the *Campylobacter* counts were converted to a logarithmic scale to approximate the results to a normal distribution. In this model, the 'Supplier' factor of the different chicken meat products was not accounted for. The R Studio version 3.1.3 software ("pscl" package) was used to apply the following equation:

$$Yi \sim \begin{cases} Poisson\ (Vi\ \lambda i), Zi = 0 \\ 0, Zi = 1 \end{cases}$$

Where Yi is the number of colonies counted from replicate i

$Zi \sim Bernoulli\ (Pi)$

$Pi =$ Probability of a zero

$Vi =$ Volume of rinse that is plated

$\lambda i =$ average colony count per milliliter (ml)

$log\lambda i = Intervention\ i : Intervention\ i$

$logit\ (Pi) = Intervention\ i : Intervention\ i$

5.3.5 Collection of samples from ruminants, wild birds and water

One hundred and five *C. jejuni* isolates were sequence typed from environmental water (recreational waterways and pre-treatment drinking water from the Manawatu-Tararua area) were collected between 2006 and 2014. Water samples were filtered through a sterile 0.45 μm filter (Millipore, USA) and the filter was transferred to 20 ml BB and

incubated at 42°C microaerobically for 48 hours, then it was subcultured onto mCCDA and incubated for 48 hours. A single colony resembling *Campylobacter* spp. on mCCDA was subcultured onto BA and incubated microaerobically at 42°C for 48 hours. Two hundred and thirteen *C. jejuni* isolates were sequence typed from wild birds in the Manawatu-Tararua area (between 2005 and 2013) and 465 *C. jejuni* isolates from ruminants faeces (cattle and sheep faeces from 30 different farms in the Manawatu-Tararua area between 2005 and 2015) were collected as part of the same long term monitoring program. Amies charcoal swabs were used for collection of the livestock faeces. The tip of the swab was cut off and placed in 15 ml of BB and incubated microaerobically at 42°C for 48 hours with loosened cap. Subculture and incubation procedure are the same as above.

Isolates from pig meat were not included due to the low prevalence of *Campylobacter* from this source (more than 650 pig meat samples were previously collected, from which only five *C. jejuni* were identified). Isolates from cat and dog samples were not included in this study because they were not collected in both period intervals.

5.3.6 Multilocus sequence typing

All *C. jejuni* isolates were subjected to MLST using seven house-keeping genes based on the method described by Dingle et al. (104). Each amplification reaction was performed in a 20 µl reaction volume containing 2 µl of the DNA preparation, 5 pmoles of both forward and reverse amplification primers and 14 µl of the PCR Mastermix (200 µl 10× PCR reaction buffer, 100 µl dNTP, 60 µl $MgCl_2$, 20 µl *Taq* polymerase and 1020 µl H_2O). Amplification was performed using 2 µl of the PCR product, 3.2 pmoles forward primer, 1 µl ABI BigDye (Applied Biosystems, USA), 2 µl of x5 buffer and H_2O to a total volume of 10 µl. Products were sequenced at ^{m}EpiLab and were read at

the Institute of Environmental Science and Research (ESR, Porirua, New Zealand) on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1.

Sequence data were collated and submitted for allele and sequence type (ST) designation with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/*campylobacter*/). Isolates yielding novel STs or alleles that did not give clear sequences were re-amplified and sequenced using the same protocol. If the sequence products were not readable, they were re-sequenced with the reverse primer and, if this failed, the locus was re-amplified and re-sequenced. New MLSTs were submitted to the online database.

5.3.7 Analysis of data

Genetic diversity

The genetic diversities of *C. jejuni* from the different sources were compared using the Simpson's and Shannon's diversity indices and their 95% bootstrap credible intervals (CrI) (calculated using PAST software version 2.17c) (151) and rarefaction analysis (performed using the package 'vegan' in R, version 3.1.3) (152).

Minimum Spanning Tree

Minimum spanning trees were implemented to visualise allelic differences between STs of isolates from the different sources, using the pairwise Hamming distance matrix. Two trees were generated comparing the period before (2005-2007) and after (2008-2015) the interventions on the poultry industry. The trees were calculated by Prim's algorithm (121) as implemented in the Bionumerics software (Applied Maths; http://applied-maths.com/bionumerics).

Modeling of *C. jejuni* sequence type abundances for source attribution

The relative contribution of different *C. jejuni* sources to human disease was estimated using a variety of tests. Tests were used to compare two periods: before, and after the food safety interventions in the poultry industry. The similarity between the frequency distribution of human *C. jejuni* STs and those of the different sources was estimated using proportional similarity indices (PSI) and their bootstrap credible intervals (CrI), as previously described (120). The PSI measures the area of intersection between two frequency distributions (119) and ranges between 0 and 1, where 0 indicates no similarity and 1 indicates identical frequency distributions. The PSI is calculated by the following equation:

$$PSI = 1 - 0.5 \sum i \mid pi - qi \mid = \sum i \min (pi,qi)$$

where pi and qi are the proportion of strains that belong to type i out of all strains typed from sources P and Q (153). Calculations were performed using R, version 3.1.3 (152).

The asymmetric island model was used to probabilistically assign each human isolate to one of the sources (chickens, ruminants, environmental water or wild birds) (111).

The Bayesian hierarchical model, based on the modified Hald model (123), was used to implement a non-parametric source attribution model to attribute human campylobacteriosis cases to sources in a Bayesian framework with source and sequence type effects ('sourceR' package https://CRAN.R-project.org/package=sourceR).

5.4 Results

5.4.1 Proportion of *C. jejuni* isolates among the different sources

A total of 7,951 samples were collected, from humans, chickens, ruminants, environmental water and wild bird sources between 2005 and 2015. A comparison

between the samples analysed in 2005-2007 and in 2008-2015 is shown in Table 5.1 and Table 5.2 respectively. The predominant species, in both periods and among all sources, was *C. jejuni,* accounting for up to 97% of the human clinical isolates (Table 5.1), followed by *C. coli,* that accounted for a maximum of 12% in chicken samples (Table 5.2).

Table 5.1: Overview of samples collected from different sources 'before the intervention' (2005-2007).

Source	Samples collected	Samples cultured positive [a]	Number of *C. jejuni*	Number of *C. coli*	Isolates not processed [b]	Number of *C. jejuni* isolates typed
Humans	774	670	652	17	1	652
Chickens	480	376	331	37	8	330
Ruminants	1058	347	236	26	85	221
Environmental Water	308	140	77	6	57	76
Wild birds	77	24	21	0	3	21
Total	2697	1548	1317	86	154	1300

[a] Number of *Campylobacter* culture-positive samples.
[b] *Campylobacter* culture-positive isolates were not further identified if the isolates were PCR negative for *C. jejuni* and *C. coli*.

Table 5.2: Overview of samples collected from different sources 'after the intervention' (2008-2015).

Source	Samples collected	Samples cultured positive [a]	Number of *C. jejuni*	Number of *C. coli*	Isolates not processed [b]	Number of *C. jejuni* isolates typed
Humans	1525	1185	1128	38	19	1040
Chickens	963	750	597	90	63	507
Ruminants	1135	503	319	41	143	244
Environmental Water	270	77	40	6	31	29
Wild birds	1361	682	354	1	327	192
Total	5254	3197	2438	176	583	2012

[a] Number of *Campylobacter* culture-positive samples.
[b] *Campylobacter* culture-positive isolates were not further identified if the isolates were PCR negative for *C. jejuni* and *C. coli*.

5.4.2 Analysis of bacterial counts

The proportion of *Campylobacter* culture-positive samples among the retail chicken was 0.785. Before the intervention, Supplier 'Others' had the highest percentage of *Campylobacter*-positive samples (91%), followed by Supplier A (84%) and Supplier B (68%). After the intervention, the percentage of *Campylobacter* contamination increased in Supplier A to 91.1% and in Supplier B to 77.3%, but decreased to 62.4% in Supplier 'Others'. The change in the proportions among Supplier A and Supplier B was not significant (based on the Fisher exact test), but there was significant change in the proportion among Supplier 'Others' where the Fisher exact test statistical value was equal to 1×10^{-16} ($p < 0.05$). Table 5.3 shows a comparison of geometric means between the numbers of *Campylobacter* colonies in the positive chicken carcasses among different suppliers. Boxplot comparisons are shown in appendices 7.3.1 and 7.3.2. The zero-inflated Poisson model findings showed that whole carcasses had higher counts in

the period before the intervention. Moreover, the model also showed that the probability of a whole carcass having a zero *Campylobacter* count is higher in the period after intervention. The results of the zero-inflated Poisson model are statistically significant ($p < 0.05$) (Appendix 7.3.3).

Table 5.3: Count level of *Campylobacter* spp. on chicken carcasses at two different time periods and among different suppliers.

	Campylobacter level (log CFU*/sample) on positive samples		
	Mean	Standard deviation	Median
Time period			
Before intervention	8.66	2.37	8.69
After intervention	7.30	2.16	6.90
Suppliers before intervention			
Supplier A	9.03	2.26	8.98
Supplier B	8.43	2.57	8.63
Supplier 'Others'	8.02	2.16	8.29
Suppliers after intervention			
Supplier A	7.63	2.41	7.09
Supplier B	7.04	1.90	6.85
Supplier 'Others'	7.20	2.05	7.09

*CFU= colony forming unit

The proportion of chicken carcasses with leaking bags among different suppliers was compared; before versus after intervention. The proportion of leaking bags dropped after the intervention from 30% to 13%, and 80% to 41% within Supplier B and Supplier 'Others', respectively. However, the proportion of leaking bags increased from 2% to 18% within Supplier A. The change in the proportion of leaking bags among all suppliers is significant based on the statistical values of the Fisher exact test ($p < 0.05$).

5.4.3 Analysis of sequence types

A total of 3,312 *C. jejuni* isolates from humans, chickens, ruminants, environmental water and wild bird sources were multilocus sequence typed between 2005 and 2015. Two hundred and thirty five different STs were detected over the same period. Before the intervention, ST-474 was the dominant ST, accounting for 17.2% of the 112 STs, followed by ST-45, ST-50 and ST-48, which accounted for 11.9%, 8% and 6.5% of the STs, respectively. After the intervention, the proportions of the major STs (ST-45, ST-50 and ST-48) did not change, except for ST-474, which dropped from 17.2% to 6.6%. The prevalence of ST-583, that belongs to CC ST-45, increased from 1.8% to 4.5% after the intervention period. Among the human clinical isolates, ST-474 declined from 28.2% (184/652) to 9.3% (97/1040), and among the chicken meat isolates from 9.7% (32/330) to 5.3% (27/507). There was no strong variation in most of the CC between the two periods, except for CC ST-48, which showed a 50% decrease, from 27% to 14.9%, and CC ST-45, that increased from 15% to 18.3%. The decrease in the CC ST-48 and the increase in the CC ST-45 were significant according to the Fisher exact test where the values were less than 0.05. Appendices 7.3.4 and 7.3.5 show the frequency of all the *C. jejuni* STs isolated in the two periods (2005-2007 and 2008-2015) from different sources with their CC designation.

5.4.4 Diversity index and rarefaction curves

The rarefaction curves of the two periods are shown in Figure 5.1 and Figure 5.2 and the comparisons of the Simpson's and Shannon's indices of diversity between two different periods are reported in Table 5.4.

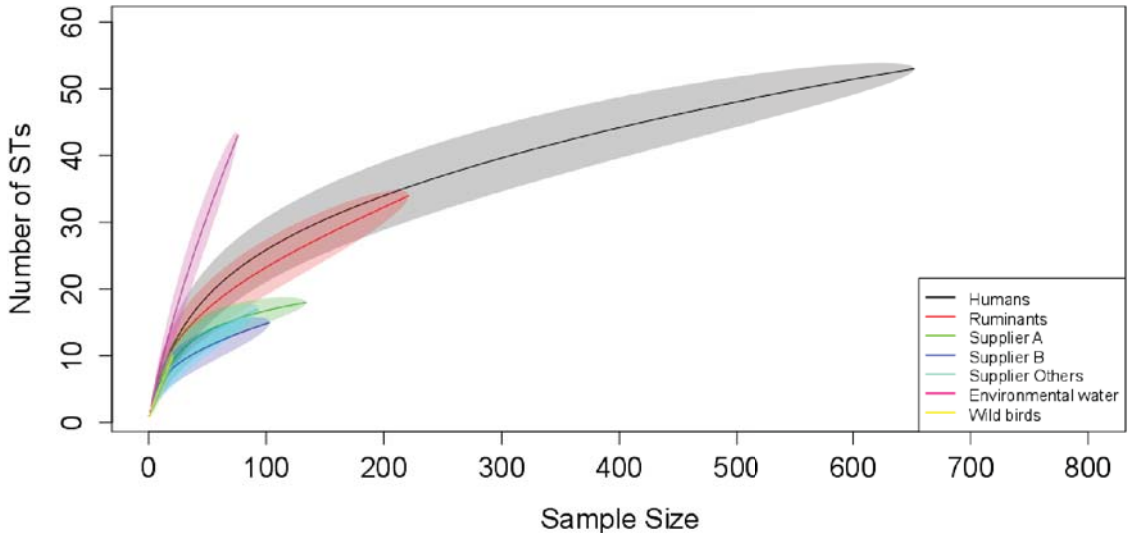


Figure 5.1: Rarefaction curves, between 2005 and 2007, of the human, chicken suppliers (A, B and 'Others'), ruminants, environmental water and wild birds *C. jejuni* STs. The shaded areas represent the 95% CrI. Note: Only ruminants' upper boundary of the 95% CrI reach the point estimate of the human curve at maximum sample size. The wild birds curve overlaps the human curve and the environmental water curve is steep.

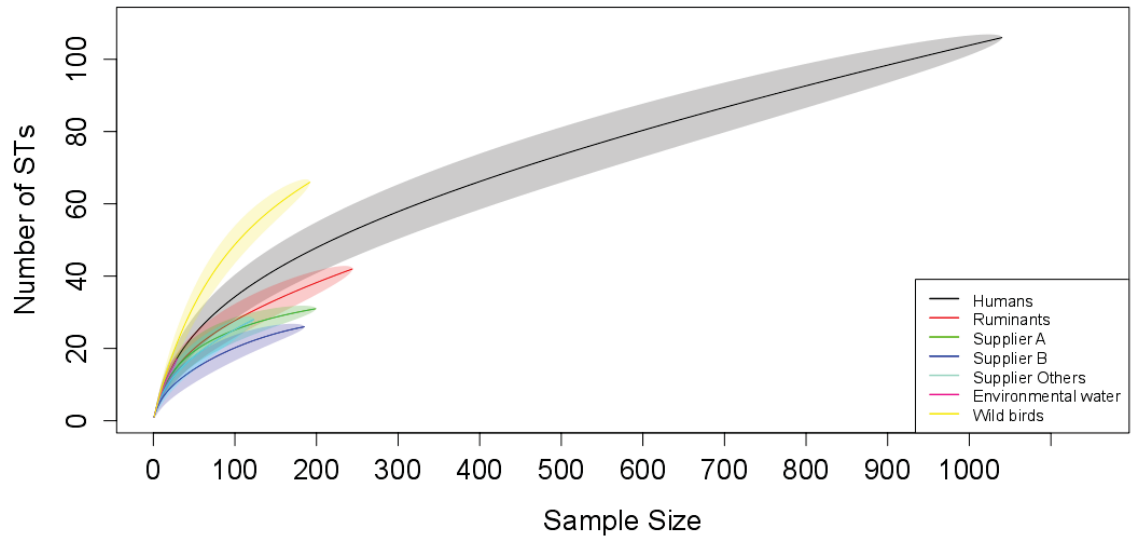


Figure 5.2: Rarefaction curves, between 2008 and 2015, of the human, chicken suppliers (A, B and 'Others'), ruminants, environmental water and wild birds *C. jejuni* STs. The shaded areas represent the 95% CrI. Note: None of the sources upper boundaries of the 95% CrI reach the point estimate of the human curve at maximum sample size.

In both periods, human *C. jejuni* showed the greatest number of STs. There was a steep rarefaction curve for the environmental water (2005-2007) and wild bird *C. jejuni* STs (2008-2015) indicating that in general the diversity in these two sources were not captured. There was a significant increase in the diversity indices of the human, Supplier A and wild birds *C. jejuni* STs from before to after the intervention.

Table 5.4: Simpson and Shannon diversity indices of different sources comparing two periods (2005-2007 and 2008-2015) with 95% CrI in brackets.

	Simpson's		Shannon's	
Source	2005-2007	2008-2015	2005-2007	2008-2015
Human	0.89 (0.87-0.90)	0.94 (0.94-0.95)	2.88 (2.7-2.97)	3.48 (3.41-3.56)
Supplier A	0.85 (0.81-0.88)	0.92 (0.91-0.93)	2.32 (2.1-2.45)	2.93 (2.82-3.03)
Supplier B	0.85 (0.82-0.87)	0.83 (0.79-0.86)	2.15 (2.0-2.30)	2.32 (2.16-2.49)
Supplier 'Others'	0.83 (0.78-0.86)	0.88 (0.85-0.91)	2.15 (1.9-2.33)	2.66 (2.52-2.84)
Ruminants	0.91 (0.89-0.92)	0.92 (0.90-0.93)	2.78 (2.6-2.88)	2.99 (2.80-3.08)
Environmental water	0.93 (0.90-0.95)	0.91 (0.85-0.92)	3.31 (3.0-3.45)	2.63 (2.20-2.69)
Wild birds	0.69 (0.46-0.83)	0.96 (0.94-0.97)	1.72 (0.97-2.02)	3.8 (3.58-3.84)

5.4.5 *C. jejuni* population structure and differentiation between sources

Two minimum spanning trees visualising ST clusters before and after food safety interventions by the poultry industry are shown in Figure 5.3 and Figure 5.4, respectively. Most of the highly abundant STs in both periods, are represented in at least three sources (humans, chickens and ruminants), with the exception of ST-48 which was represented in human clinical and chicken isolates only. STs from environmental water isolates (Figure 5.3) and wild bird isolates (Figure 5.4) generally appeared to be dissociated from the STs from other sources, with the exception of ST-61, ST-474, and ST-42, which are shared with other sources at a low prevalence. In total, ~10% of the isolates of ST-45 originated from wild birds, and this proportion remained similar in both periods.

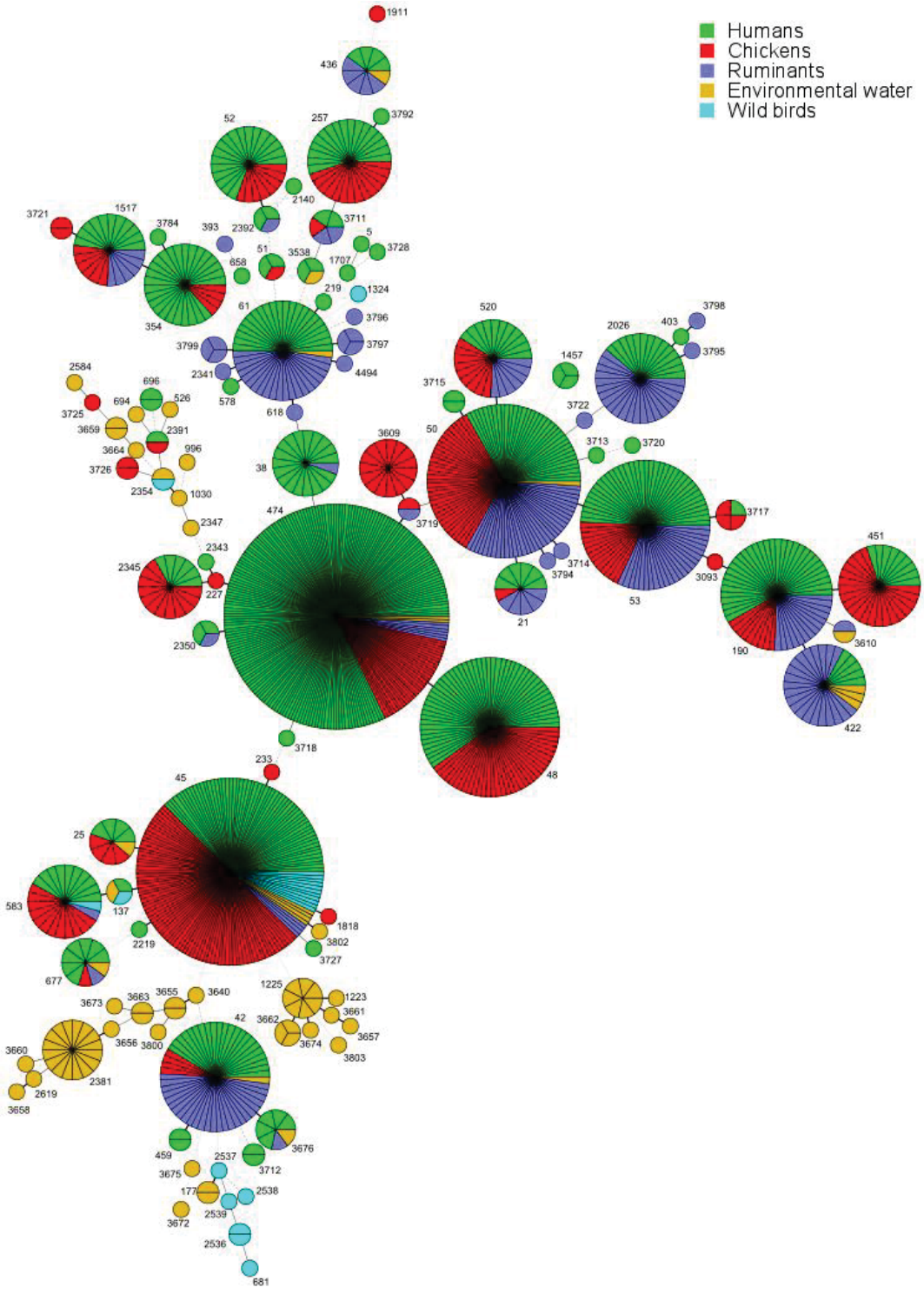


Figure 5.3: Minimum spanning tree of *C. jejuni* STs from five different sources between 2005 and 2007 (before the intervention). Each node represents a ST, and its diameter is proportional to the number of isolates. The different colours represent different sources. The thickness of the connecting lines is proportional to the similarities between STs, with the thickest connector linking single locus variants.

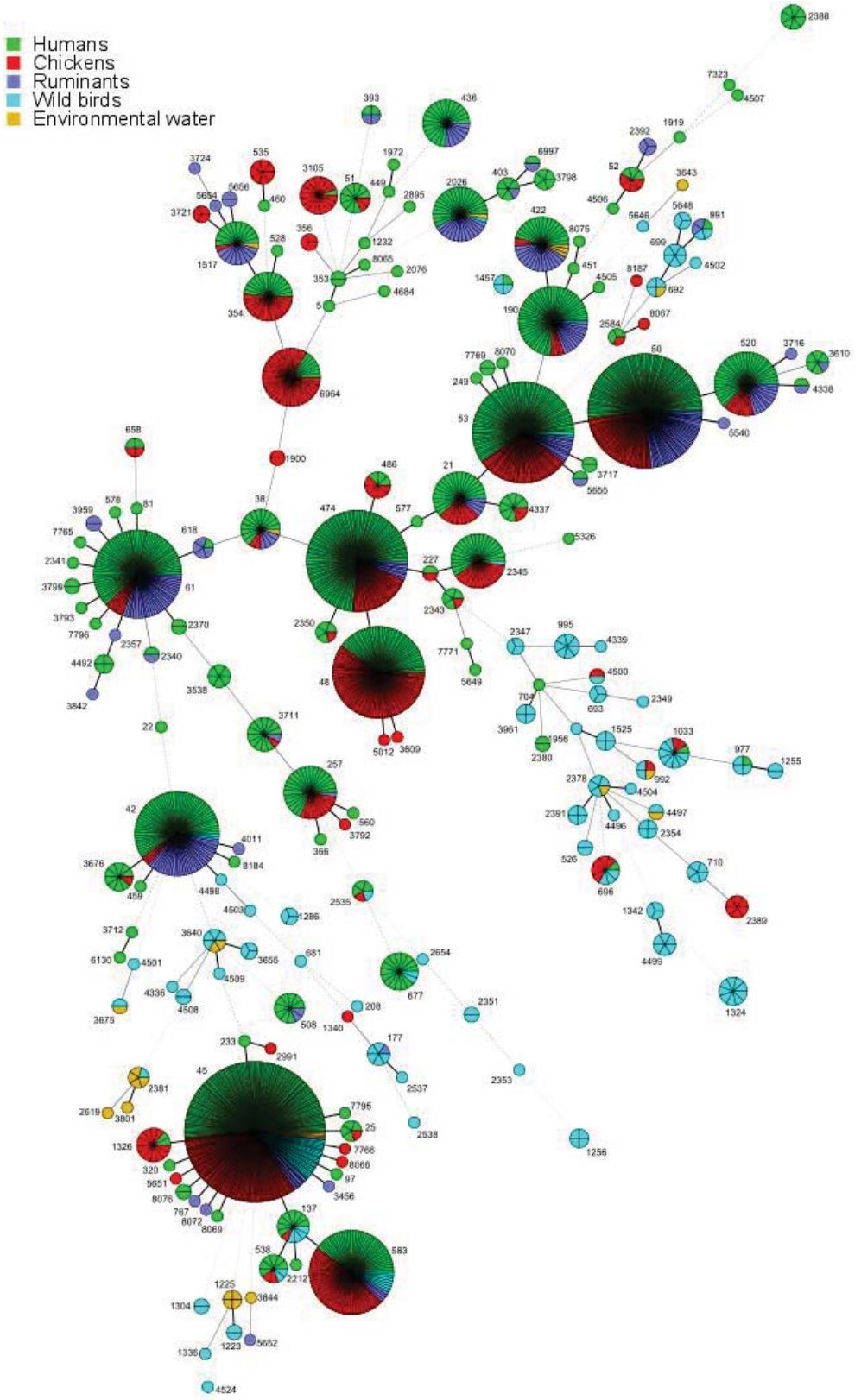


Figure 5.4: Minimum spanning tree of *C. jejuni* STs from 5 different sources between 2008 and 2015 (after intervention). Each node represents a ST, and its diameter is proportional to the number of isolates. The different colours represent different sources. The thickness of the connecting lines is proportional to the similarities between STs, with the thickest connector linking single locus variants.

5.4.6 Source attribution for human infections

The PSI comparing the two time periods and their 95% CrI are reported in Table 5.5. Before the intervention period, the PSI between Supplier A and human *C. jejuni* (PSI = 0.61, 95% CrI 0.51 – 0.66) was significantly higher than that between humans and the other sources. Supplier B, Supplier 'Others' and ruminants sources shared almost similar PSI results with overlapping 95% CrI, and the PSIs were significantly greater than the PSI between environmental water and wild bird sources and human clinical isolates. However, after the intervention period, all chicken suppliers and ruminant sources had very similar PSI with human clinical isolates, but these PSIs remained significantly greater than the PSI between environmental water and wild bird sources and the human clinical isolates. There was a significant increase, from 'before' to 'after' the intervention period, in the PSI of Supplier B, Supplier 'Others' and ruminant sources, and the human clinical isolates, with a non-significant decrease in the PSI of Supplier A, from 0.61 to 0.49 with overlapping 95% CrI.

Table 5.5: Sequence type proportional similarity index of the different *C. jejuni* sources with human clinical isolates in two different time periods (before and after intervention) (95% CrI are in brackets).

	PSI	
Source	Before intervention	After intervention
Supplier A	0.61 (0.51-0.66)	0.49 (0.42-0.52)
Supplier B	0.34 (0.27-0.38)	0.48 (0.40-0.51)
Supplier 'Others'	0.35 (0.27-0.39)	0.49 (0.39-0.52)
Ruminants	0.37 (0.32-0.41)	0.52 (0.46-0.56)
Environmental water	0.16 (0.07-0.21)	0.18 (0.06-0.19)
Wild birds	0.10 (0.07-0.12)	0.22 (0.11-0.18)

The asymmetric island model and the Bayesian hierarchical model results showed similar attribution estimates (Figure 5.5). The models attributed 59% (95% CrI 52.5-

67%) and 57% (95% CrI 47.7-67.7%) of infections to Supplier A, respectively, which was significantly identified as the main source for human infection before the intervention. In this period, ruminant sources were the second most common source of infection in the asymmetric island model and the Bayesian hierarchical model, with values of 24.1% (95% CrI 18.5-29.6%) and 20.2% (95% CrI 13.3-28.4%), respectively. 'After' the intervention period, ruminant sources became the main source for human infection attributed by both models. The asymmetric island model and the Bayesian hierarchical model attributed 45.8% (95% CrI 40.0-51.7%) and 40.0% (95%CrI 32.0-46.6%) of infections to ruminant sources, respectively. However, Supplier 'Others' had wide CrI, which overlapped with the ruminant sources in the Bayesian hierarchical model. Both models showed a significant decrease in the attribution of infections to Supplier A in the second period.

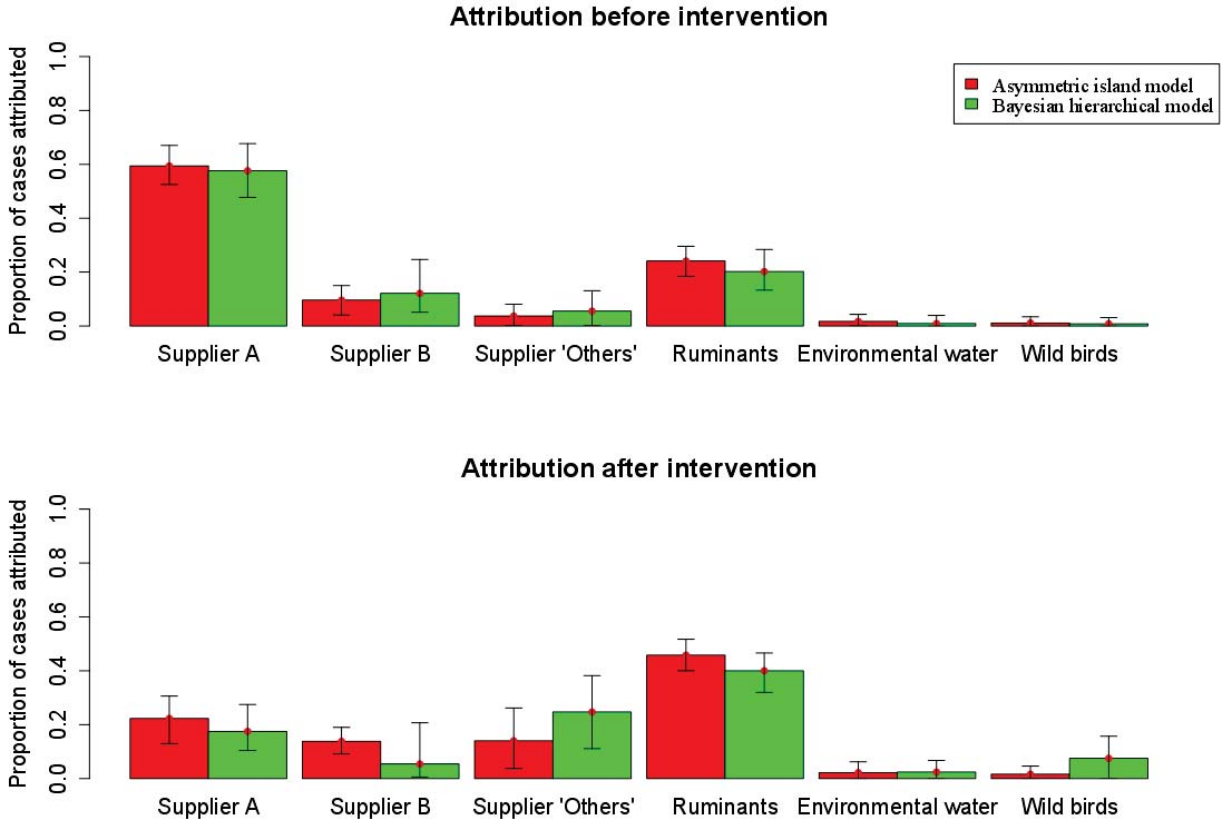


Figure 5.5: Proportion of human campylobacteriosis cases, caused by *C. jejuni*, attributable to each source based on the asymmetric island model and the Bayesian hierarchical model. Error bars represent the 95% credible interval.

Altogether, the above models attributed the majority of human campylobacteriosis infections to ruminant and chicken sources, whereas exposure to environmental water and wild bird sources appeared to contribute significantly less to the burden of disease.

5.5 Discussion

In this study we examined the changes in the molecular epidemiology of *C. jejuni* in the Manawatu sentinel surveillance site in New Zealand following food safety interventions implemented by the poultry industry. The study analysed and compared 1,300 *C. jejuni* isolates from humans and various potential sources collected before the interventions (2005-2007) with 2,012 isolates collected after the interventions (2008-2015). A limitation of this study was the arbitrary definition of the 31st of December 2007 as the last day of the pre-intervention period although, in reality, the interventions were gradually implemented between 2006 and 2008.

C. jejuni was the dominant species among all sources in both periods. It was the leading cause of bacterial gastroenteritis in humans and accounted for more than 95% of the clinical isolates, which is similar to the reports from other countries (5-7, 138). The level of *Campylobacter* contamination of chicken samples remained high during both periods (~78%) which, again, is consistent with the findings in other countries (116, 146, 183). The high level of contamination in chicken samples is consistent with consumption of chicken meat being the main risk factor for *C. jejuni* infections (24, 111). Interestingly, the interventions in the poultry industry were accompanied with a significant reduction in the number of *Campylobacter* cfu found in chicken meat products (Appendix 7.3.3). It also led to a significant drop in the number of leaking bags containing the whole carcass samples among Supplier B and Supplier 'Others'. Interestingly, Supplier A showed a significant increase in the amount of leaking bags in the period following intervention, indicating further improvements in the provision of

leak-proof packing need to be considered to prevent cross-contamination from carcasses to other surfaces.

A comparison between *C. jejuni* populations before and after the intervention revealed a marked change in the prevalence of one major clonal complex: CC ST-48, which prevalence halved, from 27% to 13.6%. The change in the prevalence of CC ST-48 was attributable to a large decrease in the isolation of the ST-474, which is internationally rare but common in New Zealand among all sources, from 17.2% (224/1300) to 6.6% (132/2012). Moreover, ST-474 was a dominant ST isolated from clinical cases before the intervention period, and it dropped from 28.2% to 9.3% after the intervention period. Likewise, ST-474 dropped from 9.7% to 5.3%, among STs isolated from chicken sources, after the food safety interventions that were applied by the poultry industry. The significant post-intervention decrease in the prevalence of ST-474 together with the significant reduction in the number of *Campylobacter* cfu found in chicken meat products might have been the major factor determining the 60% decrease in campylobacteriosis notification rate. However, it must be emphasized that no studies have confirmed that the drop in prevalence of ST-474 was a direct result of the food safety interventions. Another significant change was the increase in prevalence of the CC ST-45 (from 15% to 18.3%). This change was mainly due to an increase in the proportion of ST-583 and also the detection of 16 STs belonging to CC ST-45 that were not detected between 2005 and 2007.

The number of different STs isolated from all sources increased from 112 before the intervention, to 191 after the intervention (note: however, the period of sample collection after the intervention was longer; Figure 5.1 and Figure 5.2). We also examined the change in the diversity of *Campylobacter jejuni* STs in the New Zealand poultry supply and further investigated if chicken products were still a major contributor

to the burden of human disease, post food safety interventions by the poultry industry. By rarefaction, before the intervention, the 95% CrI of the ST richness from all chicken suppliers combined (Appendix 7.3.6) overlapped with the human clinical isolates. However, after the interventions, human clinical isolates had the greater ST diversity compared to chicken STs combined (95% CrI not overlapping). The diversity indices were in agreement with rarefaction analysis results, indicating that other sources contributed to *C. jejuni*-associated campylobacteriosis. A total of 53 different STs were isolated from clinical cases before the interventions and 106 STs were isolated after the intervention. Before the intervention, 22 of the 53 STs were also isolated from chicken sources and these STs accounted for 84.3 % of human cases. After the intervention, 37/106 STs were isolated from chicken sources, and these STs accounted for 84.1 % of human cases. Those overlaps between human and chicken-associated genotypes are consistent with poultry being an important source of infection in New Zealand (117, 143, 147, 182), in both periods.

In this study, the PSI was calculated to evaluate the similarity between ST frequency distributions before and after the interventions. Before the intervention, the largest PSI was between STs from humans and Supplier A, mainly due to the high prevalence of ST-474 among clinical cases and Supplier A, and also due to the low prevalence of ST-474 in samples from non-poultry sources (184). On the other hand, the PSI between clinical cases and Supplier B, Supplier 'Others' and ruminant sources increased significantly after intervention, and dropped among Supplier A; however, the 95% CrIs overlapped among chicken and ruminant sources. Before the intervention, the outcome of the Bayesian hierarchical model and the asymmetric island model results were consistent with the PSI outcomes, where Supplier A was the largest significant contributor to human infections (Figure 5.5). However, after the intervention, the

ruminant source became the major contributor to human infections and it increased significantly from before to after intervention unlike the Supplier A source that decreased significantly, according to the Bayesian hierarchical and asymmetric island model. However, if we combine all the suppliers as a single chicken source, the contribution to human infections is shared equally between ruminants and chickens (Appendix 7.3.7), unlike the period before the intervention, where chicken sources combined contributed between 58% and 76%, followed by ruminants (~20% to ~30%) (34). Our results from before the intervention were also consistent with previous microbiological and epidemiological studies that also indicated the poultry source as the major contributor to the burden of campylobacteriosis in industrialised countries (185, 186). However, our results for the period after the intervention differed from the results obtained after interventions in Luxembourg (187) and Denmark (188), where chicken remained the dominant risk factor for clinical infections.

In summary, the results of this study indicate that in both periods, > 95% of human campylobacteriosis cases in the study area could be attributed to infection with *C. jejuni*. Further work should focus on identifying risk factors for ruminant-associated cases, as studies have shown that animal and environmental sources, rather than consumption of contaminated food, are significant infection pathways for Shiga toxin-producing *Escherichia coli* in humans in New Zealand (189). A study in the Manawatu region showed that ruminant-associated campylobacteriosis cases are more prevalent in rural than urban areas, unlike the poultry-associated cases (182). Future studies should assess whether our results can be generalised to other New Zealand regions given that specific demographic differences between the Manawatu and other New Zealand regions may affect source attribution studies (76). Our source attribution analysis, post intervention, identified that ruminants became, along with chickens, the main infection

source unlike the situation in other countries where chickens remain the dominant risk factor followed by ruminants. Our findings showed an important change in the molecular epidemiology of *Campylobacter jejuni* following food safety interventions by the poultry industry. Therefore, it is vital that policy makers are cognisant of this change in the aetiology of *C. jejuni* infections and the information used in developing further strategies to reduce the total burden of human campylobacteriosis.

5.6 Acknowledgements

The isolates examined in this study were collected as part of the campylobacteriosis surveillance program funded by Ministry of Primary Industries, New Zealand. The authors would like to thank Rukhshana Akhter and Lynn Rogers from ^{m}EpiLab team for their laboratory work contribution and Dr Philip Carter from ESR for help with sequence typing. I am thankful for Dr Jonathan Marshall (Massey University) for the assistance in statistical analyses**.**

Chapter 6

General Discussion

This PhD project examined a number of microbiological aspects of human campylobacteriosis in New Zealand and estimated the contribution of different sources to the burden of human infection caused by the two major *Campylobacter* spp.: *C. coli* and *C. jejuni*. Data and samples used were those collected from the sentinel surveillance site established in the Manawatu region between 2005 and 2012. Data and samples collected from 2013 onwards were collected during this PhD project.

In molecular epidemiological studies, sampling and culturing methodology is vital, as any sampling bias could potentially bias the inferences. In this project, failing to recover all STs with equal probability could have biased the source attribution calculation. Therefore, the studies presented in Chapters 2 and 3 were performed to assess 1) whether the delay of culture, as commonly applied in previous source attribution studies in our laboratory, could have hindered the identification of some *Campylobacter* species or STs from human clinical cases, potentially biasing the results of these studies; and 2) whether the type of chicken retail meat analysed (whole carcasses versus drumsticks) could influence the population genetic structure of *C. jejuni* and *C. coli*, and consequently, the results of the studies. The results of both studies indicated no significant bias could have been introduced by the delay of culture of clinical samples or the type of chicken retail meat analysed. Therefore, chapters 2 and 3 did not support the working hypothesis of the existence of a bias determined by the culture and sampling methods, although there were some interesting findings in each one of the studies, which will be discussed later.

At the time of the sampling for this project, the ^{m}EpiLab was receiving once a week the *Campylobacter* ELISA-positive faecal clinical specimens from the human diagnostic

laboratory (MedLab Central). In the first study, we examined if the delay in sample processing may have affected the recovery of *Campylobacter* spp. (Chapter 2). The results indicated that the ELISA used at the diagnostic laboratory had low sensitivity and did not detect all the campylobacteriosis cases, as a relatively high number of ELISA-negative specimens gave culture-positive results. To my knowledge, after the results of this study were made known to the diagnostic laboratory management, the laboratory replaced the faecal ELISA with culture (Jan Deroles-Main, Charge scientist, Microbiology, MedLab Central, Palmerston North) for the diagnosis of campylobacteriosis. The results of our MLST analysis indicated that the ELISA was unlikely to introduce a major bias to source attribution analyses, as the PERMANOVA results presented in Chapter 2 did not show a population differentiation between the ELISA-positive and ELISA-negative samples. In spite of the fact that the previous source attribution studies were not likely to be biased by the low ELISA sensitivity, the shift from the ELISA to a culture method at MedLab central in February 2016 was an important step towards a better testing sensitivity for campylobacteriosis. The shift from ELISA to culture will increase the sample size available for future molecular epidemiological studies of campylobacteriosis and more accurately reflect the genetic variation in the population. Although the rate of campylobacteriosis at the MedLab central did not increase significantly after the shift from ELISA to culture, however the recovery rate at the ^{m}EpiLab did improve and that will increase the sample size for future analysis.

The second study (Chapter 3) was justified in view of the little published comparative data on the prevalence of different *Campylobacter* species and subtypes contaminating the different types of chicken meat products sold at retail chains. The study compared *Campylobacter* viable counts and, the population genetic structure of *C. jejuni* and *C.*

coli, between two types of chicken meat; whole carcasses and drumsticks. The analysis showed significant differences between the proportional similarity indexes (PSIs) of different chicken suppliers versus the human clinical isolates. This suggests that consumption of meat from different suppliers may pose different levels of risk. Another interesting result was the difference between the *Campylobacter* viable counts between the two retail chicken meat products: consuming meat from whole carcasses may expose consumers to higher *Campylobacter* infectious doses than consuming drumsticks (after accounting for the effects of the supplier and the weight of the sample). The finding of chicken meat samples with high *Campylobacter* counts may have implications on the human health, mainly because it increases the risk of campylobacteriosis. Moreover, given the differences between suppliers, it would be interesting to assess whether this is attributable to differences in the application of *Campylobacter* management strategies that were developed to reduce the burden of human campylobacteriosis in New Zealand (155) is being applied uniformly across all suppliers. Since the ST diversities were similar and there was no significant difference between the PSIs among the two types of products, it is likely that future molecular source attribution models would not need to take into account the type of product sampled for the study.

Molecular epidemiological studies combine the use of molecular tools for subtyping of microbial isolates, with sound and robust epidemiological method for sampling. This PhD project applied MLST to analyse the genetic relatedness of clinical *C. coli* and *C. jejuni* isolates, and isolates obtained from poultry meat, farmed ruminants faeces, environmental water and wild bird faeces collected in one study area over the same time frame, allowing inferences of source attribution. The studies on *C. jejuni and C. coli* were done separately because the relative contribution of *C. coli* to human infections,

and the sources of these infections, had not been previously examined in New Zealand. Moreover, the distribution and prevalence of *C. jejuni* and *C. coli* may differ among the sources. For example, it has been reported that the distribution of *C. jejuni* and *C. coli* differs between environmental water, food, and different animal species (140).

The study presented in Chapter 4 was justified in view of the reported differences in the ecology of *C. jejuni* and *C. coli*. The results indicated that *C. coli* accounted for ~3% of the cases of campylobacteriosis in the region between 2005 and 2014. This is a relatively low proportion of cases compared to other countries, where *C. coli* have been identified in about 10% of the cases (110, 138, 141, 175). However, calculations suggested that the incidence of *C. coli* infections could be higher in New Zealand than in other countries, due to the relatively high incidence of campylobacteriosis due to any species reported in New Zealand. Three novel *C. coli* STs (ST-7767, ST-7774 and ST-7776) were identified in this study, none of which was found in humans. ST-1581 (unassigned clonal complex) was the most prevalent ST in humans and poultry meat in this study. It occurred sporadically, but was found almost every year in humans in the ten analysed years. ST-1581 has not been reported as a common ST in humans in other countries (24, 177) which shows how the ST distribution in New Zealand is unique compared to other countries. Interestingly, in Scotland, all the STs found in environmental water sources clustered in clades 2 and 3 (170), whereas in our study they clustered in clade 1, with only one ST (ST-7774) detected in clade 2 and none in clade 3. On the other hand, human and animal *C. coli* belonged to clade 1, in accordance with the findings in Scotland. It would be interesting in future studies to increase the sample size from environmental water sources, in order to increase the probability of finding STs belonging to clades 2 and 3, if these clades are present in New Zealand.

In this study, the PSI and asymmetric island model were used to estimate the relative contribution of different *C. coli* sources to human disease burden. Both tests showed similar results, indicating that ruminant sources had a similar or slightly greater relative contribution to *C. coli* infection burden in the study region as the poultry source. In contrast, 58-76% of campylobacteriosis cases caused by *C. jejuni* were attributed to poultry, and up to 30% to ruminant sources (34). These differences indicate that interventions to control campylobacteriosis should take into consideration the differential risk of infection with different *Campylobacter* species posed by consumption of different types of meat. The results of a study in France (190) indicated the risk factors for infection with *C. jejuni* and *C. coli* differ significantly. The risk of infection with *C. coli* was higher in older persons (34.6 years) compared to *C. jejuni* (27.5 years), and travelling abroad in the preceding 2 weeks posed a higher risk of infection with *C. coli* than *C. jejuni*. In future studies, it would be interesting to assess whether there are differences in the clinical characteristics of infections with *C. jejuni* and *C. coli* in New Zealand. To the author's knowledge, the diseases caused by these two agents are undifferentiated.

Since *C. jejuni* is responsible for more than 95% of campylobacteriosis cases in New Zealand, it is essential to perform periodical attribution studies, in order to monitor possible changes in the source attribution of *C. jejuni* infections. In 2006, New Zealand had the highest annual campylobacteriosis notification rate reported in the world, with >380 cases per 100,000 population (156). A sixty percent decrease in campylobacteriosis notification was observed after the implementation of food safety interventions throughout the poultry industry. The study presented in Chapter 5 assessed the attributional changes following these interventions. The major change observed was a ~50% decrease in the prevalence of *C. jejuni* CC ST-48. This decrease could be

attributed by the ~60% decrease in the isolation of the internationally-rare ST-474 (that belongs to CC ST-48), which was the dominant ST in the region before the interventions. In the study presented in Chapter 5, ST-474 prevalence dropped from 28.2% to 9.3% among clinical isolates, and from 9.69% to 5.32% among chicken isolates (both differences were statistically significant). However, it must be emphasised that no studies have confirmed that the drop in prevalence of ST-474 was a direct result of the food safety interventions. Conversely, there was a drop in the proportion of ST-45 among chicken sources, from 23.6% in the first period, to 16.5% after 2008, but the prevalence of this ST among the clinical isolates increased from 8.8% to 12.3%.

Bacterial survival mechanisms such as the 'viable but nonculturable' state, formation of biofilm, and secretion of extracellular proteins that underpin survival under environmental stresses such as heat stress, cold stress, UV radiation stress, acid stress, aerobic stress, desiccation stress, have been reviewed in *Campylobacter* by Murphy et al. (191). With the recent advances of whole genome sequencing, it would be interesting in future work to compare gene expression of ST-474 and other STs under different conditions, in order to better understand whether there are differences in survival and adaptation mechanisms in the environment and in meat products.

The statistical methods used to estimate the relative contribution of different *C. jejuni* sources to human disease burden converged in indicating poultry Supplier A as the most important contributor to human infection burden before the intervention period. Conversely, after the intervention, the ruminant source became the major contributor, whereas the relative contribution of Supplier A decreased significantly. However, the contributions of the ruminant and chicken sources were similar when all chicken suppliers were combined as one source. Interestingly, in Luxembourg (187) and

Denmark (188), consumption of chicken meat remained the dominant risk factor for campylobacteriosis even after industry interventions.

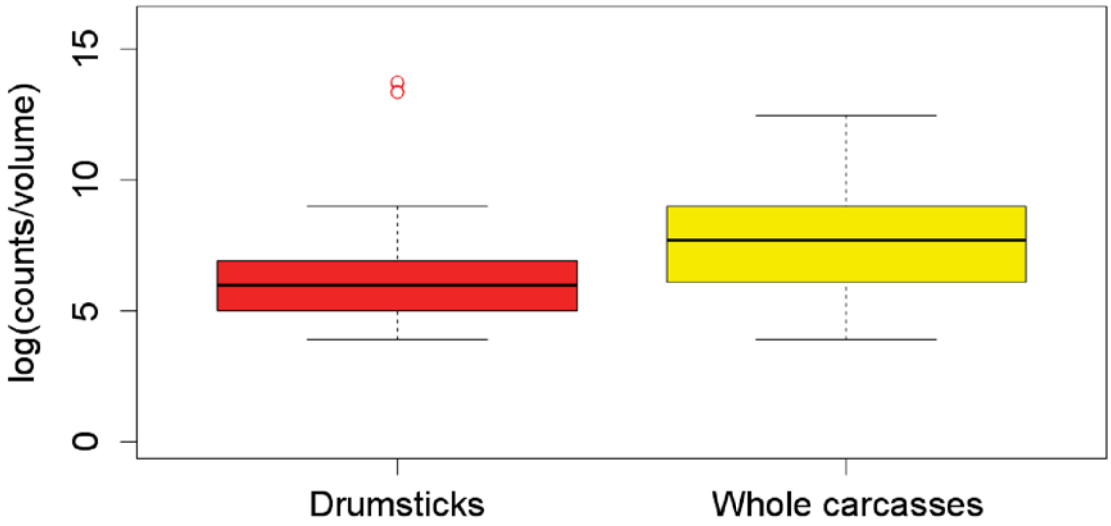
Finally, the studies presented in this thesis have limitations. Studies were performed in one geographical region, without differentiation between rural and urban areas. It would be interesting to assess whether there are difference in the dominant STs circulating in different regions. A recent report from 2015 indicated that the proportion of human cases attributed to the poultry source was higher among the urban population of the Manawatu than the rural population. *Vice versa*, the proportion of human cases attributed to ruminant sources was higher among the rural population (192). Therefore future studies should expand the geographical reach of the sampling, to account for the demographic differences between the Manawatu and other New Zealand regions (76).

In summary, this PhD project addressed several vital questions related to campylobacteriosis control at regional or national levels. The project established the proportion of infections caused by *C. coli* in the study area, and the changes observed in the proportion of infections attributed to exposure to the different *Campylobacter* sources. The findings indicate future interventions on non-poultry sources, such as the ruminant source, could further reduce campylobacteriosis incidence in New Zealand. More studies are needed in order to understand transmission routes from the ruminant source to humans.

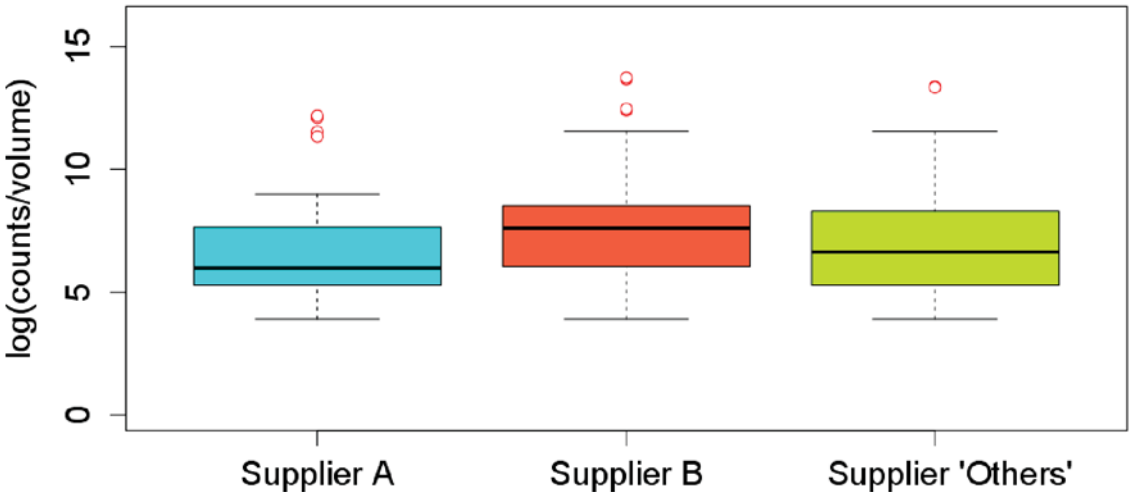
Chapter 7

Appendix

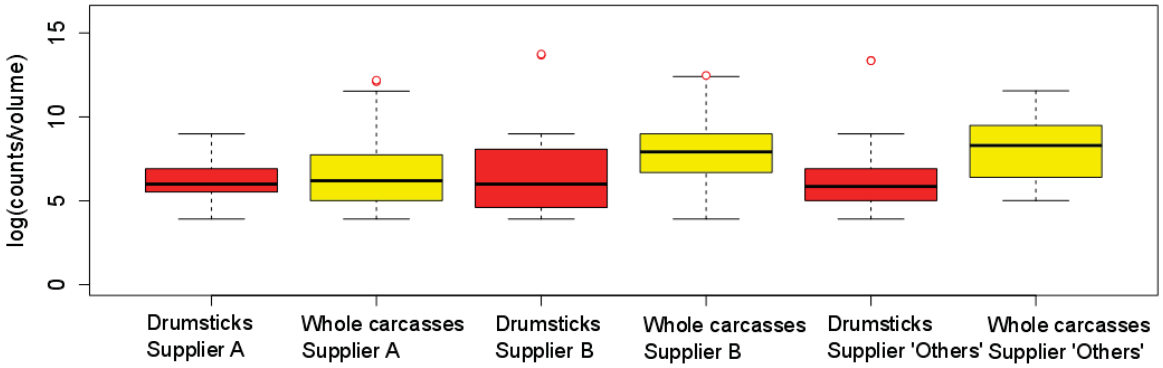
7.1 Chapter 3 supplementary information



Appendix 7.1.1: The level of *Campylobacter* contamination in drumsticks and whole carcass chicken samples.



Appendix 7.1.2: The level of *Campylobacter* contamination in Supplier A, Supplier B and Supplier 'Others'.



Appendix 7.1.3: The level of *Campylobacter* contamination in drumsticks and whole carcass chicken samples among Suppliers A, B and 'Others'.

7.2 Chapter 4 supplementary information

Appendix 7.2.1: Clonal complex (CC) assignment and the allelic profile of the 27 different *C. coli* STs detected from different sources.

		Allelic profile						
ST	CC	ASP	GLN	GLT	GLY	PGM	TKT	UNC
825	828	33	39	30	82	113	47	17
829	828	33	39	30	82	113	43	17
854	828	33	38	30	82	104	43	17
860	828	33	39	30	79	113	47	17
890	828	33	38	30	82	104	35	36
1016	828	33	38	30	82	118	43	17
1115	U/A	53	39	30	81	118	44	36
1191	828	33	39	30	82	189	47	17
1243	U/A	81	155	30	163	231	43	93
1581	U/A	129	66	30	82	189	47	17
1590	828	33	39	65	79	104	43	17
1595	828	33	38	30	79	104	43	17
1767	828	33	39	30	79	113	44	17
2256	828	33	39	30	79	189	47	17
2397	828	184	39	30	82	113	43	17
3072	828	33	39	30	82	104	173	68
3222	U/A	33	283	44	82	189	44	17
3230	828	33	39	30	322	104	85	17
3232	828	32	39	30	82	104	324	17
3233	828	33	38	30	82	104	325	36
3299	828	33	39	44	82	113	43	17
3301	U/A	86	155	69	113	276	257	67
3302	U/A	130	155	69	113	276	257	67
4009	828	33	39	65	79	104	77	17
7767	U/A	33	39	65	79	745	77	17
7774	U/A	130	535	458	612	746	129	465
7776	U/A	129	66	30	82	189	588	17

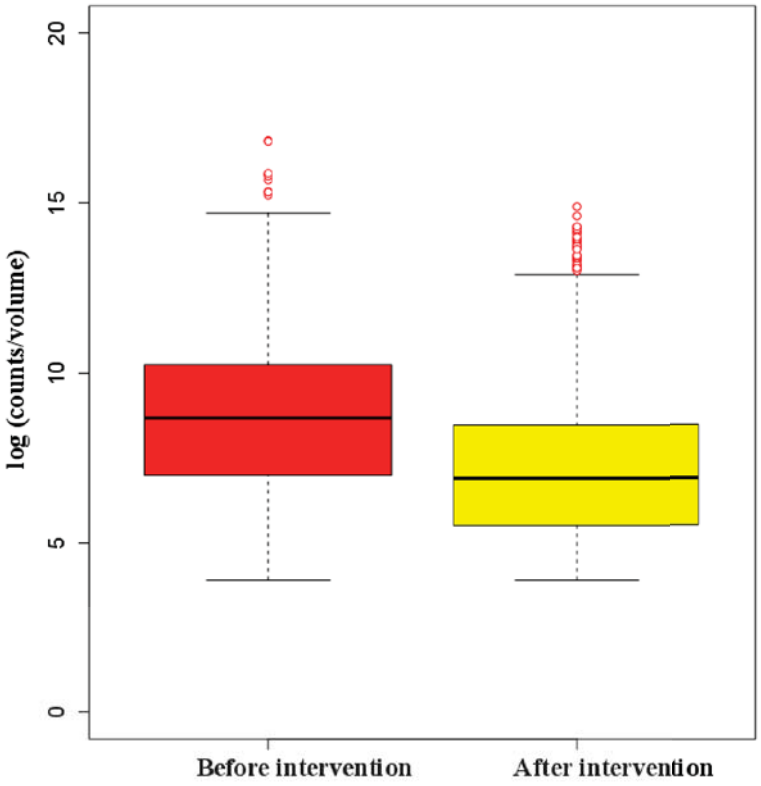
Appendix 7.2.2: Frequency of the 27 different *C. coli* STs with their clonal complex (CC) and clade designation isolated from different sources.

ST	CC	Clade	Source				Total
			Human	Poultry	Ruminants	Environmental water	
825	828	1	2	3	2	0	7
829	828	1	2	0	1	1	4
854	828	1	1	5	0	0	6
860	828	1	3	0	0	0	3
890	828	1	0	0	1	0	1
1016	828	1	1	0	0	0	1
1115	U/A	1	0	0	2	0	2
1191	828	1	0	0	1	0	1
1243	U/A	1	0	0	0	3	3
1581	U/A	1	12	44	4	0	60
1590	828	1	3	9	0	0	12
1595	828	1	0	1	0	0	1
1767	828	1	2	0	0	0	2
2256	828	1	0	5	0	0	5
2397	828	1	1	19	1	0	21
3072	828	1	9	4	12	0	25
3222	U/A	1	3	0	17	1	21
3230	828	1	0	5	0	1	6
3232	828	1	4	0	6	0	10
3233	828	1	2	0	0	0	2
3299	828	1	0	0	2	0	2
3301	U/A	1	0	0	0	1	1
3302	U/A	1	0	0	0	1	1
4009	828	1	2	5	0	0	7
7767	U/A	1	0	7	0	0	7
7774	U/A	2	0	0	0	3	3
7776	U/A	1	0	1	0	0	1
Total isolates			47	108	49	11	215

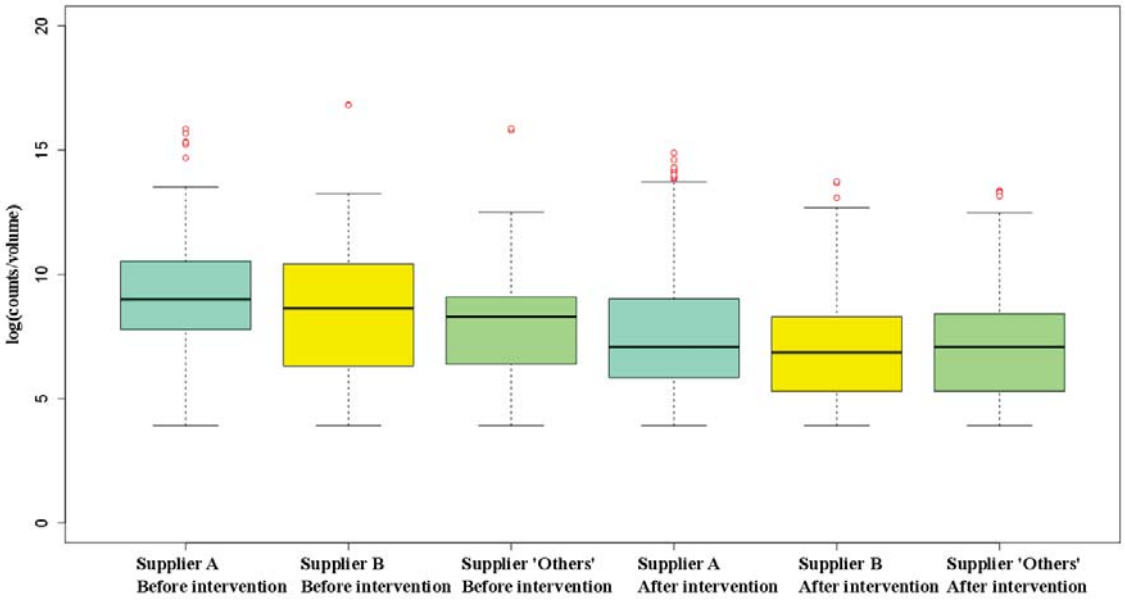
Appendix 7.2.3: Numerical indices measuring diversities, PSI and asymmetric island model in *C. coli* (95% CrI are in brackets).

	Source				
Index	Human	Sheep	Cattle	Poultry	Environmental water
Proportional similarity index	NA	0.53(0.31-0.63)	0.31(0.14-0.46)	0.46(0.30-0.56)	0.10(0.00-0.17)
Asymmetric island model output	NA	0.37(0.07-0.68)	0.20(0.01-0.50)	0.37(0.15-0.58)	0.05(0.00-0.20)
Simpson's	0.87(0.79-0.89)	0.82(0.72-0.84)	0.67(0.46-0.75)	0.78(0.70-0.82)	0.81(0.56-0.82)
Shannon's	2.32(1.89-2.40)	1.89(1.45-1.98)	1.30(0.80-1.48)	1.93(1.66-2.06)	1.80(0.95-1.84)

7.3 Chapter 5 supplementary information



Appendix 7.3.1: Comparison of the level of *Campylobacter* contamination among positive chicken samples; before versus after intervention.



Appendix 7.3.2: Comparison of the level of *Campylobacter* contamination among positive chicken suppliers; before versus after intervention.

Appendix 7.3.3: Zero-inflated Poisson model results.

Model comparing *Campylobacter* colony counts prior to and then after intervention

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)*	3.36	0.002	1192	2×10^{-16}
Before intervention	1.30	0.004	309.6	2×10^{-16}

Zero-inflation model comparing the probability of a zero *Campylobacter* colony counts prior to and then after intervention

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)*	0.16	0.03	5.23	1.6×10^{-7}
Before intervention	-0.36	0.06	-5.31	1.1×10^{-7}

*Comparison group was chicken samples from after intervention to before intervention.

Appendix 7.3.4: Frequency of the *C. jejuni* STs with their CC designation isolated from different sources 'before intervention'.

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
474	48	184	6	29	2	1	2	0	224
45	45	58	4	36	15	27	4	11	155
50	21	35	34	5	17	13	1	0	105
48	48	51	0	1	25	8	0	0	85
53	21	37	24	9	3	2	0	0	75
190	21	32	14	9	0	0	0	0	55
42	42	22	26	4	0	0	1	0	53
61	61	22	21	0	0	0	1	0	44
2026	403	14	22	0	0	0	0	0	36
257	257	17	0	9	3	2	0	0	31
354	354	26	0	3	0	1	0	0	30
451	21	9	0	0	0	21	0	0	30
422	21	5	21	0	0	0	3	0	29
520	21	11	7	5	0	4	0	0	27
52	52	18	0	7	0	1	0	0	26
583	45	10	1	8	0	4	0	1	24
1517	354	11	6	0	6	0	0	0	23
38	48	18	1	0	0	0	0	0	19
2345	206	6	0	1	11	0	0	0	18
2381	U/A	0	0	0	0	0	16	0	16
3609	48	0	0	0	15	0	0	0	15
21	21	6	5	0	1	0	0	0	12
436	U/A	4	5	0	0	0	1	0	10
677	677	7	1	0	1	0	1	0	10
25	45	4	0	2	0	2	1	0	9
1225	1275	0	0	0	0	0	7	0	7
3676	42	5	1	0	0	0	1	0	7

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ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
3711	257	2	2	0	0	1	0	0	5
3717	21	1	0	0	0	3	0	0	4
137	45	1	0	0	0	0	1	1	3
658	658	2	0	0	0	1	0	0	3
1457	U/A	3	0	0	0	0	0	0	3
2350	48	2	1	0	0	0	0	0	3
2392	52	2	1	0	0	0	0	0	3
3538	U/A	2	0	0	0	0	1	0	3
3662	1275	0	0	0	0	0	3	0	3
3797	61	0	3	0	0	0	0	0	3
3799	61	0	3	0	0	0	0	0	3
177	177	0	0	0	0	0	2	0	2
459	42	2	0	0	0	0	0	0	2
696	1332	2	0	0	0	0	0	0	2
2354	U/A	0	0	0	0	0	1	1	2
2391	1034	1	0	1	0	0	0	0	2
2536	U/A	0	0	0	0	0	0	2	2
3610	21	0	1	0	0	0	1	0	2
3655	U/A	0	0	0	0	0	2	0	2
3659	692	0	0	0	0	0	2	0	2
3663	U/A	0	0	0	0	0	2	0	2
3712	362	2	0	0	0	0	0	0	2
3715	21	2	0	0	0	0	0	0	2
3719	48	0	1	0	1	0	0	0	2
3721	354	0	0	2	0	0	0	0	2
3726	U/A	0	0	2	0	0	0	0	2
5	353	1	0	0	0	0	0	0	1
51	443	1	0	0	0	0	0	0	1
219	61	1	0	0	0	0	0	0	1
227	206	0	0	1	0	0	0	0	1

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		Source							
ST	CC	Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	Total
233	45	0	0	0	0	1	0	0	1
393	443	0	1	0	0	0	0	0	1
403	403	1	0	0	0	0	0	0	1
526	U/A	0	0	0	0	0	1	0	1
578	61	1	0	0	0	0	0	0	1
618	61	0	1	0	0	0	0	0	1
681	682	0	0	0	0	0	0	1	1
694	1034	0	0	0	0	0	1	0	1
996	U/A	0	0	0	0	0	1	0	1
1030	U/A	0	0	0	0	0	1	0	1
1223	1275	0	0	0	0	0	1	0	1
1324	U/A	0	0	0	0	0	0	1	1
1707	607	1	0	0	0	0	0	0	1
1818	45	0	0	0	1	0	0	0	1
1911	U/A	0	0	0	1	0	0	0	1
2140	574	1	0	0	0	0	0	0	1
2219	45	1	0	0	0	0	0	0	1
2341	61	0	1	0	0	0	0	0	1
2343	48	1	0	0	0	0	0	0	1
2347	U/A	0	0	0	0	0	1	0	1
2537	177	0	0	0	0	0	0	1	1
2538	U/A	0	0	0	0	0	0	1	1
2539	177	0	0	0	0	0	0	1	1
2584	692	0	0	0	0	0	1	0	1
2619	U/A	0	0	0	0	0	1	0	1
3093	21	0	0	0	0	1	0	0	1
3640	U/A	0	0	0	0	0	1	0	1
3656	U/A	0	0	0	0	0	1	0	1
3657	1275	0	0	0	0	0	1	0	1
3658	U/A	0	0	0	0	0	1	0	1

Continue on next page

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
3660	U/A	0	0	0	0	0	1	0	1
3661	1275	0	0	0	0	0	1	0	1
3664	692	0	0	0	0	0	1	0	1
3672	U/A	0	0	0	0	0	1	0	1
3673	U/A	0	0	0	0	0	1	0	1
3674	1275	0	0	0	0	0	1	0	1
3675	U/A	0	0	0	0	0	1	0	1
3713	21	1	0	0	0	0	0	0	1
3714	21	0	1	0	0	0	0	0	1
3718	48	1	0	0	0	0	0	0	1
3720	49	1	0	0	0	0	0	0	1
3722	U/A	0	1	0	0	0	0	0	1
3725	692	0	0	0	1	0	0	0	1
3727	45	1	0	0	0	0	0	0	1
3728	U/A	1	0	0	0	0	0	0	1
3784	354	1	0	0	0	0	0	0	1
3792	257	1	0	0	0	0	0	0	1
3794	21	0	1	0	0	0	0	0	1
3795	403	0	1	0	0	0	0	0	1
3796	U/A	0	1	0	0	0	0	0	1
3798	403	0	1	0	0	0	0	0	1
3800	U/A	0	0	0	0	0	1	0	1
3802	45	0	0	0	0	0	1	0	1
3803	U/A	0	0	0	0	0	1	0	1
4494	61	0	1	0	0	0	0	0	1
Total		652	221	134	103	93	76	21	1300

Appendix 7.3.5: Frequency of the *C. jejuni* STs with their CC designation isolated from different sources 'after intervention'.

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
45	45	128	8	29	24	31	4	26	226
50	21	88	39	9	24	9	0	0	169
48	48	44	0	0	63	3	0	0	136
474	48	97	8	21	2	4	0	0	132
53	21	77	11	12	19	8	0	2	127
61	61	61	30	2	5	0	0	0	100
583	45	36	4	24	6	13	0	0	90
42	42	53	31	1	2	0	0	2	87
190	21	45	12	2	0	2	0	0	66
520	21	33	11	3	2	3	0	2	52
6964	354	7	0	9	11	16	0	0	43
2026	403	22	14	0	0	0	1	0	38
422	21	17	15	0	0	2	3	0	37
2345	206	22	0	12	2	0	0	3	37
257	257	24	1	7	2	1	0	0	35
21	21	21	4	0	1	8	0	0	34
354	354	15	0	11	0	5	0	0	31
436	U/A	20	7	0	0	0	0	0	27
1517	354	12	9	0	0	1	1	4	23
38	48	13	4	0	2	0	1	0	20
677	677	15	0	0	0	0	0	1	17
3105	U/A	1	0	13	1	2	0	0	17
1326	45	2	0	9	0	3	0	1	14
3711	257	12	1	0	1	0	0	0	14
51	443	9	0	2	0	0	0	0	11
508	508	9	2	0	0	0	0	0	11
1033	1034	1	0	1	0	1	0	2	11

Continue on next page

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
538	45	6	0	2	0	0	0	0	10
3676	42	9	0	0	0	1	0	0	10
137	45	8	0	1	0	0	0	4	9
696	1332	1	0	4	0	1	0	6	9
1324	U/A	0	0	0	0	0	0	0	9
4337	21	7	0	0	2	0	0	0	9
486	21	3	0	0	5	0	0	0	8
52	52	3	0	4	0	0	0	0	7
535	460	0	0	5	2	0	0	2	7
995	U/A	0	0	0	0	0	0	8	7
2388	U/A	7	0	0	0	0	0	0	7
403	403	5	1	0	0	0	0	0	6
699	692	0	0	0	0	0	0	0	6
2389	U/A	0	0	3	2	1	0	4	6
3538	U/A	6	0	0	0	0	0	0	6
3610	21	5	1	0	0	0	0	4	6
3640	U/A	0	0	0	0	0	2	0	6
4499	U/A	0	0	0	0	0	0	1	6
25	45	4	0	1	0	0	0	0	5
177	177	0	1	0	0	0	0	5	5
618	61	1	4	0	0	0	0	0	5
710	U/A	0	0	0	0	0	0	0	5
991	692	1	2	0	0	0	0	2	5
2343	48	4	0	0	0	1	0	1	5
2350	48	4	0	0	1	0	0	2	5
2378	1034	0	0	0	0	0	1	0	5
2381	U/A	0	0	0	0	0	4	0	5
2535	U/A	3	0	0	0	1	0	1	5
3798	403	5	0	0	0	0	0	0	5
393	443	2	2	0	0	0	0	0	4

Continue on next page

		Source							
ST	CC	Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	Total
658	658	2	0	2	0	0	0	2	4
692	692	0	0	0	0	0	1	3	4
977	1034	1	0	0	0	0	0	2	4
992	U/A	0	0	1	0	0	1	7	4
1225	1275	0	0	0	0	0	4	0	4
1256	1034	0	0	0	0	0	0	3	4
1457	U/A	1	0	0	0	0	0	0	4
1525	U/A	0	0	0	0	0	0	0	4
2354	U/A	0	0	0	0	0	0	0	4
2391	1034	0	0	0	0	0	0	0	4
3961	U/A	0	0	0	0	0	0	0	4
4492	61	4	0	0	0	0	0	1	4
227	206	1	0	0	0	1	0	0	3
356	353	0	0	3	0	0	0	0	3
693	U/A	0	0	0	0	0	0	3	3
1286	U/A	0	0	0	0	0	0	2	3
1342	U/A	0	0	0	0	0	0	3	3
2347	U/A	0	0	0	0	0	0	1	3
2392	52	0	3	0	0	0	0	1	3
2584	692	2	0	0	0	1	0	0	3
3655	U/A	0	0	0	0	0	0	1	3
3721	354	0	0	3	0	0	0	0	3
5648	692	0	0	0	0	0	0	0	3
353	353	2	0	0	0	0	0	0	2
526	U/A	0	0	0	0	0	0	0	2
1223	1275	0	0	0	0	0	0	0	2
1255	1034	0	0	0	0	0	0	4	2
1304	1304	0	0	0	0	0	0	9	2
1900	658	0	0	0	2	0	0	0	2
2340	61	1	1	0	0	0	0	0	2

Continue on next page

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
2351	U/A	0	0	0	0	0	0	1	2
2370	61	2	0	0	0	0	0	4	2
2380	U/A	2	0	0	0	0	0	1	2
3675	U/A	0	0	0	0	0	1	0	2
3717	21	2	0	0	0	0	0	0	2
3799	61	2	0	0	0	0	0	0	2
3959	61	0	2	0	0	0	0	4	2
4338	21	1	1	0	0	0	0	1	2
4497	U/A	0	0	0	0	0	1	1	2
4500	U/A	0	0	1	0	0	0	1	2
4508	U/A	0	0	0	0	0	0	1	2
5655	21	1	1	0	0	0	0	0	2
5656	354	0	2	0	0	0	0	0	2
6997	403	1	1	0	0	0	0	0	2
7769	21	2	0	0	0	0	0	0	2
8076	45	2	0	0	0	0	0	0	2
5	353	1	0	0	0	0	0	0	1
22	22	1	0	0	0	0	0	0	1
81	61	1	0	0	0	0	0	0	1
97	45	1	0	0	0	0	0	0	1
233	45	1	0	0	0	0	0	0	1
249	21	1	0	0	0	0	0	0	1
320	45	1	0	0	0	0	0	0	1
366	257	1	0	0	0	0	0	0	1
449	U/A	1	0	0	0	0	0	0	1
451	21	1	0	0	0	0	0	0	1
459	42	1	0	0	0	0	0	0	1
460	460	1	0	0	0	0	0	0	1
528	354	1	0	0	0	0	0	0	1
560	257	1	0	0	0	0	0	0	1

Continue on next page

ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
577	21	1	0	0	0	0	0	0	1
578	61	1	0	0	0	0	0	7	1
681	682	0	0	0	0	0	0	3	1
704	U/A	1	0	0	0	0	0	5	1
767	45	0	1	0	0	0	0	3	1
1232	353	1	0	0	0	0	0	2	1
1336	1275	0	0	0	0	0	0	0	1
1340	177	0	0	0	0	1	0	3	1
1919	52	1	0	0	0	0	0	1	1
1956	1034	0	0	0	0	0	0	0	1
1972	U/A	1	0	0	0	0	0	1	1
2076	353	1	0	0	0	0	0	0	1
2212	45	1	0	0	0	0	0	0	1
2341	61	1	0	0	0	0	0	0	1
2349	U/A	0	0	0	0	0	0	0	1
2353	U/A	0	0	0	0	0	0	4	1
2357	61	0	1	0	0	0	0	0	1
2537	177	0	0	0	0	0	0	1	1
2538	U/A	0	0	0	0	0	0	0	1
2619	U/A	0	0	0	0	0	1	1	1
2654	U/A	0	0	0	0	0	0	0	1
2895	574	1	0	0	0	0	0	0	1
2991	45	0	0	1	0	0	0	0	1
3456	45	0	1	0	0	0	0	0	1
3609	48	0	0	0	1	0	0	0	1
3643	U/A	0	0	0	0	0	1	3	1
3712	362	1	0	0	0	0	0	0	1
3716	21	0	1	0	0	0	0	0	1
3724	U/A	0	1	0	0	0	0	0	1
3792	257	0	0	0	1	0	0	0	1

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ST	CC	Source							Total
		Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	
3793	61	1	0	0	0	0	0	0	1
3801	U/A	0	0	0	0	0	1	0	1
3842	61	0	1	0	0	0	0	0	1
3844	U/A	0	0	0	0	0	1	0	1
4011	42	0	1	0	0	0	0	1	1
4336	U/A	0	0	0	0	0	0	0	1
4339	U/A	0	0	0	0	0	0	0	1
4496	U/A	0	0	0	0	0	0	1	1
4498	42	0	0	0	0	0	0	6	1
4501	U/A	0	0	0	0	0	0	1	1
4502	692	0	0	0	0	0	0	1	1
4503	42	0	0	0	0	0	0	1	1
4504	U/A	0	0	0	0	0	0	0	1
4505	21	1	0	0	0	0	0	0	1
4506	52	1	0	0	0	0	0	0	1
4507	U/A	1	0	0	0	0	0	2	1
4509	U/A	0	0	0	0	0	0	1	1
4524	U/A	0	0	0	0	0	0	0	1
4684	U/A	1	0	0	0	0	0	0	1
5012	48	0	0	0	1	0	0	0	1
5326	U/A	1	0	0	0	0	0	0	1
5540	21	0	1	0	0	0	0	1	1
5646	952	0	0	0	0	0	0	3	1
5649	21	1	0	0	0	0	0	0	1
5651	45	0	0	1	0	0	0	0	1
5652	U/A	0	1	0	0	0	0	0	1
5654	354	0	1	0	0	0	0	0	1
6130	362	1	0	0	0	0	0	0	1
7323	U/A	1	0	0	0	0	0	0	1
7765	61	1	0	0	0	0	0	0	1

Continue on next page

		Source							
ST	CC	Human	Ruminants	Supplier A	Supplier B	Supplier 'Others'	Environmental water	Wild birds	Total
7766	45	0	0	0	0	1	0	0	1
7771	21	1	0	0	0	0	0	0	1
7795	45	1	0	0	0	0	0	0	1
7796	61	1	0	0	0	0	0	0	1
8065	353	1	0	0	0	0	0	0	1
8066	45	0	0	0	0	1	0	0	1
8067	692	0	0	0	0	1	0	0	1
8069	45	1	0	0	0	0	0	0	1
8070	21	1	0	0	0	0	0	0	1
8072	45	0	1	0	0	0	0	0	1
8075	21	1	0	0	0	0	0	0	1
8184	42	1	0	0	0	0	0	0	1
8187	U/A	0	0	0	1	0	0	0	1
208	682	0	0	0	0	0	0	1	0
Total		1040	244	199	185	123	29	192	2012

Appendix 7.3.6: Simpson and Shannon diversity indices of different sources (The chicken suppliers are combined as one chicken source) comparing two periods (2005-2007 and 2008-2015) with 95% CrI in brackets.

	Simpson's		Shannon's	
Source	2005-2007	2008-2015	2005-2007	2008-2015
Human	0.89 (0.87-0.90)	0.94 (0.94-0.95)	2.88 (2.7-2.97)	3.48 (3.41-3.56)
Chickens	0.89 (0.87-0.91)	0.92 (0.91-0.93)	2.70 (2.58-2.80)	3.01 (2.94-3.13)
Ruminants	0.91 (0.89-0.92)	0.92 (0.90-0.93)	2.78 (2.6-2.88)	2.99 (2.80-3.08)
Environmental water	0.93 (0.90-0.95)	0.91 (0.85-0.92)	3.31 (3.0-3.45)	2.63 (2.20-2.69)
Wild birds	0.69 (0.46-0.83)	0.96 (0.94-0.97)	1.72 (0.97-2.02)	3.8 (3.58-3.84)

Appendix 7.3.7: Numerical indices measuring PSI, asymmetric island model and Bayesian hierarchical model for *C. jejuni* (95% CrI are in brackets) after the intervention sources (The chicken suppliers are combined as one chicken source).

	Source				
Index	Human	Chickens	Ruminants	Environmental water	Wild birds
Proportional similarity index	NA	0.52 (0.47-0.55)	0.51 (0.44-0.54)	0.17 (0.06-0.18)	0.21 (0.16-0.23)
Asymmetric island model output	NA	0.48 (0.42-0.53)	0.47 (0.41-0.52)	0.02 (0.00-0.06)	0.02 (0.00-0.05)
Bayesian hierarchical model	NA	0.44 (0.35-0.52)	0.39 (0.31-0.50)	0.10 (0.00-0.19)	0.04 (0.00-0.11)

Chapter 8

Bibliography

1. **Sebald M, Veron M.** 1963. Teneur en bases de l'ADN et classification des vibrions. Ann Inst Pasteur (Paris) **105:**897-910.
2. **Euzeby JP.** List of prokaryotic names with standing in nomenclature. http://www.bacterio.net/campylobacter.html. Accessed 2016.
3. **Van TTH, Elshagmani E, Gor MC, Scott PC, Moore RJ.** 2016. *Campylobacter hepaticus* sp. nov., isolated from chickens with spotty liver disease. Int J Syst Evol Microbiol doi:10.1099/ijsem.0.001383.
4. **Piccirillo A, Niero G, Calleros L, Perez R, Naya H, Iraola G.** 2016. *Campylobacter geochelonis* sp. nov. isolated from the western Hermann's tortoise (Testudo hermanni hermanni). Int J Syst Evol Microbiol **66:**3468-3476.
5. **Horrocks SM, Anderson RC, Nisbet DJ, Ricke SC.** 2009. Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. Anaerobe **15:**18-25.
6. **WHO.** 2011. *Campylobacter*. http://www.who.int/mediacentre/factsheets/fs255/en/. Accessed 2016.
7. **Moore JE, Corcoran D, Dooley JS, Fanning S, Lucey B, Matsuda M, McDowell DA, Megraud F, Millar BC, O'Mahony R, O'Riordan L, O'Rourke M, Rao JR, Rooney PJ, Sails A, Whyte P.** 2005. *Campylobacter*. Vet Res **36:**351-382.
8. **Penner JL.** 1988. The genus *Campylobacter*: a decade of progress. Clin Microbiol Rev **1:**157-172.
9. **Garénaux A, Luchetti-Miganeh C, Ermel G, Barloy-Hubler F, De Jonge R, Newell DG, Payot S, Federighi M, Tresse O, Guillou S, Ritz M.** 2008. Better understanding of the *Campylobacter* conundrum. Nova Science Publishers, Inc.

10. **Wassenaar TM, Newell DG.** 2006. The Genus *Campylobacter*, vol 7. Springer New York.

11. **Nachamkin I, Yang XH, Stern NJ.** 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks analysis with flagellar mutants. Appl Environ Microbiol. **59**.

12. **Bullman S, Corcoran D, O'Leary J, Lucey B, Byrne D, Sleator RD.** 2011. *Campylobacter ureolyticus*: an emerging gastrointestinal pathogen? FEMS Immunol Med Microbiol **61:**228-230.

13. **Vandamme P, Goossens H.** 1992. Taxonomy of *Campylobacter*, *Arcobacter*, and *Helicobacter*: a review. Zentralbl Bakteriol **276:**447-472.

14. **Rossi M, Debruyne L, Zanoni RG, Manfreda G, Revez J, Vandamme P.** 2009. *Campylobacter avium* sp. nov., a hippurate-positive species isolated from poultry. Int J Syst Evol Microbiol **59:**2364-2369.

15. **Hwang M-N, Ederer GM.** 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. J Clin Microbiol **1:**114-115.

16. **Walder M, Sandstedt K, Ursingt J.** 1983. Phenotypic characteristics of thermotolerant *Campylobacter* from human and animal sources. Curr Microbiol **9:**291-296.

17. **Park SF.** 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. Int J Food Microbiol **74:**177-188.

18. **FDA BAM.** 1998. Bacteriological Analytical Manual. Chapter 7, *Campylobacter*, 8th ed.

19. **World Organisation for Animal Health (OIE).** 2008. *Campylobacter jejuni* and *Campylobacter coli*. OIE Terrestrial Manual 2008.

20. **Guevara CLd, Pérez-Pomata MT, Agulla A, Merino FJ, Villasante PA, Velasco AC.** 1989. Recovery of *Campylobacter* from human faeces stored at 4 °C. Epidemiol Infect **102:**281-285.

21. **Cox NA, Richardson LJ, Buhr RJ, Northcutt JK, Bailey JS, Cray PF, Hiett KL.** 2007. Recovery of *Campylobacter* and *Salmonella* serovars from the spleen, liver and gallbladder, and ceca of six-and eight-week-old commercial broilers. J Appl Poult Res **16:**477-480.

22. **Shinha T.** 2015. Fatal Bacteremia Caused by *Campylobacter gracilis*, United States. Emerg Infect Diseases **21:**1084-1085.

23. **Etoh Y, Dewhirst FE, Paster BJ, Yamamoto A, Goto N.** 1993. *Campylobacter showae* sp. nov., isolated from the human oral cavity. Int J Syst Bacteriol **43:**631-639.

24. **Kittl S, Heckel G, Korczak BM, Kuhnert P.** 2013. Source attribution of human *Campylobacter* isolates by MLST and fla-typing and association of genotypes with quinolone resistance. PLoS One **8:**e81796.

25. **Mughini Gras L, Smid JH, Wagenaar JA, de Boer AG, Havelaar AH, Friesema IH, French NP, Busani L, van Pelt W.** 2012. Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. PLoS One **7:**e42599.

26. **French NP, Midwinter A, Holland B, Collins-Emerson J, Pattison R, Colles F, Carter P.** 2009. Molecular epidemiology of *Campylobacter jejuni* isolates from wild-bird fecal material in children's playgrounds. Appl Environ Microbiol **75:**779-783.

27. **Newell DG.** 2002. The ecology of *Campylobacter jejuni* in avain and human hosts and in the environment. Int J Infect Dis **6:**S16-S21.

28. **Oliver JD.** 2005. The viable but nonculturable state in bacteria. J Microbiol **43:**93-100.

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

30. **Kwan PS, Barrigas M, Bolton FJ, French NP, Gowland P, Kemp R, Leatherbarrow H, Upton M, Fox AJ.** 2008. Molecular epidemiology of *Campylobacter jejuni* populations in dairy cattle, wildlife, and the environment in a farmland area. Appl Environ Microbiol **74:**5130-5138.

31. **Williams A, Oyarzabal OA.** 2012. Prevalence of *Campylobacter* spp. in skinless, boneless retail broiler meat from 2005 through 2011 in Alabama, USA. BMC Microbiol **12**.

32. **Scott W, Scott H, Lake R, Baker MG.** 2000. Economic cost to New Zealand of foodborne infectious disease. New Zeal Med J **113:**281-284.

33. **Lake R, Adlam B, Perera S.** 2009. Acute gastrointestinal illness (AGI) study: final study report. Institute of Environmental Science and Research (ESR), New Zealand.

34. **Mullner P, Spencer SE, Wilson DJ, Jones G, Noble AD, Midwinter AC, Collins-Emerson JM, Carter P, Hathaway S, French NP.** 2009. Assigning the source of human campylobacteriosis in New Zealand: a comparative genetic and epidemiological approach. Infect Genet Evol **9:**1311-1319.

35. **Sheppard SK, Colles F, Richardson J, Cody AJ, Elson R, Lawson A, Brick G, Meldrum R, Little CL, Owen RJ, Maiden MC, McCarthy ND.** 2010. Host association of *Campylobacter* genotypes transcends geographic variation. Appl Environ Microbiol **76:**5269-5277.

36. **Colles FM, Ali JS, Sheppard SK, McCarthy ND, Maiden MC.** 2011. *Campylobacter* populations in wild and domesticated Mallard ducks (Anas platyrhynchos). Environ Microbiol Rep **3:**574-580.

37. **Black R, Levine M, Clements M, Hughes T, Blaser M.** 1988. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis **157:**472-479.

38. **Medema GJ, Teunis PF, Havelaar AH, CN H.** 1996. Assessment of the dose-response relationship of *Campylobacter jejuni*. Int J Food Microbiol **30:**101-111.

39. **Ang CW, De Klerk MA, Endtz HP, Jacobs BC, Laman JD, van der Meche FG, van Doorn PA.** 2001. Guillain-Barre syndrome- and Miller Fisher syndrome-associated *Campylobacter jejuni* lipopolysaccharides induce anti-GM1 and anti-GQ1b antibodies in rabbits. Infect Immun **69:**2462-2469.

40. **Jacobs BC, Rothbarth PH, van der Meché FG, Herbrink P, Schmitz PI, de Klerk MA, van Doorn PA.** 1998. The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. Neurology **51:**1110-1115.

41. **Rees JH, Soudain SE, Gregson NA, Hughes RAC.** 1995. *Campylobacter jejuni* infection and Guillain-Barré syndrome. N Engl J Med **333:**1374-1379.

42. **Allos BM.** 1997. Association between *Campylobacter i*nfection and Guillain-Barre´ Syndrome. J Infect Dis **176:**S125–128.

43. **Peterson MC.** 1994. Rheumatic manifestations of *Campylobacter jejuni* and *C. fetus* infections in adults. Scand J Rheumatol **23:**167-170.

44. **Strachan NJ, Rotariu O, MacRae M, Sheppard SK, Smith-Palmer A, Cowden J, Maiden MC, Forbes KJ.** 2013. Operationalising factors that explain the emergence of infectious diseases: a case study of the human campylobacteriosis epidemic. PLoS One **8:**e79331.

45. **Levesque S, Fournier E, Carrier N, Frost E, Arbeit RD, Michaud S.** 2013. Campylobacteriosis in urban versus rural areas: a case-case study integrated with molecular typing to validate risk factors and to attribute sources of infection. PLoS One **8:**e83731.

46. **Stafford R, Tenkate T, McCall B.** 1996. A five year review of *Campylobacter* infection in Queensland. Comm Dis Intell **20:**478-482.

47. **S. Yan, Pendrak ML, Foley SL, Powers JH.** 2005. *Campylobacter* infection and Guillain–Barré syndrome: public health concerns from a microbial food safety perspective. Clin Appl Immunol Rev **5:**285-305.

48. **Gallay A, Prouzet-Mauléion V, Kempf I, Lehours P, Labadi L, Camou C, Denis M, Valk Hd, Desenclos J-C, Mégraud F.** 2007. *Campylobacter* antimicrobial drug resistance among humans, broiler chickens, and pigs, France. Emerg Infect Dis **13**.

49. **WHO.** 2015. WHO estimates of the global burden of foodborne diseases. WHO estimates of the global burden of foodborne diseases.

50. **Batz MB, Hoffmann S, Morris JG, Jr.** 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. J Food Prot **75:**1278-1291.

51. **CDC.** 2015. Preliminary incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. Sites, 2006–2014. CDC, USA.

52. **Hoffmann S, Batz MB, Morris JG, Jr.** 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot **75:**1292-1302.

53. **Arsenault J, Berke O, Michel P, Ravel A, Gosselin P.** 2012. Environmental and demographic risk factors for campylobacteriosis: do various geographical scales tell the same story? BMC Infect Dis **12:**318.

54. **Keegan VA, Majowicz SE, Pearl DL, Marshall BJ, Sittler N, Knowles L, Wilson JB.** 2009. Epidemiology of enteric disease in C-EnterNet's pilot site – Waterloo region, Ontario, 1990 to 2004. Can J Infect Dis Med Microbiol **20:**79-87.

55. **Benoit SR, Lopez B, Arvelo W, Henao O, Parsons MB, Reyes L, Moir JC, Lindblade K.** 2014. Burden of laboratory-confirmed *Campylobacter* infections in Guatemala 2008-2012: results from a facility-based surveillance system. J Epidemiol Glob Health **4:**51-59.

56. **Workman SN, Sobers SJ, Mathison GE, Lavoie MC.** 2006. Human *Campylobacter*-associated enteritis on the Caribbean island of Barbados. Am J Trop Med Hyg **74:**623-627.

57. **Kaakoush NO, Castano-Rodriguez N, Mitchell HM, Man SM.** 2015. Global Epidemiology of *Campylobacter* Infection. Clin Microbiol Rev **28:**687-720.

58. **Havelaar AH, Ivarsson S, Lofdahl M, Nauta MJ.** 2013. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. Epidemiol Infect **141:**293-302.

59. **Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, Gray JJ, Letley LH, Rait G, Tompkins DS, O'Brien SJ.** 2012. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut **61:**69-77.

60. **Hauri AM, Just M, McFarland S, Schweigmann A, Schlez K, J. K.** 2013. Campylobacteriosis outbreaks in the state of Hesse, Germany, 2005-2011: raw milk yet again. Dtsch Med Wochenschr **138:**357-361.

61. **Stingl K, Knuver MT, Vogt P, Buhler C, Kruger NJ, Alt K, Tenhagen BA, Hartung M, Schroeter A, Ellerbroek L, Appel B, Kasbohrer A.** 2012. Quo vadis? - Monitoring *Campylobacter* in Germany. Eur J Microbiol Immunol (Bp) **2:**88-96.

62. **Sadkowska-Todys M, Kucharczyk B.** 2012. Campylobacteriosis in Poland in 2010. Przegl Epidemiol **66:**255-258.

63. **Bouwknegt M, van Pelt W, Havelaar AH.** 2013. Scoping the impact of changes in population age-structure on the future burden of foodborne disease in the Netherlands, 2020-2060. Int J Environ Res Public Health **10:**2888-2896.

64. **Havelaar AH, Nauta MJ, Mangen M-JJ, Koeijer AGd, Bogaardt M-J, Evers EG, Jacobs-Reitsma WF, van Pelt W, Wagenaar JA, de Wit GA, van der Ze H.** 2005. Costs and benefits of controlling *Campylobacter* in the Netherlands—integrating risk analysis, epidemiology and economics. National Institute for Public Health and the Environment (RIVM), Bilthoven, Netherlands.

65. **Wang J, Guo YC, Li N.** 2013. Prevalence and risk assessment of *Campylobacter jejuni* in chicken in China. Biomed Environ Sci **26:**243-248.

66. **Kubota K, Kasuga F, Iwasaki E, Inagaki S, Sakurai Y, Komatsu M, Toyofuku H, Angulo FJ, Scallan E, Morikawa K.** 2011. Estimating the burden of acute gastroenteritis and foodborne illness caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* by using population-based telephone survey data, Miyagi Prefecture, Japan, 2005 to 2006. J Food Prot **74:**1592-1598.

67. **Weinberger M, Lerner L, Valinsky L, Moran-Gilad J, Nissan I, Agmon V, Peretz C.** 2013. Increased incidence of *Campylobacter* spp. infection and high rates among children, Israel. Emerg Infect Dis **19:**1828-1831.

68. **Harrison D, Corry JE, Tchorzewska MA, Morris VK, Hutchison ML.** 2013. Freezing as an intervention to reduce the numbers of *campylobacters* isolated from chicken livers. Lett Appl Microbiol **57:**206-213.

69. **Georgsson F, Thornorkelsson AE, Geirsdottir M, Reiersen J, Stern NJ.** 2006. The influence of freezing and duration of storage on *Campylobacter* and indicator bacteria in broiler carcasses. Food Microbiol **23:**677-683.

70. **OzFoodNet Working Group.** 2012. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2010. Commun Dis Intell Q Rep **36:**E213-E241.

71. **OzFoodNet Working Group.** 2010. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2009. Commun Dis Intell Q Rep **34:**396-426.

72. **OzFoodNet Working Group.** 2009. Monitoring the incidence and causes of diseases potentially transmitted by food in Australia: annual report of the OzFoodNet network, 2008. Commun Dis Intell Q Rep **33:**389-413.

73. **ESR.** 2014. Notifiable diseases in New Zealand: annual report 2014. Institute of Environmental Science and Research Ltd, Porirua, New Zealand.

74. **Statistics New Zealand.** 2013. Number of people counted. http://www.stats.govt.nz/Census/2013-census/profile-and-summary-reports/quickstats-about-a-place.aspx?request_value=14263&parent_id=14181&tabname=&gclid=CI_Go_-I2cYCFUtwvAodd44Peg#14263. Accessed

75. **Manawatu-Wanganui Regional Council.** 2013. Community statistics as at the 2013 census. http://www.localcouncils.govt.nz/lgip.nsf/wpg_url/Profiles-Councils-Manawatu-Wanganui-Regional-Council-main. Accessed

76. **Bolwell CF, Gilpin BJ, Campbell D, French NP.** 2015. Evaluation of the representativeness of a sentinel surveillance site for campylobacteriosis. Epidemiol Infect **143:**1990-2002.

77. **Mayr E.** 1982. Speciation and macroevolution. Evol **36:**1119-1132.

78. **Wanscher JH.** 1975. The history of Wilhelm Johannsen's genetical terms and concepts from the period 1903 to 1926. Centaurus **19:**125-147.

79. **Fitzgerald C, Helsel LO, Nicholson MA, Olsen SJ, Swerdlow DL, Flahart R, Sexton J, Fields PI.** 2001. Evaluation of methods for subtyping *Campylobacter jejuni* during an outbreak involving a food handler. J Clin Microbiol **39:**2386-2390.

80. **Jackson CJ, Fox AJ, Wareing DRA, Hutchinson DN, Jones DM.** 1996. The application of genotyping techniques to the epidemiological analysis of *Campylobacter jejuni*. Epidemiol Infect **117:**233-244.

81. **On SLW.** 2013. Isolation, identification and subtyping of *Campylobacter*: where to from here? J Microbiol Methods **95:**3-7.

82. **Klena J.** 2001. A survey of phenotypic and genetic methods used to identify and differentiate thermotolerant *Campylobacter* spp. strains. A report to the Ministry of Health, New Zealand.

83. **Bolton FJ, Holt AV, Hutchinson DN.** 1984. *Campylobacter* biotyping scheme of epidemiological value. J Clin Pathol **37:**677-681.

84. **Steele M, McNab B, Fruhner L, DeGrandis S, Woodward D, Odumeru JA.** 1998. Epidemiological typing of *Campylobacter* isolates from meat processing

plants by pulsed-field gel electrophoresis, fatty acid profile typing, serotyping, and biotyping. Appl Environ Microbiol **64:**2346-2349.

85. **Penner JL, Hennessy JN.** 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J Clin Microbiol **12:**732-737.

86. **Lior H, Woodward DL, Edgar JA, Laroche LJ, Gill P.** 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J Clin Microbiol **15:**761-768.

87. **Leonard EE, Takata T, Blaser MJ, Falkow S, Tompkins LS, Gaynor EC.** 2003. Use of an open-reading frame–specific *Campylobacter jejuni* DNA microarray as a new genotyping tool for studying epidemiologically related isolates. J Infect Dis **187:**691-694.

88. **Woodward DL, Rodgers FG.** 2002. Identification of *Campylobacter* Heat-Stable and Heat-Labile antigens by combining the Penner and Lior serotyping schemes. J Clin Microbiol **40:**741-745.

89. **Patton CM, Nicholson MA, Ostroff SM, Ries AA, Wachsmuth IK, Tauxe RV.** 1993. Common somatic O and heat-labile serotypes among *Campylobacter* strains from sporadic infections in the United States. J Clin Microbiol **31:**1525-1530.

90. **Wilson GS, Atkinson D.** 1945. Typing of *Staphylococci* by the bacteriophage method. Lancet **1:**647-649.

91. **Ward LR, de Sa JDH, Rowe B.** 1987. A phage-typing scheme for *Salmonella enteritidis*. Epidemiol Infect **99:**291-294.

92. **Coward C, Grant AJ, Swift C, Philp J, Towler R, Heydarian M, Frost JA, Maskell DJ.** 2006. Phase-variable surface structures are required for infection of

Campylobacter jejuni by bacteriophages. Appl Environ Microbiol **72:**4638-4647.

93. **Li W, Raoult D, Fournier PE.** 2009. Bacterial strain typing in the genomic era. FEMS Microbiol Rev **33:**892-916.

94. **Stucki U, Frey J, Nicolet J, Burnens AP.** 1995. Identification of *Campylobacter jejuni* on the basis of a species-specific gene that encodes a membrane protein. J Clin Microbiol **33:**855-859.

95. **On SLW, Brandt SM, Cornelius AJ, Fusco V, Quero GM, Maćkiw E, Hout K, Bilbao A, Diaz AI, Benejat L, Megraud F, Collins-Emerson J, French NP, Gotcheva V, Angelov A, Alakomi HL, Saarela M, Paulin SM.** 2013. PCR revisited: a case for revalidation of PCR assays for microorganisms using identification of *Campylobacter* species as an exemplar. Qual Assur Saf Crop **5:**49-62.

96. **Gonzalez I, Grant KA, Richardson PT, Park SF, Collins MD.** 1997. Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceu*E gene encoding a putative virulence determinant. J Clin Microbiol **35:**759-763.

97. **Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, Woodward DL, Rodgers FG.** 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J Clin Microbiol **40:**4744-4747.

98. **Nassonova ES.** 2008. Pulsed field gel electrophoresis: Theory, instruments and application. Cell tissue biol **2:**557-565.

99. **De Boer P, Duim B, Rigter A, van Der Plas J, Jacobs-Reitsma WF, Wagenaar JA.** 2000. Computer-assisted analysis and epidemiological value of

genotyping methods for *Campylobacter jejuni* and *Campylobacter coli*. J Clin Microbiol **38:**1940-1946.

100. **Newell DG, Frost JA, Duim B, Wagenaar JA, Madden RH, van Der Plas J, On SLW.** 2000. New developments in the subtyping of *Campylobacter* species. American Society for Microbiology, Washington.

101. **Urwin R, Maiden MCJ.** 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol **11:**479-487.

102. **Sullivan CB, Diggle MA, Clarke SC.** 2005. Multilocus sequence typing data analysis in clinical microbiology and public health. Mol Biotechnol **29:**245-254.

103. **Colles FM, Jones K, Harding RM, Maiden MCJ.** 2003. Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Appl Environ Microbiol **69:**7409-7413.

104. **Dingle KE, Colles FM, Wareing DR, Ure R, Fox AJ, Bolton FE, Bootsma HJ, Willems RJ, Urwin R, Maiden MC.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J Clin Microbiol **39:**14-23.

105. **Baker M, Wilson N, McIntyre M, McLean M.** 2005. Findings and methodological lessons from a small case-control study into campylobacteriosis in Wellington. N Z Med J **118:**U1622.

106. **Eberhart-Phillips J, Walker N, Garrett N, Bell D, Sinclair D, Rainger W, Bates M.** 1997. Campylobacteriosis in New Zealand: results of a case-control study. J Epidemiol Community Health **51:**686-691.

107. **Ikram R, Chambers S, Mitchell P, Brieseman MA, Ikam OH.** 1994. A case control study to determine risk factors for *Campylobacter* infection in Christchurch in the summer of 1992-3. N Z Med J **107:**430-432.

108. **Domingues AR, Pires SM, Halasa T, Hald T.** 2012. Source attribution of human campylobacteriosis using a meta-analysis of case-control studies of sporadic infections. Epidemiol Infect **140:**970-981.

109. **Doorduyn Y, Van Den Brandhof WE, Van Duynhoven YT, Breukink BJ, Wagenaar JA, Van Pelt W.** 2010. Risk factors for indigenous *Campylobacter jejuni* and *Campylobacter coli* infections in the Netherlands: a case-control study. Epidemiol Infect **138:**1391-1404.

110. **Sheppard SK, Dallas JF, Strachan NJ, MacRae M, McCarthy ND, Wilson DJ, Gormley FJ, Falush D, Ogden ID, Maiden MC, Forbes KJ.** 2009. *Campylobacter* genotyping to determine the source of human infection. Clin Infect Dis **48:**1072-1078.

111. **Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Fearnhead P, Hart CA, Diggle PJ.** 2008. Tracing the source of campylobacteriosis. PLoS Genet **4:**e1000203.

112. **Haan CPd, Kivistö RI, Hakkinen M, Corander J, Hänninen M-L.** 2010. MLST of Finnish bovine *Campylobacter jejuni* isolates and their attribution to human infections. BMC Microbiol **10**.

113. **Strachan NJ, Gormley FJ, Rotariu O, Ogden ID, Miller G, Dunn GM, Sheppard SK, Dallas JF, Reid TM, Howie H, Maiden MC, Forbes KJ.** 2009. Attribution of *Campylobacter* infections in northeast Scotland to specific sources by use of multilocus sequence typing. J Infect Dis **199:**1205-1208.

114. **Gilpin BJ, Scholes P, Robson B, Savill MG.** 2008. The transmission of thermotolerant *Campylobacter* spp. to people living or working on dairy farms in New Zealand. Zoonoses Public Health **55:**352-360.

115. **Rosenquist H, Nielsen NL, Sommer HM, Nørrung B, Christensen BB.** 2003. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. Int J Food Microbiol **83:**87-103.

116. **Kramer JM, Frost JA, Bolton FJ, Wareing DR.** 2000. *Campylobacter* contamination of raw meat and poultry at retail sale: identification of multiple types and comparison with isolates from human infection. J Food Prot **63:**1654-1659.

117. **Mullner P, Collins-Emerson JM, Midwinter AC, Carter P, Spencer SE, van der Logt P, Hathaway S, French NP.** 2010. Molecular epidemiology of *Campylobacter jejuni* in a geographically isolated country with a uniquely structured poultry industry. Appl Environ Microbiol **76:**2145-2154.

118. **Sears A, Baker MG, Wilson N, Marshall J, Muellner P, Campbell DM, Lake RJ, French NP.** 2011. Marked campylobacteriosis decline after interventions aimed at poultry, New Zealand. Emerg Infect Dis **17:**1007-1015.

119. **Rosef O, Kapperud G, Lauwers S, Gondrosen B.** 1985. Serotyping of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis* from domestic and wild animals. Appl Environ Microbiol **49:**1507-1510.

120. **Garrett N, Devane ML, Hudson JA, Nicol C, Ball A, Klena JD, Scholes P, Baker MG, Gilpin BJ, Savill MG.** 2007. Statistical comparison of *Campylobacter jejuni* subtypes from human cases and environmental sources. J Appl Microbiol **103:**2113-2121.

121. **Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M.** 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol **60:**1136-1151.

122. **Wright S.** 1931. Evolution in mendelian populations. Genetics **16:**97-159.

123. **Mullner P, Jones G, Noble A, Spencer SE, Hathaway S, French NP.** 2009. Source attribution of food-borne zoonoses in New Zealand: a modified Hald model. Risk Anal **29:**970-984.

124. **Skarp CP, Hanninen ML, Rautelin HI.** 2016. Campylobacteriosis: the role of poultry meat. Clin Microbiol Infect **22:**103-109.

125. **Lawes JR, Vidal A, Clifton-Hadley FA, Sayers R, Rodgers J, Snow L, Evans SJ, Powell LF.** 2012. Investigation of prevalence and risk factors for *Campylobacter* in broiler flocks at slaughter: results from a UK survey. Epidemiol Infect **140:**1725-1737.

126. **Agunos A, Waddell L, Leger D, Taboada E.** 2014. A systematic review characterizing on-farm sources of *Campylobacter* spp. for broiler chickens. PLoS One **9:**e104905.

127. **Siemer BL, Nielsen EM, On SL.** 2005. Identification and molecular epidemiology of *Campylobacter coli* isolates from human gastroenteritis, food, and animal sources by amplified fragment length polymorphism analysis and Penner serotyping. Appl Environ Microbiol **71:**1953-1958.

128. **Strachan NJ, MacRae M, Thomson A, Rotariu O, Ogden ID, Forbes KJ.** 2012. Source attribution, prevalence and enumeration of *Campylobacter* spp. from retail liver. Int J Food Microbiol **153:**234-236.

129. **Moore JE, Barton MD, Blair IS, Corcoran D, Dooley JS, Fanning S, Kempf I, Lastovica AJ, Lowery CJ, Matsuda M, McDowell DA, McMahon A, Millar BC, Rao JR, Rooney PJ, Seal BS, Snelling WJ, Tolba O.** 2006. The epidemiology of antibiotic resistance in *Campylobacter*. Microbes Infect **8:**1955-1966.

130. **Baker M, Kvalsvig A, Zhang J, Lake R, Sears A, Wilson N.** 2012. Declining Guillain-Barre syndrome after campylobacteriosis control, New Zealand, 1988-2010. Emerg Infect Dis **18:**226-233.

131. **Denis M, Refrégier-Petton J, Laisney M-J, Ermel G, Salvat G.** 2001. *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and *Camp. coli*. J Appl Microbiol **91:**255-267.

132. **Parikh R, Mathai A, Parikh S, Sekhar GC, Thomas R.** 2008. Understanding and using sensitivity, specificity and predictive values. Indian J Ophthalmol **56**.

133. **Newcombe R.** 1998. Two-sided confidence intervals for the single proportion comparison of seven methods. Stat Med **17:**857-872.

134. **Anderson MJ.** 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol **26:**32-46.

135. **McArdle BH, Anderson J.** 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecol **82:**290-297.

136. **Anderson MJ, Gorley RN, Clarke KR.** 2008. PERMANOVA+ for PRIMER: Guide to software and statistical methods. Plymouth.

137. **Nylen G, Dunstan F, Palmer SR, Andersson Y, Bager F, Cowden J, Feierl G, Galloway Y, Kapperud G, Megraud F, Molbak K, Petersen LR, Ruutu P.** 2002. The seasonal distribution of *Campylobacter* infection in nine European countries and New Zealand. Epidemiol Infect **128**.

138. **Schielke A, Rosner BM, Stark K.** 2014. Epidemiology of campylobacteriosis in Germany – insights from 10 years of surveillance. BMC Infect Dis **14**.

139. **Hearnden M, Skelly C, Eyles R, Weinstein P.** 2003. The regionality of campylobacteriosis seasonality in New Zealand. Int J Environ Res Publ **13:**337-348.

140. **Tam CC, O'Brien SJ, Adak GK, Meakins SM, Frost JA.** 2003. *Campylobacter coli*—an important foodborne pathogen. J Infect **47:**28-32.

141. **Gillespie IA, O'Brien SJ, Frost JA, Adak GK, Horby P, Swan AV, Painter MJ, Neal KR.** 2002. A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. Emerg Infect Dis **8**.

142. **Baker M, Sneyd E, Wilson NA.** 2007. Is the major increase in notified campylobacteriosis in New Zealand real? Epidemiol Infect **135:**163-170.

143. **Eberhart-Phillips J, Walker N, Garrett N, Bell D, Sinclair D, Rainger W, Bates M.** 1997. Campylobacteriosis in New Zealand results of a case-control study. J Epidemiol Community Health **51:**686-691.

144. **Wong TL, Hollis L, Cornelius A, Nicol C, Cook R, Hudson JA.** 2007. Prevalence, numbers, and subtypes of *Campylobacter jejuni* and *Campylobacter coli* in uncooked retail meat samples. J Food Prot **70:**566-573.

145. **Habib I, Sampers I, Uyttendaele M, Berkvens D, De Zutter L.** 2008. Baseline data from a Belgium-wide survey of *Campylobacter* species contamination in chicken meat preparations and considerations for a reliable monitoring program. Appl Environ Microbiol **74:**5483-5489.

146. **Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. Appl Environ Microbiol **67:**5431-5436.

147. **Baker M, Wilson N, Ikram R, Chambers S, Shoemack P, Cook G.** 2006. Regulation of chicken contamination is urgently needed to control New Zealand's serious campylobacteriosis epidemic. New Zeal Med J **119**.

148. **Lake R, Cressey P.** 2013. Risk profile: *Campylobacter jejuni*/*coli* in poultry (whole and pieces). Institute of Environmental Science and Research, New Zealand.

149. **Sasaki Y, Maruyama N, Zou B, Haruna M, Kusukawa M, Murakami M, Asai T, Tsujiyama Y, Yamada Y.** 2013. *Campylobacter* cross-contamination of chicken products at an abattoir. Zoonoses Public Health **60:**134-140.

150. **Inglis GD, Kalischuk LD.** 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl Environ Microbiol **69:**3435-3447.

151. **Hammer Ø, Harper DAT, Ryan PD.** 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron **4:**9.

152. **R Development Core Team.** 2005. R: A language and environment for statistical computing. Accessed

153. **Feinsinger P, Spears EE, Poole RW.** 1981. A simple measure of niche breadth. Ecol **62:**27-32.

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

155. **New Zealand Food Safety Authority.** 2008. *Campylobacter* Risk Management Strategy 2008-2011. http://www.foodsafety.govt.nz/elibrary/industry/Campylobacter_Risk-Aims_Acheive.pdf. Accessed 01/11/2016.

156. **ESR.** 2006. Notifiable and other diseases in New Zealand: annual report 2006. Institute of Environmental Science and Research Ltd, Porirua, New Zealand.

157. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM.** 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis **17:**7-15.

158. **Food Standards Agency.** 2000. A report of the study of infectious intestinal disease in England. London: The Stationery Office, England.

159. **CDC.** 2012. National antimicrobial resistance system, enteric bacteria, human isolates final report 2010. CDC, Atlanta, Georgia: U.S. Department of Health and Human Services, USA.

160. **Buzby J, Roberts T.** 1997. Economic costs and trade impacts of microbial foodborne illness. World Health Stat Q **50:**57-66.

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

162. **Dingle KE, Colles FM, Falush D, Maiden MC.** 2005. Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J Clin Microbiol **43:**340-347.

163. **Roux F, Sproston E, Rotariu O, Macrae M, Sheppard SK, Bessell P, Smith-Palmer A, Cowden J, Maiden MC, Forbes KJ, Strachan NJ.** 2013. Elucidating the aetiology of human *Campylobacter coli* infections. PLoS One **8**.

164. **Litrup E, Torpdahl M, Nielsen EM.** 2007. Multilocus sequence typing performed on *Campylobacter coli* isolates from humans, broilers, pigs and cattle originating in Denmark. J Appl Microbiol **103:**210-218.

165. **Sheppard SK, Didelot X, Jolley KA, Darling AE, Pascoe B, Meric G, Kelly DJ, Cody A, Colles FM, Strachan NJ, Ogden ID, Forbes K, French NP,**

Carter P, Miller WG, McCarthy ND, Owen R, Litrup E, Egholm M, Affourtit JP, Bentley SD, Parkhill J, Maiden MC, Falush D. 2013. Progressive genome-wide introgression in agricultural *Campylobacter coli*. Mol Ecol **22:**1051-1064.

166. **Sheppard SK, McCarthy ND, Falush D, Maiden MCJ.** 2008. Convergence of *Campylobacter* species: implications for bacterial evolution. Science **320:**237-239.

167. **Denis M, Soumet C, Rivoal K, Ermel G, Blivet D, Salvat G, Colin P.** 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett Appl Microbiol **29:**406-410.

168. **Kimura M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16:**111-120.

169. **Gascuel O.** 1997. BIONJ an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol **14:**685-695.

170. **Sheppard SK, Dallas JF, Wilson DJ, Strachan NJC, McCarthy ND, Jolley KA, Colles FM, Rotariu O, Ogden ID, Forbes KJ, Maiden MCJ.** 2010. Evolution of an agriculture-associated disease causing *Campylobacter coli* clade: evidence from national surveillance data in Scotland. PLoS One **5**.

171. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol **30:**2725-2729.

172. **Mughini-Gras L, Smid JH, Wagenaar JA, A DEB, Havelaar AH, Friesema IH, French NP, Graziani C, Busani L, Van Pelt W.** 2014. Campylobacteriosis

in returning travellers and potential secondary transmission of exotic strains. Epidemiol Infect **142:**1277-1288.

173. **Miller WG, Englen MD, Kathariou S, Wesley IV, Wang G, Pittenger-Alley L, Siletz RM, Muraoka W, Fedorka-Cray PJ, Mandrell RE.** 2006. Identification of host-associated alleles by multilocus sequence typing of *Campylobacter coli* strains from food animals. Microbiology **152:**245-255.

174. **Wright SL, Carver DK, Siletzky RM, Romine S, Morrow WEM, Kathariou S.** 2008. Longitudinal study of prevalence of *Campylobacter jejuni* and *Campylobacter coli* from turkeys and swine grown in close proximity. J Food Prot **71:**1791-1796.

175. **Cody AJ, McCarthy NM, Wimalarathna HL, Colles FM, Clark L, Bowler IC, Maiden MC, Dingle KE.** 2012. A longitudinal 6-year study of the molecular epidemiology of clinical *Campylobacter* isolates in Oxfordshire, United Kingdom. J Clin Microbiol **50:**3193-3201.

176. **Lake RJ, Adlam SB, Perera S, Campbell DM, Baker MG.** 2010. The disease pyramid for acute gastrointestinal illness in New Zealand. Epidemiol Infect **138:**1468-1471.

177. **Sopwith W, Birtles A, Matthews M, Fox A, Gee S, James S, Kempster J, Painter M, Edwards-Jones V, Osborn K, Regan M, Syed Q, Bolton E.** 2010. Investigation of food and environmental exposures relating to the epidemiology of *Campylobacter coli* in humans in Northwest England. Appl Environ Microbiol **76:**129-135.

178. **Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Hart CA, Diggle PJ, Fearnhead P.** 2009. Rapid evolution and the

importance of recombination to the gastroenteric pathogen *Campylobacter jejuni*. Mol Biol Evol **26:**385-397.

179. **Nohra A, Grinberg A, Midwinter AC, Marshall JC, Collins-Emerson JM, French NP.** 2016. Molecular epidemiology of *Campylobacter coli* isolated from different sources in New Zealand between 2005 and 2014. Appl Environ Microbiol **82:**4363-4370.

180. **Friedman CJ, Neiman J, Wegener HC, Tauxe RV.** 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialised nations. ASM Press, Washington, D.C.

181. **Kapperud G.** 2003. Factors associated with increased and decreased risk of *Campylobacter* infection: a prospective case-control study in Norway. Am J Epidemiol **158:**234-242.

182. **Muellner P, Marshall JC, Spencer SE, Noble AD, Shadbolt T, Collins-Emerson JM, Midwinter AC, Carter PE, Pirie R, Wilson DJ, Campbell DM, Stevenson MA, French NP.** 2011. Utilizing a combination of molecular and spatial tools to assess the effect of a public health intervention. Prev Vet Med **102:**242-253.

183. **Stern NJ, Line JE.** 1992. Comparison of three methods for recovery of *Campylobacter* spp. from broiler carcasses. J Food Prot **55:**663-666.

184. **French N.** 2008. Enhancing surveillance of potentially foodborne enteric diseases in New Zealand: human campylobacteriosis in the Manawatu. Institute of Veterinary, Animal and Biomedical Sciences, New Zealand, New Zealand.

185. **Wagenaar JA, Mevius DJ, Havelaar AH.** 2006. *Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis. Rev Sci Tech Int OFF Epiz **25:**581-594.

186. **Gormley FJ, Macrae M, Forbes KJ, Ogden ID, Dallas JF, Strachan NJ.** 2008. Has retail chicken played a role in the decline of human campylobacteriosis? Appl Environ Microbiol **74:**383-390.

187. **Mossong J, Mughini-Gras L, Penny C, Devaux A, Olinger C, Losch S, Cauchie HM, van Pelt W, Ragimbeau C.** 2016. Human campylobacteriosis in Luxembourg, 2010-2013: A case-control study combined with multilocus sequence typing for source attribution and risk factor analysis. Sci Rep **6:**20939.

188. **Boysen L, Rosenquist H, Larsson JT, Nielsen EM, Sorensen G, Nordentoft S, Hald T.** 2014. Source attribution of human campylobacteriosis in Denmark. Epidemiol Infect **142:**1599-1608.

189. **Jaros P, Cookson AL, Campbell DM, Besser TE, Shringi S, Mackereth GF, Lim E, Lopez L, Dufour M, Marshall JC, Baker MG, Hathaway S, Prattley DJ, French NP.** 2013. A prospective case–control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. BMC Infect Dis **13**.

190. **Bessede E, Lehours P, Labadi L, Bakiri S, Megraud F.** 2014. Comparison of characteristics of patients infected by *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter fetus*. J Clin Microbiol **52:**328-330.

191. **Murphy C, Carroll C, Jordan KN.** 2006. Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. J Appl Microbiol **100:**623-632.

192. **Marshall J, Wilkinson D, French N.** 2016. Completion of sequence typing of human and poultry isolates and source attribution modelling. Institute of Veterinary, Animal and Biomedical Sciences, New Zealand.

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Molecular Epidemiology of *Campylobacter coli* Strains Isolated from Different Sources in New Zealand between 2005 and 2014

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ABSTRACT

Campylobacteriosis is one of the most important foodborne diseases worldwide and a significant health burden in New Zealand. *Campylobacter jejuni* is the predominant species worldwide, accounting for approximately 90% of human cases, followed by *Campylobacter coli*. Most studies in New Zealand have focused on *C. jejuni*; hence, the impact of *C. coli* strains on human health is not well understood. The aim of this study was to genotype *C. coli* isolates collected in the Manawatu region of New Zealand from clinical cases, fresh poultry meat, ruminant feces, and environmental water sources, between 2005 and 2014, to study their population structure and estimate the contribution of each source to the burden of human disease. *Campylobacter* isolates were identified by PCR and typed by multilocus sequence typing. *C. coli* accounted for 2.9% ($n = 47/1,601$) of *Campylobacter* isolates from human clinical cases, 9.6% ($n = 108/1,123$) from poultry, 13.4% ($n = 49/364$) from ruminants, and 6.4% ($n = 11/171$) from water. Molecular subtyping revealed 27 different sequence types (STs), of which 18 belonged to clonal complex ST-828. ST-1581 was the most prevalent *C. coli* sequence type isolated from both human cases ($n = 12/47$) and poultry ($n = 44/110$). When classified using cladistics, all sequence types belonged to clade 1 except ST-7774, which belonged to clade 2. ST-854, ST-1590, and ST-4009 were isolated only from human cases and fresh poultry, while ST-3232 was isolated only from human cases and ruminant sources. Modeling indicated ruminants and poultry as the main sources of *C. coli* human infection.

IMPORTANCE

We performed a molecular epidemiological study of *Campylobacter coli* infection in New Zealand, one of few such studies globally. This study analyzed the population genetic structure of the bacterium and included a probabilistic source attribution model covering different animal and water sources. The results are discussed in a global context.

The genus *Campylobacter* currently includes 26 species and 11 subspecies (1). *Campylobacter jejuni* is the predominant species isolated from cases of campylobacteriosis worldwide, followed by *Campylobacter coli* (2–4). In 2006, New Zealand had the highest annual campylobacteriosis notification rate in the world, with >380 cases per 100,000 population (5). In 2014, the rate was 150 cases per 100,000 population, and, despite the observed 60% decrease compared to the 2006 rate, campylobacteriosis remained the most commonly notified gastrointestinal infection, accounting for ~45% of all notifications (6). In comparison, campylobacteriosis was the third most important bacterial foodborne disease in the United States (7), with an incidence of laboratory-confirmed cases of 13.5 cases per 100,000 population in 2014 (8). Estimates in the United States indicate that *C. coli* accounts for ~9% of campylobacteriosis cases (9). Considering an estimated incidence of ~845,000 cases of campylobacteriosis per year (7), the incidence of *C. coli* infections therefore amounts to ~84,000 cases per year in the United States alone.

Most epidemiological studies of campylobacteriosis that applied bacterial identification in New Zealand focused on *C. jejuni* (10, 11). Consumption of chicken meat is considered the main risk factor for *C. jejuni* infections in New Zealand and in a number of other countries (10, 12). However, there is little information about the sources and risk factors for *C. coli* infections. Extrapolating source attribution data from *C. jejuni* to *C. coli* is problematic, as different *Campylobacter* species may have different eco-epidemiological features and infection risk factors may also differ (13). For instance, in a case-control study in the Netherlands (14), the main risk factor for *C. jejuni* enteritis was consumption of chicken meat, whereas *C. coli* infections had different risk factors: swimming in open water or sea and consumption of game and tripe. Molecular subtyping by means of multilocus sequence typing (MLST) plays an important role in measuring the contributions of different infection sources to the burden of human disease caused by *C. jejuni*, and the same MLST scheme can be applied to *C. coli* (15). MLST was applied to *C. coli* isolates from humans and several other animal sources and water in studies in Scotland (16), Denmark (17), and Switzerland (12). Sheppard et al. (18, 19) carried out phylogenetic analyses of *C. coli* from Scotland using concatenated sequences obtained by MLST and showed that *C. coli* could be divided into three distinct clades. All the *C. coli* isolates from human clinical cases belonged to clade 1, which was dominated by strains isolated from farm animal sources, whereas clades

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2 and 3 were mainly composed of environmental water strains. Hence, unlike the case-control study in the Netherlands (where environmental water sources appeared to be a major source of *C. coli* infection), this study indicated a major role of animal sources in the transmission of *C. coli* to humans in Scotland.

Most cases of human campylobacteriosis in New Zealand are attributable to *C. jejuni*. However, the relative contribution of *C. coli* and the source of these infections had not been previously examined. Therefore, the objectives of this study were, first, to assess the relative contribution of *C. coli* to campylobacteriosis morbidity by identifying to the species level 1,601 *Campylobacter* clinical isolates collected between 2005 and 2014 from human cases in one New Zealand region and, second, to analyze the genetic relatedness of the clinical *C. coli* isolates to isolates obtained from poultry meat, farmed ruminants, and environmental water sources in the same study area and time frame using MLST, in order to infer possible transmission pathways of this important pathogen.

MATERIALS AND METHODS

Isolates and molecular typing. (i) Collection of human clinical *Campylobacter* spp. and identification of *C. coli*. The study used *Campylobacter* spp. isolated from human feces in the Manawatu Health District of New Zealand between March 2005 and December 2014. The region had a population of ~223,000 and its main city, Palmerston North, had a population of ~80,000 (20, 21). All the human fecal specimens that were submitted to the main medical laboratory (MedLab Central, Palmerston North) during the sampling period and tested positive for *Campylobacter* spp. by enzyme-linked immunosorbent assay (ELISA; ProSpecT, Remel, USA) were delivered for bacterial culture to the Molecular Epidemiology and Public Health laboratory (^{m}EpiLab) of Massey University (MU). The feces were delivered weekly on Amies charcoal transport swabs (Copan, Brescia, Italy). Fecal swabs were first streaked on modified cefoperazone charcoal deoxycholate agar (mCCDA) plates (Fort Richard, Auckland, New Zealand) and then immersed in 3 ml of Bolton broth (BB) (Lab M, Bury, United Kingdom) in a loose-capped bijou bottle and incubated at 42°C microaerobically (85% N_2, 10% CO_2, and 5% O_2) for 48 h (MACS-VA500 microaerophilic workstation; Don Whitley Scientific, West Yorkshire, United Kingdom). A single colony resembling *Campylobacter* spp. on mCCDA was subcultured onto Columbia horse blood agar (BA) (Fort Richard, Auckland, New Zealand) and incubated microaerophilically at 42°C for 48 h. If the mCCDA plate was negative for colonies resembling *Campylobacter* spp., a subculture was made from BB onto another mCCDA plate and the plate was incubated in a microaerobic atmosphere as described above. Pure cultures were frozen in 15% glycerol broth (Oxoid, United Kingdom) at −80°C for future reference.

Campylobacter identification to species level was done by molecular methods. Briefly, DNA was extracted by boiling a freshly grown culture of frozen isolates for 10 min in 1 ml of 2% Chelex (Bio-Rad, USA) in sterile Milli-Q water, followed by centrifugation (12,470 × *g* for 3 min) to remove cell debris and the Chelex. The supernatant was transferred to a fresh tube and used for PCR for species confirmation and multilocus sequence typing. Isolates were first identified through the use of the *mapA* gene for the detection of *C. jejuni* (22), employing the following primers: *map*A-F (5′-CTTGGCTTGAAATTTGCTTG-3′) and *map*A-R (5′-GCTTGGTGCGGATTGTAAA-3′). All the non-*jejuni* isolates were tested by a *Campylobacter* genus-specific PCR using the following primers: C412-F (5′-GGATGACACTTTTCGGAGC-3′) and C1288-R (5′-CATTGTAGCACGTGTGTC-3′) (23). Subsequently, all the non-*jejuni Campylobacter* spp. were subject to a *C. coli* species-specific PCR for detection of the *ceuE* gene (24). Primers were *ceu*E-F (5′-AATTGAAAATTGCTCCAACTATG-3′) and *ceu*E-R (5′-TGATTTTATTATTTGTAGCAGCG-3′). Amplification was performed in a 20-μl reaction volume containing 0.2 μl of Platinum *Taq* polymerase (Invitrogen, USA), 2 μl of 10× PCR buffer (Invitrogen), 1 μl of deoxynucleoside triphosphate (dNTP; Bioline, United Kingdom) (2 mM), 0.6 μl of $MgCl_2$, 2 μl of each forward and reverse primer (2 pmol/μl), 6.2 μl of H_2O, and 2 μl of DNA. The reactions were carried out in a thermocycler (SensoQuest, Germany) with denaturation at 96°C for 2 min, followed by 35 cycles of 96°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension of 72°C for 2 min. Electrophoresis in a 1% agarose gel plus staining with ethidium bromide was used to visualize PCR products. The presence of an ~462-bp product indicated *C. coli* (25).

(ii) Collection of poultry meat samples. Over the same sampling period (2005 to 2014), fresh whole poultry carcasses (chicken and turkey samples) from different poultry suppliers were sampled each month from supermarkets in Palmerston North. Carcasses were washed and massaged in 200 ml of sterile buffered peptone water (BPW) (Difco, USA). The liquid from the wash was centrifuged (16,000 × *g* and 4°C for 30 min) and the resultant pellet resuspended in 5 ml of BPW. This suspension was added to 90 ml of BB and incubated microaerophilically as described above, after which it was subcultured onto mCCDA and incubated for 2 days. Two colonies resembling *Campylobacter* spp. on mCCDA were subcultured to separate BA and incubated microaerophilically at 42°C for 2 days, and pure cultures were frozen at −80°C. Identification of *C. jejuni* and *C. coli* proceeded as for the human clinical isolates.

(iii) Collection of ruminant and water samples. Water samples were filtered through a sterile 0.45-μm filter (Millipore, USA), and the filter was transferred to 20 ml of BB and incubated at 42°C microaerobically for 2 days; then it was subcultured onto mCCDA and incubated for 2 days. A single colony resembling *Campylobacter* spp. on mCCDA was subcultured onto BA and incubated microaerophilically at 42°C for 2 days. Eleven *C. coli* isolates from environmental water (recreational waterways and pretreatment drinking water; nine from Manawatu-Tararua area and two [ST-3302 and ST-7774] from the North Island of New Zealand between 2006 and 2014) and 49 *C. coli* isolates from ruminant feces (nine different farms in the Manawatu-Tararua area between 2006 and 2011) were collected as part of the same long-term monitoring program. These were kept frozen in our laboratory for future reference and were also included in this study.

Isolates from pig meat were not included in the study due to the low prevalence of *Campylobacter* organisms from this source (more than 650 pig meat samples were previously collected, from which only one *C. coli* isolate was identified).

***C. coli* subtyping by MLST.** All *C. coli* isolates were subjected to MLST using seven housekeeping genes based on the method described by Dingle et al. (26). Each amplification reaction was performed in a 20-μl reaction volume containing 2 μl of the DNA preparation, 5 pmol of both forward and reverse amplification primers, and 14 μl of the PCR master mix (200 μl of 10× PCR buffer, 100 μl of dNTP, 60 μl of $MgCl_2$, 20 μl of Platinum *Taq* polymerase, and 1,020 μl of H_2O). Sequencing reactions were performed using 2 μl of the PCR product, 3.2 pmol of forward primer, 1 μl of ABI BigDye (Applied Biosystems, USA), 2 μl of 5× buffer, and H_2O to a total volume of 10 μl. Products were sequenced at the Institute of Environmental Science and Research (ESR, Porirua, New Zealand) on an ABI 3130XL automated DNA sequencer using ABI BigDye v3.1.

Sequence data were collated and submitted for allele and sequence type (ST) designation with their corresponding clonal complex (CC) online using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/). Isolates yielding novel STs or alleles that did not give clear sequences were reamplified and sequenced using the same protocol. If the sequence products were not readable, they were resequenced with the reverse primer and, if this failed, the locus was reamplified and resequenced. New MLSTs were submitted to the online database.

Data analysis. (i) Assessment of *C. coli* population structure and differentiation. The relatedness between human, poultry, ruminant, and environmental water *C. coli* isolates was assessed using multiple methods. Minimum spanning trees were implemented to visualize allelic differences between STs of isolates from the different sources using the pairwise

Hamming distance matrix. The trees were calculated by Prim's algorithm (27) as implemented in the Bionumerics software (Applied Maths).

A maximum likelihood (ML) method based on the Kimura 2-parameter model was used to infer the evolutionary history (28) using the concatenated DNA sequences composed of 3,309 nucleotide positions across the seven loci. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (29) to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. Previous phylogenetic work done in Scotland identified 3 *C. coli* clades of closely related STs associated with different sources (30); we examined the position of our STs in the same clades. Therefore, the ML method was implemented using 36 nucleotide sequences representing the different STs found in this study ($n = 27$) with the addition of 9 STs taken at random (4 STs from clade 2 [ST-3304, ST-3122, ST-3180, and ST-3182] and 5 STs from clade 3 [ST-2485, ST-2681, ST-3109, ST-3123, and ST-3124]) from two clades described in Scotland and not present in our study. Clade 1 had 10 shared STs with the Scottish study, so no additional STs were sampled from this clade. These analyses were conducted using MEGA6 software (31).

A different approach to assess genetic relatedness between *C. coli* utilized permutational multivariate analysis of variance (PERMANOVA) (32, 33). This analysis was implemented using PERMANOVA+, an "add in" to the PRIMER 6 software (34). Finally, the genetic diversities of *C. coli* from the different sources were compared using Simpson's and Shannon's diversity indices and their 95% bootstrap credible intervals (CrIs) (calculated using PAST software, version 2.17c) (35) and rarefaction analysis (performed using the package 'vegan' in R, version 3.1.3) (36).

(ii) *C. coli* source attribution. Although human-to-human spread may be implicated in some outbreaks (37), we assume that human-to-human spread as a cause of sporadic cases is negligible in common with other source attribution studies and therefore do not consider anthroponotic spread (11, 30). The relative contributions of different *C. coli* sources to the human disease burden were estimated using a number of tests. The similarity between the frequency distribution of human *C. coli* STs and those of the different sources was estimated using proportional similarity indices (PSI) and their bootstrap CrIs, as previously described (38). The PSI measures the area of intersection between two frequency distributions (39) and ranges between 0 and 1, where 0 indicates no similarity and 1 indicates identical frequency distributions. The PSI is calculated by the equation $PSI = 1 - 0.5 \sum i \mid pi - qi \mid = \sum i \min (pi,qi)$, where pi and qi are the proportions of strains that belong to type i out of all strains typed from sources P and Q (40). Calculations were performed using R, version 3.1.3.

The asymmetric island model was used to probabilistically assign each human isolate to one of the source populations (poultry, ruminants, or environmental water) (41).

RESULTS

Proportion of *C. coli* in ELISA-positive fecal specimens and ST diversity. A total of 2,009 *Campylobacter* ELISA-positive human fecal specimens were submitted by the medical laboratory for culture to *m*EpiLab between March 2005 and December 2014, and 1,601 (~80%) were *Campylobacter* positive by culture. The dominant species was *C. jejuni*, accounting for 1,552/1,601 (~96%) isolates, followed by *C. coli*, accounting for 47/1,601 isolates (~2.9%). Two isolates (<0.2%) were not identified as either *C. jejuni* or *C. coli* and were not further analyzed. The annual numbers of *C. coli* isolates were 9 (2005), 6 (2006), 2 (2007), 6 (2008), 0 (2009), 4 (2010), 2 (2011), 6 (2012), 7 (2013), and 5 (2014). A total of 1,123 *Campylobacter* isolates were isolated from poultry (chicken, $n = 1,074$; turkey, $n = 49$, of which 6 were *C. coli*) meat samples. *C. jejuni* accounted for 980/1,123 (87.3%) and *C. coli* for 108/1,123 (9.6%) of the isolates. The *Campylobacter* species remained unidentified in 29/1,123 (<3%) isolates. The proportion of *C. coli* was significantly higher in the poultry meat samples than in human feces (chi-square, $P < 0.01$).

Two hundred fifteen *C. coli* isolates from all sources were subtyped by MLST (47 from human feces, 49 from ruminant feces [32 sheep feces and 17 cattle feces], 108 from poultry meat, and 11 from environmental water). There were 27 different *C. coli* STs. Eighteen STs (66%) belonged to the ST-828 CC, which was the predominant CC in our data and accounted for 54% (116/215) of the isolates. The remaining STs could not be classified into any known CC. Three previously unidentified STs (ST-7767, ST-7774, and ST-7776) were detected. ST-7767 and ST-7776 were isolated from poultry and ST-7774 from environmental water. The most prevalent sequence type, ST-1581, which was identified in 60/215 (28%) of the isolates (12 from human feces, 44 from poultry meat, 4 from ruminant feces, and 0 from water), is not currently assigned to any CC. The second most prevalent ST was ST-3072 (25/215 [~11.6%]), and the third most prevalent STs were ST-3222 and ST-2397 (with 21 isolates each). These were not predominant STs in other countries (12, 16, 17, 30).

***C. coli* population structure and differentiation between sources.** The minimum spanning tree visualizing the ST clusters is shown in Fig. 1. Except for the environmental water isolates, the diagram shows no distinctive partition of *C. coli* STs between the sources, with most of the highly abundant STs represented in at least three sources. The diagram also shows a large cluster of closely related STs. However, four environmental water *C. coli* STs (ST-7774, ST-3302, ST-3301, and ST-1243) appear dissociated from this cluster, and two of these STs (ST-3301 and ST-3302) are single-locus variants.

Ten out of 27 STs found in this study were previously reported by Sheppard et al. in Scotland (30) and belong to clade 1. Hence, in order to compare the phylogenetic relatedness of *C. coli* from New Zealand and Scotland, we generated an ML diagram with the addition of 9 Scottish STs belonging to clades 2 and 3 but not found in our study (Fig. 2). Interestingly, 26/27 of the New Zealand STs clustered within the Scottish clade 1, only one ST of an environmental water *C. coli* clustered in clade 2 (ST-7774), and no STs clustered in clade 3 (Fig. 2). The frequencies of the different STs along with their clade designation are provided in supplemental material (see Table S1).

PERMANOVA was used to formally test for significant population differentiation, comparing mean pairwise Hamming distances of *C. coli* isolates from different sources. The pseudo-F statistic was 8.92 ($P = 0.001$), indicating that at least one of the populations differed from the other three. A nonmetric multidimensional scaling (NMDS) plot was used to assess which populations differed (see Fig. S1 in the supplemental material). NMDS together with the minimum spanning tree indicated that most *C. coli* isolates from water sources were differentiated from the strains circulating in the other sources, although this was not obvious from the results of the maximum likelihood phylogenetic analysis.

Source attribution for human infections. The proportional similarity indices (PSI) and their 95% CrIs are reported in Table 1. The PSI between ruminant and human *C. coli* isolates (PSI = 0.50; 95% CrI = 0.31 to 0.59) was similar to that observed between poultry and human isolates (PSI = 0.46; 95% CrI = 0.30 to 0.56), and the 95% CrI overlapped. However, in relation to human *C. coli*, the ruminant and poultry PSI were significantly greater than

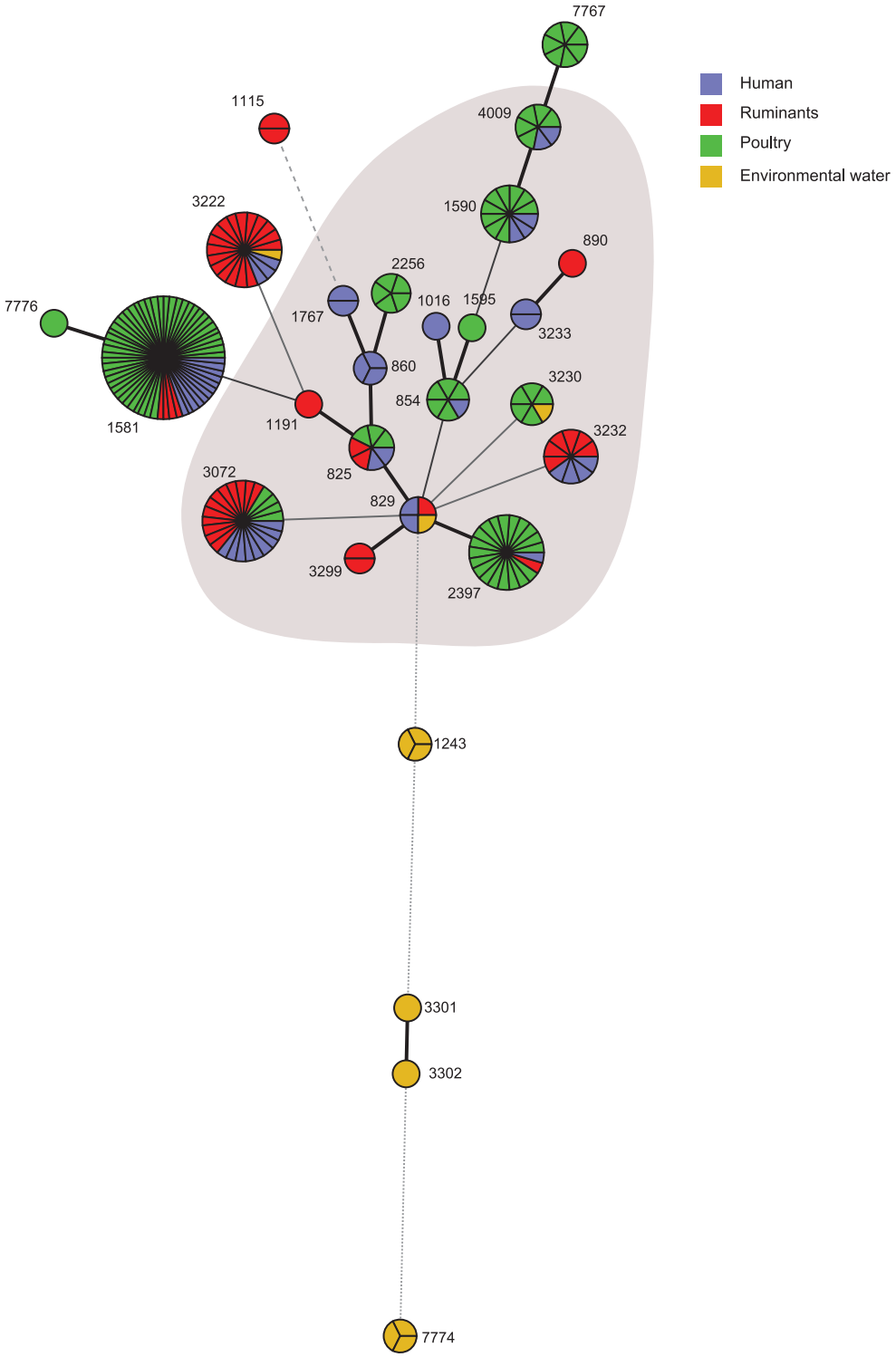

FIG 1 Minimum spanning tree of *C. coli* STs from 4 different sources. Each node represents an ST; its size is proportional to the frequency of isolation and the colors represent the different source types. The thickness of the connecting lines is proportional to the similarities between STs, with the thickest connector linking single-locus variants. The shaded area represents members of the ST-828 CC.

PSI obtained with environmental water *C. coli* (PSI = 0.10; 95% CrI = 0 to 0.17).

The asymmetric island model results were consistent with the PSI (Table 1), where ruminants and poultry were identified by both as the main sources for human infection. The model attributed 55% (95% CrI = 31 to 79%) and 38% (95% CrI = 16 to 58%) of the infections to ruminants and poultry sources, respectively, but the 95% CrIs of the estimated contributions of these sources overlapped widely, whereas the environmental water source accounted for only 7% (95% CrI = 0 to 24%) of infections, and the

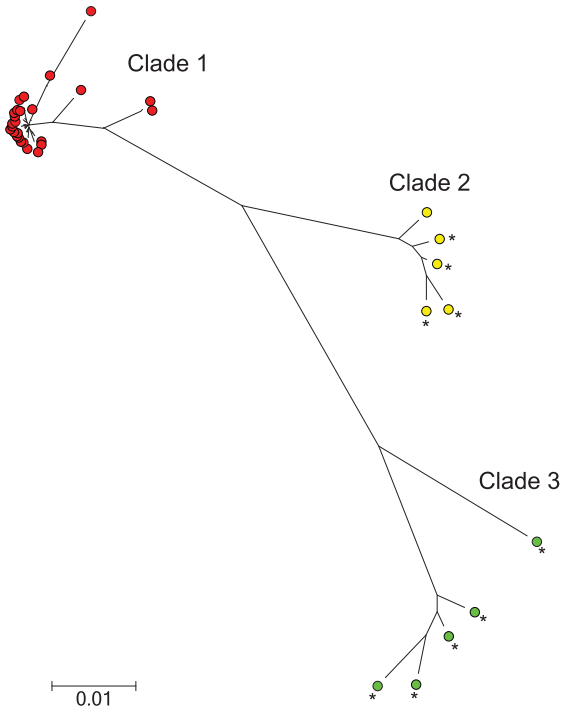

FIG 2 Molecular phylogenetic analysis by maximum likelihood method. The 27 STs found in this study and 9 STs from clades 2 and 3 from Sheppard et al. (30) were used. Clade 1 is indicated in red, clade 2 in yellow, and clade 3 in green. *C. coli* STs in the work of Sheppard et al. (30) are indicated with an asterisk. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar represents a genetic distance of 0.01 (i.e., 1% of the nucleotides differ).

CrI overlapped only slightly with that of the ruminant source. As a whole, these results attributed the majority of the infections to ruminant and poultry sources, whereas exposure to environmental water sources appeared to contribute less to the burden of disease.

Simpson's and Shannon's indices of diversity are reported in Table 1, and rarefaction curves are shown in Fig. 3. Human *C. coli* isolates showed the greatest number of STs ($n = 14$), followed by isolates from poultry ($n = 12$), ruminants ($n = 11$), and environmental water ($n = 7$). Some human STs (ST-854, ST-1590, and ST-4009) were found only in poultry, and one (ST-3232) was isolated only from ruminants. The diversity indices of human *C. coli* STs were the greatest among all the sources (Table 1), with 95% CrIs overlapping. Consistent with the diversity indices, rarefaction curves indicated a greater ST richness of human *C. coli* than for the other sources (except the environmental water source, the curve of which overlapped the human curve). When the ruminant, poultry, and environmental water data were combined in one rarefaction curve, this curve did not differ from the human rarefaction curve (data not shown).

DISCUSSION

In this paper, we report a molecular source attribution study of *C. coli* campylobacteriosis in New Zealand, one of few such studies globally. The study analyzed 215 *C. coli* isolates gathered from 2,009 *Campylobacter* ELISA-positive human fecal specimens collected between 2005 and 2014. Our study did not include pork meat, as only one *C. coli* isolate was from this source. Although in some studies, *C. coli* has been found at a relatively high prevalence in pig feces (42, 43), a low rate of isolation of *C. coli* from pork meat has also been observed in a study in the greater Washington, DC, area (44). A limitation of this study was an inability to assess variability statistically between years, due to the modest number of *C. coli* organisms identified each year. However, aggregation of the samples into 3-year intervals did not provide any evidence of temporal changes in dominant STs (data not shown).

C. coli accounted for ~3% of the human isolates, a relatively low proportion compared to the situation in other countries, where *C. coli* has been identified in 10% of the cases (13, 45–47). In the last 6 years the average annual number of campylobacteriosis cases in New Zealand was ~7,000, or 155 cases per 100,000 population (https://surv.esr.cri.nz/surveillance/surveillance.php). By extrapolation to the whole country without accounting for possible regional differences (48), there would be an average of ~200 *C. coli* infections per year. Estimation of the true incidence of *C. coli* infections in the population is hindered by significant underreporting. For example, in New Zealand it has been estimated that for every reported case of acute gastrointestinal illness (from all causes) there could be 222 unreported cases. However, the majority of these cases could be due to viral infections (49). One estimate in the United Kingdom suggested that the number of cases of campylobacteriosis in the population could be 7-fold the number of notifications (50). If we extrapolate the United Kingdom estimate to New Zealand, there could be ~1,400 (200 × 7) *C. coli* infections per year, equivalent to an incidence rate of ~31 cases per 100,000 population. This rate is high compared with an estimate of 8.5 cases per 100,000 population in the United States, even though the relative contribution of *C. coli* to the campylobacteriosis burden is approximately three times lower in New Zealand than in the United States (9). This is due to the relatively high incidence rate of campylobacteriosis (due to any species) in New Zealand.

A comparison between *C. coli* populations cycling in New Zealand and elsewhere revealed a number of key features: STs belonging to CC ST-828 were predominant in our study (54% of the isolates), and interestingly, also predominant in Scotland (94% of

TABLE 1 Numerical indices measuring diversities, PSI, and asymmetric island model in *C. coli*

Source	Value for index (95% CrI)			
	PSI	Asymmetric island model output	Simpson's index	Shannon's index
Humans	NA[a]	NA	0.87 (0.79–0.89)	2.32 (1.89–2.41)
Ruminants	0.50 (0.31–0.59)	0.55 (0.31–0.79)	0.79 (0.69–0.84)	1.88 (1.49–2.03)
Poultry	0.46 (0.30–0.56)	0.38 (0.16–0.58)	0.78 (0.70–0.83)	1.93 (1.66–2.06)
Environmental water	0.10 (0.00–0.17)	0.07 (0.00–0.24)	0.81 (0.56–0.83)	1.80 (0.99–1.85)

[a] NA, not available.

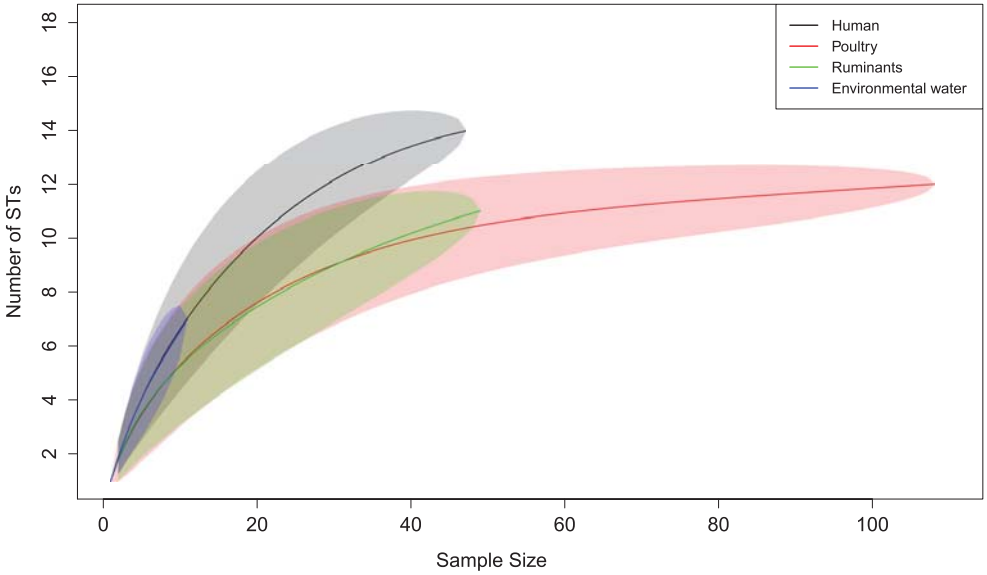

FIG 3 Rarefaction curves of the human, poultry, ruminant, and environmental water *C. coli* STs. The shaded areas represent the 95% CrI. Note that the poultry and ruminant upper boundary of the 95% CrI does not reach the point estimate of the human curve at maximum sample size. The environmental water curve overlaps the human curve.

the isolates) (45) and Switzerland (71%) (12). Ten out of 27 STs identified in the New Zealand study have also been reported in Scotland. What determines the apparent global distribution and predominance of STs belonging to CC ST-828 is not currently well understood and may require more samples and the application of higher-resolution genotyping using whole-genome sequencing to resolve. Three novel STs (ST-7767, ST-7774, and ST-7776) were identified in our study, but none of these were detected in humans. Rarefaction analysis and diversity indices were in broad agreement and indicated that human clinical isolates had the highest ST diversity (Fig. 3; Table 1). However, when the rarefaction curve for human isolates was compared to that for all other sources combined, the rarefaction curves were very similar, providing evidence that the increased diversity in clinical isolates could be due to multiple sources contributing to *C. coli*-associated campylobacteriosis.

Most human isolates (32/47) belonged to CC ST-828 (see Table S1 in the supplemental material). However, the most prevalent ST in humans and poultry meat (ST-1581) did not belong to any known CC and appeared sporadically but with regularity in humans in the past 10 years. Another ST that appeared with regularity in humans was ST-3072. The remaining 12 STs found in humans either were detected sporadically in a single year or disappeared and reappeared after several years. For example, ST-3232 was detected in 2005 and disappeared and reappeared in 2010. The predominance of ST-1581 in humans has not been reported elsewhere. ST-1581 was detected in Denmark from a clinical case, but it was not the dominant strain (17). In a study in Switzerland, none of the 616 *C. coli* isolates collected between 2002 and 2012 from poultry, pigs, and humans belonged to ST-1581. In that study, the dominant human ST was ST-827, which belongs to CC ST-828 but was not detected in our study (12). Another study in England did not detect ST-1581 in the 175 human and water *C. coli* isolates examined (51).

There were 10 STs shared between our study and a study conducted in Scotland (30). These STs belonged to clade 1 as defined by Sheppard et al. (30), and in the ML tree they clustered with 16 other STs that were not reported in Scotland (Fig. 2). Conversely, a previously unreported ST (ST-7774) isolated from environmental water clustered with Scottish STs belonging to clade 2, and none clustered with the Scottish clade 3. The finding of human and animal *C. coli* sources harboring STs from clade 1 is consistent with the findings in Scotland. However, in Scotland all the STs found in environmental water sources clustered in clades 2 and 3 rather than clade 1 (30), whereas in our study we did not find any clade 3 STs in environmental water. Further, the *C. coli* STs isolated from water samples collected in northwest England also belonged to clades 2 and 3 (51). It is possible that increasing the sample size from environmental water sources will result in the identification of clade 2 and clade 3 STs in New Zealand.

In spite of the fact that the ML analysis did not well differentiate the environmental water *C. coli* from isolates obtained from other sources, the PERMANOVA results indicated a significant differentiation of these isolates from *C. coli* cycling in humans, poultry, and ruminants. The PSI was calculated to evaluate the similarity between ST frequency distributions and the highest PSI was observed between STs from humans and ruminant sources, but the CrI overlapped widely with the CrI of the PSI between human and poultry *C. coli*. The asymmetric island model results supported the PSI and indicated that ruminant and poultry sources were the largest contributor to human infections, again with overlapping 95% CrIs (Table 1). In contrast, up to 76% of *C. jejuni* infections were attributed to poultry sources in New Zealand and ruminants were the second source, accounting for up to 20% of the cases (10). In Scotland, the major source of *C. coli* campylobacteriosis, based on the asymmetric island model, was poultry (57%), and 41% of cases were attributed to the ruminant source (30).

Although it is possible that a mutation could occur during culture, and this could result in a change in an allele and the ST, such events are likely to occur at very low frequencies due to the low intrinsic rate of mutation in *Campylobacter* housekeeping

genes (52). In fact, only one new ST occurred as a singleton in our study (ST-7776), and the inclusion and exclusion of this isolate in the analyses did not affect the conclusions.

In summary, our results indicate that ~3% of human campylobacteriosis cases in the study area could be attributed to infection with *C. coli*. This relative contribution of *C. coli* appears to be smaller than the contribution estimated in other countries. However, calculations suggest that the incidence rate of *C. coli* infection remained higher than in other countries due to the higher incidence of campylobacteriosis in general. Future studies should assess whether these results can be generalized to the whole country given that specific demographic differences between the Manawatu and other New Zealand regions may affect source attribution studies (48). The population genetic structure of *C. coli* in the study area is reminiscent of the structure described in the United Kingdom, with the predominance of CC ST-828 and the presence of many shared STs. As in Scotland, our source attribution analysis identified ruminants and poultry as the main infection sources, as well as a smaller contribution from surface water sources. Unlike the situation with *C. jejuni*, our results suggest that the ruminant sources might have a greater relative contribution to *C. coli* infection burden than poultry. These results highlight the need to consider each *Campylobacter* species separately when designing public health interventions.

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REFERENCES

1. **Euzeby JP.** List of prokaryotic names with standing in nomenclature. Genus *Campylobacter*. http://www.bacterio.net/campylobacter.html.
2. **Horrocks SM, Anderson RC, Nisbet DJ, Ricke SC.** 2009. Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. Anaerobe **15:**18–25. http://dx.doi.org/10.1016/j.anaerobe.2008.09.001.
3. **WHO.** 2011. *Campylobacter*. WHO, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs255/en/.
4. **Moore JE, Corcoran D, Dooley JS, Fanning S, Lucey B, Matsuda M, McDowell DA, Megraud F, Millar BC, O'Mahony R, O'Riordan L, O'Rourke M, Rao JR, Rooney PJ, Sails A, Whyte P.** 2005. *Campylobacter*. Vet Res **36:**351–382. http://dx.doi.org/10.1051/vetres:2005012.
5. **ESR.** 2006. Notifiable and other diseases in New Zealand: annual report 2006. Institute of Environmental Science and Research Ltd, Porirua, New Zealand.
6. **ESR.** 2014. Notifiable diseases in New Zealand: annual report 2014. Institute of Environmental Science and Research Ltd, Porirua, New Zealand.
7. **Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM.** 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis **17:**7–15. http://dx.doi.org/10.3201/eid1701.P11101.
8. **CDC.** 2015. Preliminary incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006–2014. CDC, Atlanta, GA.
9. **CDC.** 2012. National antimicrobial resistance system, enteric bacteria, human isolates final report 2010. CDC, Atlanta, GA.
10. **Mullner P, Spencer SE, Wilson DJ, Jones G, Noble AD, Midwinter AC, Collins-Emerson JM, Carter P, Hathaway S, French NP.** 2009. Assigning the source of human campylobacteriosis in New Zealand: a comparative genetic and epidemiological approach. Infect Genet Evol **9:**1311–1319. http://dx.doi.org/10.1016/j.meegid.2009.09.003.
11. **Müllner P, Collins-Emerson JM, Midwinter AC, Carter P, Spencer SE, van der Logt P, Hathaway S, French NP.** 2010. Molecular epidemiology of *Campylobacter jejuni* in a geographically isolated country with a uniquely structured poultry industry. Appl Environ Microbiol **76:**2145–2154. http://dx.doi.org/10.1128/AEM.00862-09.
12. **Kittl S, Heckel G, Korczak BM, Kuhnert P.** 2013. Source attribution of human *Campylobacter* isolates by MLST and fla-typing and association of genotypes with quinolone resistance. PLoS One **8:**e81796. http://dx.doi.org/10.1371/journal.pone.0081796.
13. **Gillespie IA, O'Brien SJ, Frost JA, Adak GK, Horby P, Swan AV, Painter MJ, Neal KR.** 2002. A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. Emerg Infect Dis **8:**937–942. http://dx.doi.org/10.3201/eid0809.010817.
14. **Doorduyn Y, Van Den Brandhof WE, Van Duynhoven YT, Breukink BJ, Wagenaar JA, Van Pelt W.** 2010. Risk factors for indigenous *Campylobacter jejuni* and *Campylobacter coli* infections in the Netherlands: a case-control study. Epidemiol Infect **138:**1391–1404. http://dx.doi.org/10.1017/S095026881000052X.
15. **Dingle KE, Colles FM, Falush D, Maiden MC.** 2005. Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J Clin Microbiol **43:**340–347. http://dx.doi.org/10.1128/JCM.43.1.340-347.2005.
16. **Roux F, Sproston E, Rotariu O, Macrae M, Sheppard SK, Bessell P, Smith-Palmer A, Cowden J, Maiden MC, Forbes KJ, Strachan NJ.** 2013. Elucidating the aetiology of human *Campylobacter coli* infections. PLoS One **8**(5):e64504. http://dx.doi.org/10.1371/journal.pone.0064504.
17. **Litrup E, Torpdahl M, Nielsen EM.** 2007. Multilocus sequence typing performed on *Campylobacter coli* isolates from humans, broilers, pigs and cattle originating in Denmark. J Appl Microbiol **103:**210–218. http://dx.doi.org/10.1111/j.1365-2672.2006.03214.x.
18. **Sheppard SK, Didelot X, Jolley KA, Darling AE, Pascoe B, Meric G, Kelly DJ, Cody A, Colles FM, Strachan NJ, Ogden ID, Forbes K, French NP, Carter P, Miller WG, McCarthy ND, Owen R, Litrup E, Egholm M, Affourtit JP, Bentley SD, Parkhill J, Maiden MC, Falush D.** 2013. Progressive genome-wide introgression in agricultural *Campylobacter coli*. Mol Ecol **22:**1051–1064. http://dx.doi.org/10.1111/mec.12162.
19. **Sheppard SK, McCarthy ND, Falush D, Maiden MCJ.** 2008. Convergence of *Campylobacter* species: implications for bacterial evolution. Science **320:**237–239. http://dx.doi.org/10.1126/science.1155532.
20. **Statistics New Zealand.** 2013. 2013 Census QuickStats about a place: Palmerston North City. Number of people counted. Statistics New Zealand, Wellington, New Zealand. http://www.stats.govt.nz/Census/2013-census/profile-and-summary-reports/quickstats-about-a-place.aspx?request_value=14263&parent_id=14181&tabname=&gclid=CI_Go_-I2cYCFUtwvAodd44Peg#14263.
21. **Department of Internal Affairs, New Zealand Government.** 2013. Manawatu-Wanganui Regional Council. Community statistics as at the 2013 census. Department of Internal Affairs, New Zealand Government, Wellington, New Zealand. http://www.localcouncils.govt.nz/lgip.nsf/wpg_url/Profiles-Councils-Manawatu-Wanganui-Regional-Council-main.
22. **Stucki U, Frey J, Nicolet J, Burnens AP.** 1995. Identification of *Campylobacter jejuni* on the basis of a species-specific gene that encodes a membrane protein. J Clin Microbiol **33:**855–859.
23. **Inglis GD, Kalischuk LD.** 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl Environ Microbiol **69:**3435–3447. http://dx.doi.org/10.1128/AEM.69.6.3435-3447.2003.
24. **Gonzalez I, Grant KA, Richardson PT, Park SF, Collins MD.** 1997. Specific identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* by using a PCR test based on the *ceu*E gene encoding a putative virulence determinant. J Clin Microbiol **35:**759–763.
25. **Denis M, Soumet C, Rivoal K, Ermel G, Blivet D, Salvat G, Colin P.** 1999. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett Appl Microbiol **29:**406–410. http://dx.doi.org/10.1046/j.1472-765X.1999.00658.x.
26. **Dingle KE, Colles FM, Wareing DR, Ure R, Fox AJ, Bolton FE, Bootsma HJ, Willems RJ, Urwin R, Maiden MC.** 2001. Multilocus sequence typing

system for *Campylobacter jejuni*. J Clin Microbiol **39:**14–23. http://dx.doi.org/10.1128/JCM.39.1.14-23.2001.
27. **Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden MC, Ochman H, Achtman M.** 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol **60:**1136–1151. http://dx.doi.org/10.1111/j.1365-2958.2006.05172.x.
28. **Kimura M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol **16:**111–120. http://dx.doi.org/10.1007/BF01731581.
29. **Gascuel O.** 1997. BIONJ an improved version of the NJ algorithm based on a simple model of sequence data. Mol Biol Evol **14:**685–695. http://dx.doi.org/10.1093/oxfordjournals.molbev.a025808.
30. **Sheppard SK, Dallas JF, Wilson DJ, Strachan NJC, McCarthy ND, Jolley KA, Colles FM, Rotariu O, Ogden ID, Forbes KJ, Maiden MCJ.** 2010. Evolution of an agriculture-associated disease causing *Campylobacter coli* clade: evidence from national surveillance data in Scotland. PLoS One 5(12):e15708.
31. **Tamura K, Stecher G, Peterson D, Filipski A, Kumar S.** 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol **30:**2725–2729. http://dx.doi.org/10.1093/molbev/mst197.
32. **Anderson MJ.** 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol **26:**32–46. http://dx.doi.org/10.1111/j.1442-9993.2001.01070.pp.x.
33. **McArdle BH, Anderson J.** 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecol **82:**290–297. http://dx.doi.org/10.1890/0012-9658(2001)082[0290:FMMTCD]2.0.CO;2.
34. **Anderson MJ, Gorley RN, Clarke KR.** 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth, United Kingdom.
35. **Hammer Ø, Harper DAT, Ryan PD.** 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron **4:**9.
36. **R Core Team.** 2005. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
37. **Mughini-Gras L, Smid JH, Wagenaar JA, De Boer A, Havelaar AH, Friesema IH, French NP, Graziani C, Busani L, Van Pelt W.** 2014. Campylobacteriosis in returning travellers and potential secondary transmission of exotic strains. Epidemiol Infect **142:**1277–1288. http://dx.doi.org/10.1017/S0950268813002069.
38. **Garrett N, Devane ML, Hudson JA, Nicol C, Ball A, Klena JD, Scholes P, Baker MG, Gilpin BJ, Savill MG.** 2007. Statistical comparison of *Campylobacter jejuni* subtypes from human cases and environmental sources. J Appl Microbiol **103:**2113–2121. http://dx.doi.org/10.1111/j.1365-2672.2007.03437.x.
39. **Rosef O, Kapperud G, Lauwers S, Gondrosen B.** 1985. Serotyping of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter laridis* from domestic and wild animals. Appl Environ Microbiol **49:**1507–1510.
40. **Feinsinger P, Spears EE, Poole RW.** 1981. A simple measure of niche breadth. Ecology **62:**27–32. http://dx.doi.org/10.2307/1936664.
41. **Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Fearnhead P, Hart CA, Diggle PJ.** 2008. Tracing the source of campylobacteriosis. PLoS Genet **4:**e1000203. http://dx.doi.org/10.1371/journal.pgen.1000203.
42. **Miller WG, Englen MD, Kathariou S, Wesley IV, Wang G, Pittenger-Alley L, Siletz RM, Muraoka W, Fedorka-Cray PJ, Mandrell RE.** 2006. Identification of host-associated alleles by multilocus sequence typing of *Campylobacter coli* strains from food animals. Microbiology **152:**245–255. http://dx.doi.org/10.1099/mic.0.28348-0.
43. **Wright SL, Carver DK, Siletzky RM, Romine S, Morrow WEM, Kathariou S.** 2008. Longitudinal study of prevalence of *Campylobacter jejuni* and *Campylobacter coli* from turkeys and swine grown in close proximity. J Food Prot **71:**1791–1796.
44. **Zhao C, Ge B, De Villena J, Sudler R, Yeh E, Zhao S, White DG, Wagner D, Meng J.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, DC, area. Appl Environ Microbiol **67:**5431–5436. http://dx.doi.org/10.1128/AEM.67.12.5431-5436.2001.
45. **Sheppard SK, Dallas JF, Strachan NJ, MacRae M, McCarthy ND, Wilson DJ, Gormley FJ, Falush D, Ogden ID, Maiden MC, Forbes KJ.** 2009. *Campylobacter* genotyping to determine the source of human infection. Clin Infect Dis **48:**1072–1078. http://dx.doi.org/10.1086/597402.
46. **Schielke A, Rosner BM, Stark K.** 2014. Epidemiology of campylobacteriosis in Germany—insights from 10 years of surveillance. BMC Infect Dis **14:**30. http://dx.doi.org/10.1186/1471-2334-14-30.
47. **Cody AJ, McCarthy NM, Wimalarathna HL, Colles FM, Clark L, Bowler IC, Maiden MC, Dingle KE.** 2012. A longitudinal 6-year study of the molecular epidemiology of clinical *Campylobacter* isolates in Oxfordshire, United Kingdom. J Clin Microbiol **50:**3193–3201. http://dx.doi.org/10.1128/JCM.01086-12.
48. **Bolwell CF, Gilpin BJ, Campbell D, French NP.** 2015. Evaluation of the representativeness of a sentinel surveillance site for campylobacteriosis. Epidemiol Infect **143:**1990–2002. http://dx.doi.org/10.1017/S0950268814003173.
49. **Lake RJ, Adlam SB, Perera S, Campbell DM, Baker MG.** 2010. The disease pyramid for acute gastrointestinal illness in New Zealand. Epidemiol Infect **138:**1468–1471. http://dx.doi.org/10.1017/S0950268810000397.
50. **Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, Gray JJ, Letley LH, Rait G, Tompkins DS, O'Brien SJ.** 2012. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut **61:**69–77. http://dx.doi.org/10.1136/gut.2011.238386.
51. **Sopwith W, Birtles A, Matthews M, Fox A, Gee S, James S, Kempster J, Painter M, Edwards-Jones V, Osborn K, Regan M, Syed Q, Bolton E.** 2010. Investigation of food and environmental exposures relating to the epidemiology of *Campylobacter coli* in humans in Northwest England. Appl Environ Microbiol **76:**129–135. http://dx.doi.org/10.1128/AEM.00942-09.
52. **Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Hart CA, Diggle PJ, Fearnhead P.** 2009. Rapid evolution and the importance of recombination to the gastroenteric pathogen *Campylobacter jejuni*. Mol Biol Evol **26:**385–397. http://dx.doi.org/10.1093/molbev/msn264.