Swansea University
Prifysgol Abertawe

Understanding the Seasonality of *Campylobacter* Infection among Commercial Broiler Chickens in the United Kingdom

Daniel Phillips

Submitted to Swansea University in fulfilment of the requirements for the Degree of Doctor of Philosophy

Swansea University

2023

a

Summary/Abstract

Campylobacter spp. are responsible for more cases of gastroenteritis than any other bacteria in humans. Up to 80% of cases originate from poultry. Infections in both chickens and humans follow a seasonal pattern, with an increase in incidence during warmer months. This study aims to determine which causal factors are associated with the seasonal onset of *Campylobacter* infection in housed chickens.

Eleven farms around Herefordshire, UK, were assessed daily for *Campylobacter* spp. presence by quantitative PCR on swabs taken inside one chicken house per farm. Weather, farm performance indicators and background information about each farm were recorded and used in statistical models to determine the strength of association between parameters and *Campylobacter* presence. Increased detections of *Campylobacter* were associated with wooden house construction, and how well temperature and humidity were managed within the house.

A subset of four farms was observed for a further seven production cycles, with the same sampling regime as before, along with paired daily swabs of the external farm environment. At slaughter, sections of ilea were collected, tested for *Campylobacter* presence by PCR and for gut damage using histopathology, and caecal contents were collected for community 16S rRNA gene analysis. Damage to ileal villi was observed primarily in summer months. The diversity of caecal bacteria increased with *Campylobacter* infection and during summer months.

Campylobacter infection of chickens was found to be unlikely to originate from the farmyard environment. Changes to the chicken gut were identified as varying with season, in similar patterns as observed under *Campylobacter* infection. This study identifies risk factors associated with *Campylobacter* infection that will guide how future chicken farms may be constructed to improve control of *Campylobacter* contamination risk factors, and proposes interaction between chicken gut microbiota and the environment inside the chicken house as being a potential explanation of *Campylobacter* seasonality.

Declarations and Statements

DECLARATION:

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed..... [redacted] ..... Date.....31/03/2023

STATEMENT 1:

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed..... [redacted] .....Date.....31/03/2023

STATEMENT 2:

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed..... [redacted] ..... Date.....31/03/2023

Table Of Contents

Acknowledgements

I would like to thank my supervision team: Prof. Martin Sheldon, Prof. Tom Wilkinson, Dr. Lisa Williams, and Keith Warner. Without their patience, guidance, encouragement and words of advice, I would not have been able to complete this work. I would additionally like to extend my thanks to Prof. Tom Humphrey and Rob Barker, under whom I began this project and without whom none of this opportunity would have been made real.

A massive thanks is in order for all of the farm managers, assistants and staff who have welcomed me onto their farms, collected samples on my behalf and diligently worked over many months to help me realise this thesis. None of this would have been possible without them. I would also like to thank all the laboratory, development and quality assurance staff at Avara Foods Ltd. for their continued support in sample collection, provision of data and help.

Additionally, I would like to thank Dr. Kayleigh Rose for her statistical insight, Dr. Matt Hitchings for kindly performing 16S sequencing of caecal contents and help with subsequent analyses, and Kate Murphy for taking the time to prepare and photograph histological specimens. An extra thank you goes out to the technicians, Celeste Felion and Stephen Leary, for all of their expertise in the laboratory.

To all the staff and students within Microbiology and Infectious Diseases Group, especially Dr. Llinos Harris, Dr. Rowena Jenkins, Amy Hillier, Matt Lewis and Dr. Phil Butterick, I thank you for making me so welcome, offering me advice as needed and providing support in my work. An extra special thanks goes to Dr. Heather Chick for putting up with sitting next to me in the office for so long and still being my friend throughout that time.

No list of thanks would be complete without recognising the eternal support of my mother, father and siblings, who have kept me going with their encouragement whether I am near or far.

This thesis is dedicated both to my son, Rowan, and in loving memory of my Nain, Greta.

List of Figures

List of Tables

Abbreviations

16S	16 Svedberg Units
ANOVA	Analysis of Variance
bp	Base Pair
C. coli	*Campylobacter coli*
C. hepaticus	*Campylobacter hepaticus*
C. jejuni	*Campylobacter jejuni*
CDT	Cytolethal Distending Toxin
cfu	Colony-Forming Unit
cfu/g	Colony-Forming Units per Gram
cm	Centimetre
cm^2	Square Centimetre
C_q	Quantification Cycle
CRAN	Comprehensive R Archive Network
C_t	Threshold Cycle
df	Degrees of Freedom
DNA	Deoxyribose Nucleic Acid
EPEF	European Production Efficiency Factor
EU	European Union
FSA	Food Standards Agency
g	Grams
GAM	Generalised Additive Model
G/C	Guanine/Cytosine
H&E	Haematoxylin and Eosin
HSD	Honestly Significant Difference
kg	Kilogram
kg/m^2	Kilograms per Square Metre
km^2	Square Kilomere
LAMP	Loop-Mediated Isothermal Amplification

LPG	Liquefied Petroleum Gas
m^2	Square Metre
Mbp	Megabase pair
mg/l	Milligrams per Litre
ml	Millilitre
µl	Microlitre
mm	Millimetre
µm	Micrometre
NHS	National Health Service
ng/µl	Nanograms per Microlitre
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal Ribose Nucleic Acid
SDI	Shannon Diversity Index
SIMPER	Similarity Percentages
UK	United Kingdom
VBNC	Viable But Non-Culturable
x g	Multiple of Earth's Gravitational Force

1 Literature review

1.1 General characteristics of *Campylobacter*

1.1.1 History of *Campylobacter*

Early historical observations of *Campylobacter* spp. are attributed to Theodor Escherich, who noted spiral-shaped organisms in microscope observations of infant gastrointestinal illness (Escherich, 1886). Whilst it had been known as a veterinary pathogen in various livestock species since the start of the 20th century, it had been classified as '*Vibrio*-like' rather than as an independent species (McFadyean and Stockman, 1913; Smith and Taylor, 1919). Cases of *Campylobacter*-linked enteritis were recorded as early as 1938 (Levy, 1946) and a link with diarrhoea was known in literature within a few decades (Wheeler and Borchers, 1961), but it was not a commonly reported human pathogen nor was it able to be isolated from the faeces of patients until later in the 1960s (Butzler, 2004).

The first known successful isolation of *Campylobacter* from a clinical case via faecal sample took place in 1968 from a patient suffering acute diarrhoea (Dekeyser *et al.*, 1972). It was identified as being the origin of the pathology presented and offered the first viable method of isolating and culturing *Campylobacter* spp. directly from gastrointestinal tract contents (Dekeyser *et al.*, 1972). Improved isolation from faecal samples of diarrhoea patients led to a more widespread acceptance of *Campylobacter*-induced enteritis testing and international epidemiological studies (Skirrow, 1977).

The genus of *Campylobacter* (named from the Greek for 'curved rod') was first described in 1963 (Sebald and Véron, 1963) as a means of better classifying the then-disparate species of '*Vibrio*-like' organisms known, and was later elaborated upon (Véron and Chatelain, 1973) to include specific species previously described as *Vibrio*, such as *Campylobacter jejuni* and *Campylobacter coli*. However, it was not until 1991 that the modern definition of the family *Campylobacteraceae* that still broadly stand today was codified (Vandamme *et al.*, 1991). It includes the *Campylobacter* and *Helicobacter* genera and forms the basic list of species traits that are used to characterise members of the family.

The recognition of *Campylobacter* as a family distinct from other agents which cause similar disease states, alongside methods to identify, isolate and culture it, led to its recognition as an important family of pathogens in humans and livestock.

1.1.2 Characteristics and biology

Members of the *Campylobacter* genus are spiralling rod-shaped, oxidase-positive, Gram-negative bacteria, 0.5-5.0 µm in length and 0.2-0.5 µm in diameter (Vandamme *et al.*, 1991; On *et al.*, 2017). They are microaerophilic and thermophilic, with optimum conditions of 3-15% O_2 at 42°C, and usually possess at least one polar flagellum for motility (Ketley, 1997). Genome sizes are usually 1.6-1.7 Mbp, with a G/C ratio of around 30% (Parkhill *et al.*, 2000).

Unlike most bacteria, *Campylobacter* spp. are unable to utilise common carbohydrates such as glucose in their metabolic reactions, with few genes in their genomes enabling them to degrade and utilise carbohydrates (Parkhill *et al.*, 2000). Instead, their primary metabolites are amino acids and some short chain fatty acids (Wright *et al.*, 2009), acquired both from the gut lumen contents of the host organism, and from gut epithelial cells that the bacterium invades.

Iron is an extremely important micronutrient for *Campylobacter* spp. and its growth is severely inhibited without its bioavailability (Palyada, Threadgill and Stintzi, 2004). This is due to the role of iron-sulfur complexes within several key metabolic enzymes (Stahl, Butcher and Stintzi, 2012) and as a result *Campylobacter* spp. have a large range of iron acquisition mechanisms at their disposal (Palyada, Threadgill and Stintzi, 2004; Miller *et al.*, 2008; Stahl, Butcher and Stintzi, 2012).

Campylobacter spp. are highly sensitive to environmental stressors, such as osmotic stress or low temperatures. However, they are capable of entering a state known as Viable but Non-Culturable (VBNC), whereby the bacteria decrease their metabolic functions and undergo a morphological shift to a coccoid state (Rollins and Colwell, 1986). This dormant phase allows the cells to cope with many different external pressures that would normally result in cell death (Chaveerach *et al.*, 2003; Cook and Bolster, 2007). Whilst alive, VBNC cells are incapable of division and this may have been a confounding factor in many attempts to isolate *Campylobacter* (Rollins and Colwell,

1986) while remaining prominent as a pathogen in the wild where it can be resuscitated *in vivo* (Baffone *et al.*, 2006).

1.1.3 Phylogeny and species distribution

There are 37 currently known species within the *Campylobacter* genus (Encyclopedia Of Life, 2020). The broader family *Campylobacteraceae* contains three members: *Campylobacter, Acrobacter* and *Sulfurospirillum*; they are closely related to the family *Helicobacteraceae* (On *et al.*, 2017). Both family and genus have proven to be relatively dynamic, with *Arcobacter* proposed as a species within the past two decades (Vandamme *et al.*, 1991) and *Helicobacter* only being designated as part of its own family outside of *Campylobacteraceae* within the past few years (Garrity, Bell and Lilburn, 2015). Both *Campylobacteraceae* and *Helicobacteraceae* are part of the class Epsilonproteobacteria, a genetically distinct lineage of Proteobacteria (On *et al.*, 2017).

Campylobacter jejuni, followed by *Campylobacter coli*, is the most prevalent *Campylobacter* spp. internationally, both in terms of human clinical cases and in livestock testing (BIOHAZ, 2010; Kaakoush *et al.*, 2015). *C. jejuni* is most prominent in domestic poultry and cattle populations, while *C. coli* is more often isolated from domestic swine (Horrocks *et al.*, 2009). Many other *Campylobacter* species, also capable of causing human disease, have previously been thought of as 'emerging' pathogens (Man, 2011; Kaakoush *et al.*, 2015) but have not become more prevalent than 6.5% of European cases of campylobacteriosis combined (EFSA and ECDC, 2018). These usually arise from sources besides commercial livestock; for example, *Campylobacter lari* is primarily associated with wild birds (Waldenstrom *et al.*, 2002) and *Campylobacter upsaliensis* is most common in dogs (Horrocks *et al.*, 2009).

1.1.4 Genomics

The archetypical *Campylobacter* reference strain has, for most experiments, been *C. jejuni* NCTC 11168, first isolated in 1977 (Skirrow, 1977) as part of the initial research into the species as a human pathogen, and was the first strain to be whole genome sequenced in 2000 (Parkhill *et al.*, 2000). It has around 170 protein-coding genes that are functionally essential for its survival (Mandal, Jiang and Kwon, 2017) out of a total gene number of 1643 (Gundogdu *et al.*, 2007). Compared to most bacterial genomes,

Campylobacter spp. has an unusually low number of insertion or phage-associated sequences (Parkhill *et al.*, 2000), which could be indicative of a cell capsule derived protection that *Campylobacter* possesses against phage attack (Young, Davis and DiRita, 2007).

One of the most unique structures present in the *C. jejuni* genome are hypervariable regions on the chromosome which confer a high level of genomic plasticity; this enables the organism to adapt rapidly to multiple host environments (Parkhill *et al.*, 2000; Stahl and Stintzi, 2011; Woodcock *et al.*, 2017). The function of these regions is in part evidenced by the fact that most of the hypervariable regions cover clusters of genes strongly associated with virulence, such as flagellar modification and cell surface polysaccharide genes (Parkhill *et al.*, 2000; Duong and Konkel, 2009). The inherent genetic diversity of *Campylobacter* spp. has enabled it to be one of the most successful targets for using Multilocus Sequence Typing as a tool for speciation and genomic analysis (Dingle *et al.*, 2001; Duong and Konkel, 2009).

1.1.5 Virulence factors

The genes necessary for the survival of *Campylobacter* once inside the gut of its host remain relatively unknown when compared to those recognised for pathogens such as *Salmonella* (Bolton, 2015), although some have been identified relating to motility, cell adhesion and invasion, and toxin production (Bang *et al.*, 2004).

Campylobacter motility is unusual due to its polar flagellae and helical shape, enabling it to move easily in a corkscrew fashion through viscous gut contents (Ferrero and Lee, 1988). The gene for the extracellular filament multimer *flaA* is highly conserved throughout *Campylobacter* species and is key for motility (Nachamkin, Yang and Stern, 1993; Jones *et al.*, 2004; Bolton, 2015). Upregulation of motility-related genes has been observed during colonisation of the avian gut (Hendrixson and DiRita, 2004).

Adhesion to gut epithelial cells is imperative for their invasion by *Campylobacter*. This is primarily initiated through a bacterial cell surface protein encoded by the highly conserved gene *cadF*, which binds to fibronectin, a glycoprotein found on gut epithelial cell membranes (Konkel *et al.*, 1997), and causes a gene expression cascade resulting in cell invasion (Ziprin *et al.*, 1999). The flagellum appears to have an important secondary

role during the invasion process, particularly through utilising the flagellar export apparatus to excrete a set of extracellular proteins which appear vital for invasion, the *Campylobacter* invasion antigens (Konkel *et al.*, 2000, 2004; Christensen, Pacheco and Konkel, 2009).

Campylobacter spp. have been observed producing many toxins (Wassenaar, 1997), the most well-known and well-studied of which is Cytolethal Distending Toxin (CDT) (Bang *et al.*, 2004). Some strains also produce lipo-oligosaccharides, which trigger the human immune response in such a way as to bring about Guillian-Barré Syndrome (Yuki *et al.*, 2004; Godschalk *et al.*, 2007), a rare side-effect of *Campylobacter* infection.

1.2 *Campylobacter* in humans

1.2.1 *Campylobacter* as a pathogen

Campylobacter infection in humans usually results in a disease known as campylobacteriosis. The main symptom is acute diarrhoea, accompanied by abdominal pain and occasionally fever, which is self-limiting and usually lasts around one week. More infrequent symptoms include headache, muscular ache, blood in the diarrhoea and vomiting (Blaser *et al.*, 1979; Blaser, 1997; Allos, 2001; Zilbauer *et al.*, 2008; Wilson and Wilson, 2021). There is potential for these symptoms to persist up to several weeks, but asymptomatic infections are not unknown (Allos, 2001; Wilson and Wilson, 2021). These clinical manifestations are the result of acute inflammation caused by bacterial invasion of the intestinal epithelium (Zilbauer *et al.*, 2008).

1.2.2 Prevalence and impact on public health

Campylobacter spp. infection is probably responsible for most cases of bacterial gastroenteritis observed internationally, particularly in the developed world (Wagenaar, French and Havelaar, 2013; Kaakoush *et al.*, 2015; EFSA and ECDC, 2021). There are an estimated 250,000 cases of campylobacteriosis in the UK every year (McCarthy *et al.*, 2021), 1.3 million in the United States (Scallan *et al.*, 2011) and 9 million in the European Union (EFSA, 2014), although actual rate of reporting in all countries is much lower than these estimates (Scallan *et al.*, 2011; EFSA, 2014; Kaakoush *et al.*, 2015). Disease caused by *Campylobacter* is an enormous strain on public health and economies internationally; in the European Union alone, an estimated €2.4 billion are lost in productivity each year

due to workplace absence as a result of *Campylobacter* infection (EFSA, 2014). There were over 20,000 reported hospitalisations due to campylobacteriosis in the European Union in 2017, with 45 reported deaths (EFSA and ECDC, 2018). Over 80% of hospital admissions in the UK for food poisoning and 80,000 annual medical consultations are due to *Campylobacter*, with a national cost of £133 million from the organism in 2000 (Humphrey, 2006; Wilson and Wilson, 2021).

1.2.3 Sources of infection in humans

Campylobacteriosis is primarily a foodborne zoonosis (Humphrey, 2006; Silva *et al.*, 2011; Wagenaar, French and Havelaar, 2013; Skarp, Hänninen and Rautelin, 2016; Wilson and Wilson, 2021), acquired from consumption of, or cross-contamination from, undercooked contaminated chicken (Cody *et al.*, 2010).

Human *Campylobacter* infection overwhelmingly arises from the poultry reservoir as a whole (Wilson *et al.*, 2008; Sheppard *et al.*, 2009; BIOHAZ, 2010; Wagenaar, French and Havelaar, 2013; Skarp, Hänninen and Rautelin, 2016; Rosner *et al.*, 2017; Duarte *et al.*, 2019). Up to 80% of European human incidences of campylobacteriosis can be attributed to poultry (BIOHAZ, 2010) and up to 70% of cases globally (Wilson and Wilson, 2021). Transmission from other livestock, such as cattle and swine, is also possible and attested to in clinical cases, but less prevalent than from poultry (Wilson *et al.*, 2008; Sheppard *et al.*, 2009; Wagenaar, French and Havelaar, 2013). A minority of cases may also be acquired from environmental sources such as water and soil, although these too are usually associated with the presence of livestock (Jones *et al.*, 2017; Sanderson *et al.*, 2018).

1.2.4 Treatment of human infection

Since the infection is usually self-clearing after around one week, most treatment involves rehydration and electrolyte management until symptoms pass (Allos, 2001; Wilson and Wilson, 2021). Antibiotics will only be considered for unusually severe or long-lasting symptoms, or for immunocompromised patients (Allos, 2001). However, most cases do not require specific treatment.

1.2.5 Post-infection complications

Campylobacter infection is a common precursor to some rare, but more severe and long-lasting conditions. These include Guillain-Barré Syndrome, Miller-Fisher Syndrome, *Campylobacter* bacteraemia and Reactive Arthritis (McCarthy, 2001; Hannu *et al.*, 2002; Humphrey, 2006; Louwen *et al.*, 2012; Hansson *et al.*, 2016). Guillain-Barré Syndrome is highly associated with prior *Campylobacter* infection, with a *C. jejuni* infection providing an antecedent to the onset of the syndrome in 25-30% of observed cases and raising the chance of acquisition 100-fold compared to the general population (Allos, 2001; McCarthy, 2001; Hansson *et al.*, 2016).

1.3 *Campylobacter* epidemiology in broilers

1.3.1 *Campylobacter* in avian species

Although capable of colonising the gut of most homeothermic organisms, *Campylobacter* spp. appear to be preferentially adapted to colonise the gastrointestinal tracts of avian species, as their optimal growth conditions match well with environments observed inside avian guts (Newell, 2001, 2002). Domestic poultry species, including ducks, turkeys, and chickens, are especially prone to infection (Weber *et al.*, 2014).

1.3.1.1 *Campylobacter* in chickens

Of all poultry species, the domesticated chicken is perhaps the one most associated with *Campylobacter* (Sahin *et al.*, 2015). A 2008 Europe-wide survey of broiler chicken flocks (EFSA, 2010) determined that over 71.2% of batches sampled at point of slaughter were contaminated with *Campylobacter* (n = 10,132). Though highly prevalent in mature flocks, *Campylobacter* is rarely detected in chickens under the age of around two weeks (Newell and Fearnley, 2003), in part due to the protective presence of maternal antibodies (Sahin *et al.*, 2003). Infection is primarily acquired through the faecal-oral route (Sahin, Morishita and Zhang, 2002) and therefore rapidly spreads through commercial chicken production facilities, where birds are kept in proximity with shared bedding and feed, usually infecting every bird within a few days of onset (Newell and Fearnley, 2003; Awad, Hess and Hess, 2018; Sandilands *et al.*, 2018).

Campylobacter is usually contained to the chickens' caeca and small intestines but can also spread to other tissues, such as the liver, in the case of the most virulent strains

(Meade *et al.*, 2009; Williams *et al.*, 2013). Infection actively damages the chicken gut, with observable injury occurring within seven days of exposure to *Campylobacter*, irrespective of breed or production system (Humphrey *et al.*, 2014; Awad *et al.*, 2015).

1.3.1.2 Commensal or pathogen?

For much of the history of the study of *Campylobacter* in non-human species, it has been considered by some authors to primarily be a commensal in chickens (Newell, 2002; Newell and Fearnley, 2003; Lee and Newell, 2006; Sahin *et al.*, 2015). However, in the last decade, the consensus scientific opinion has been shifting towards *Campylobacter* having a pathogenic effect, particularly in commercial chicken populations (Smith *et al.*, 2005; Humphrey *et al.*, 2014; Wigley, 2015; Pielsticker *et al.*, 2016). Chickens produce a marked immune response upon infection (Smith *et al.*, 2005; Humphrey *et al.*, 2014; John *et al.*, 2017) as their gut epithelial cells are invaded (Van Deun *et al.*, 2008; Humphrey *et al.*, 2014), resulting in inflammation of the intestinal mucosa and caeca.

1.3.1.3 Environmental prevalence on farm

Campylobacter spp., despite their apparent fastidiousness, are often isolated from environmental samples such as soil, sand, or water, though they are incapable of active propagation under such circumstances (Bronowski, James and Winstanley, 2014; Gölz *et al.*, 2018). Although keeping chickens in houses offers a degree of protection from environmental *Campylobacter* compared to free-ranging birds (Näther *et al.*, 2009; Allen *et al.*, 2011), numerous parts of the farm environment surrounding chicken houses can be shown to act as potential reservoirs for infection. Nearby livestock and bodies of water, wild animals such as rodents and birds, tarmac road surfaces, chicken transport crates and vehicles, chicken house anterooms and even the air surrounding the chicken houses can all contain detectable *Campylobacter* which may be horizontally transferred to the chickens (Newell and Fearnley, 2003; Bull *et al.*, 2006; Ridley *et al.*, 2011; Ellis-Iversen *et al.*, 2012; Robyn *et al.*, 2015; Battersby, Whyte and Bolton, 2016b; Frosth *et al.*, 2020).

1.3.1.4 Flock infection risk factors

As there are diverse possible environmental reservoirs for *Campylobacter* in a farmyard setting, it is perhaps unsurprising that many anthropogenic factors relating to farm practice are considered risk factors for flock infection.

The practice of 'thinning' is commonplace in poultry production. Approximately one third of the birds will be taken for slaughter at a first harvest, allowing the rest to grow larger in the remaining space without overcrowding for a second harvest at a later date. This allows for a range of carcass sizes available at retail and increases the number of kilogrammes of meat that can be produced by a given size of poultry house floor area. However, it does introduce a major breach in biosecurity when vehicles, equipment and personnel move in and out of the poultry house as part of the catching process, all of which have been shown to be potential vectors for *Campylobacter* (Allen *et al.*, 2008; Newell *et al.*, 2011; Ridley *et al.*, 2011; Battersby, Whyte and Bolton, 2016a). For this reason, numerous studies have implicated thinning as a major route of horizontal transmission for *Campylobacter* (Hald, Rattenborg and Madsen, 2001; Koolman, Whyte and Bolton, 2014; Higham *et al.*, 2018).

Certain chicken farming styles, such as free-range, have been observed as being more conducive to *Campylobacter* acquisition than others, perhaps due to the reduced barrier between the chickens and potential external contaminants (Näther *et al.*, 2009; Allen *et al.*, 2011), but this is not reflected in carcass contamination seen at UK retail (Jorgensen *et al.*, 2019, 2021) or in strains of *Campylobacter* seen in sympatric wild and domesticated birds (Griekspoor *et al.*, 2013).

1.4 *Campylobacter* in other species

Campylobacter spp. are not only present in humans and birds, but in a range of other species as well. Cattle have been noted as a prominent source of *C. jejuni* infection in humans, second only to chickens (Wilson *et al.*, 2008; Kaakoush *et al.*, 2015), with the additional risk of contaminated dairy products contributing to observed cases (Fernandes *et al.*, 2015; EFSA and ECDC, 2018, 2019, 2021). Swine and pork products are also often associated with *Campylobacter*, but with *C. coli* as the dominant species isolated from contaminated specimens (Horrocks *et al.*, 2009; Kaakoush *et al.*, 2015).

Domestic companion animals, such as dogs and cats, are also known to be carriers of *Campylobacter*, but primarily harbour species such as *C. upsaliensis* and *C. helveticus* which do not contribute greatly to human disease (Horrocks *et al.*, 2009; Kaakoush *et al.*, 2015).

It has been shown that a variety of animals can potentially carry *Campylobacter* to environments where they can cause infections in humans or chickens, including bats (Hazeleger *et al.*, 2018) and rodents (Ellis-Iversen *et al.*, 2012; Kaakoush *et al.*, 2015). However, transmission and carriage amongst wild mammals remains relatively unexplored in current literature, with little evidence presented in research for a prominent role in their contributions to infections in chickens or humans (Kaakoush *et al.*, 2015).

1.5 Seasonality of *Campylobacter* incidence

1.5.1 Seasonal trends in humans

Campylobacteriosis is endemic in every country for which data is available (Kaakoush *et al.*, 2015). Monitoring by public health bodies has revealed a widespread and distinctive seasonal pattern in human infection, collated by Lake et al. (2019) using Europe-wide data. The pattern is broadly temperature-linked, with warmer summer months corresponding with elevated infection rates, and colder winter months being associated with reduced infection numbers. This pattern has also been observed outside of Europe, with associations between high temperatures and high numbers of cases of campylobacteriosis being found in areas such as Canada, Australia, and New Zealand (Nylen *et al.*, 2002; Sari Kovats *et al.*, 2005; David *et al.*, 2017). However, despite many years of monitoring and study, the primary driving forces behind the seasonal trend in cases has not been made clear and remains a topic of speculation (Nylen *et al.*, 2002; Patrick *et al.*, 2004; Meldrum *et al.*, 2005; Sari Kovats *et al.*, 2005; Jore *et al.*, 2010; Strachan *et al.*, 2013).

In the UK, the peak of human campylobacteriosis typically occurs in June (Louis *et al.*, 2005; Meldrum *et al.*, 2005; Nichols *et al.*, 2012; Lake *et al.*, 2019). Overall case numbers have increased over the course of decades of monitoring (Nichols *et al.*, 2012),

which has given rise to fears that temperature rises resulting from anthropogenic climate change will cause an increase in *Campylobacter* infections (Lake, 2017).

1.5.2 Seasonal trends in broiler chicken flock infection

Much like the seasonal patterns observed with humans, *Campylobacter* infection in broiler chicken flocks follows a predictable and recurrent annual pattern, corresponding with temperature (Sari Kovats *et al.*, 2005; Jore *et al.*, 2010). Human and broiler chicken *Campylobacter* case rates tend to align well in parallel time-series studies (Patrick *et al.*, 2004; Hartnack *et al.*, 2009; Jore *et al.*, 2010), suggesting that both are influenced by the same seasonal factors. Warm, summer months correspond with heightened *Campylobacter* infection amongst chickens in every EU country observed (Patrick *et al.*, 2004; Hartnack *et al.*, 2009; Jonsson *et al.*, 2010, 2012; Jore *et al.*, 2010; Chowdhury *et al.*, 2013; Weber *et al.*, 2014), the United Kingdom (McDowell *et al.*, 2008; Jorgensen *et al.*, 2011) and Japan (Ishihara *et al.*, 2017). Seasonal peaks and dips in broiler chicken *Campylobacter* infection have also been observed in tropical countries without distinct temperature-driven seasons, such as Sri Lanka and Thailand (Prachantasena *et al.*, 2017; Kalupahana *et al.*, 2018). The principal causative agents behind these seasonal trends, however, are equally as unidentified as those seen in human cases (Hansson *et al.*, 2016).

1.6 Defining the Problem

1.6.1 Understanding broiler chicken production in the United Kingdom

The UK poultry industry is a large and growing branch of agriculture, with over 1.4 billion eggs set to hatch for use as meat chickens in 2021 alone (DEFRA, 2022). Modern broiler chickens, as used in the UK, are a fast-growing breed capable of reaching an optimal slaughter weight in five weeks (Bennett *et al.*, 2018). The average Briton consumes around 30 kg of poultry meat per capita per annum, a trend that has been increasing in recent years (AVEC, 2021). Although the precise percentage changes with trends in global trade, the UK is 93-97% self-sufficient in terms of poultry meat production (AVEC, 2021). A UK broiler chicken farm will undergo around 7-8 production cycles per year (ADAS, 2019) and operates on an ‘all in, all out’ policy (Aviagen, 2018; DEFRA, 2018), where for each production cycle, no new chicks are added after their

initial introduction to the house, and no chickens are left remaining at the end. Figure 1.1 represents the interior of a typical UK broiler house.

Figure 1.1: Interior photograph of a wooden posted poultry house on a UK broiler chicken farm in Herefordshire. Annotated features are Liquefied Petroleum Gas (LPG) heating system (A), ventilation inlets (B), windows for natural light (C), feed dispensers (D) and water nipples (E).

1.6.1.1 UK geography and climate

According to the Köppen-Meier climate classification index, the UK is considered a warm-temperate region, with a fully humid precipitation pattern and warm summer

(Kottek *et al.*, 2006). This means that annual average minimum temperatures are above -3°C and rainfall occurs year-round. Considering its location in the northern hemisphere, the longest days and highest temperatures tend to fall around July, and the shortest days and lowest temperatures around December (National Geographic, 2022).

The area of the UK of particular importance to the presented body of work is the county of Herefordshire and its immediate surroundings. This region, located in England at the southern edge of the Welsh borders, sits at approximately 75 metres above sea level and at a latitude of approximately 52° North (MET Office, 2022). Figure 1.2 presents the recorded climate in the region between 1991 and 2020.

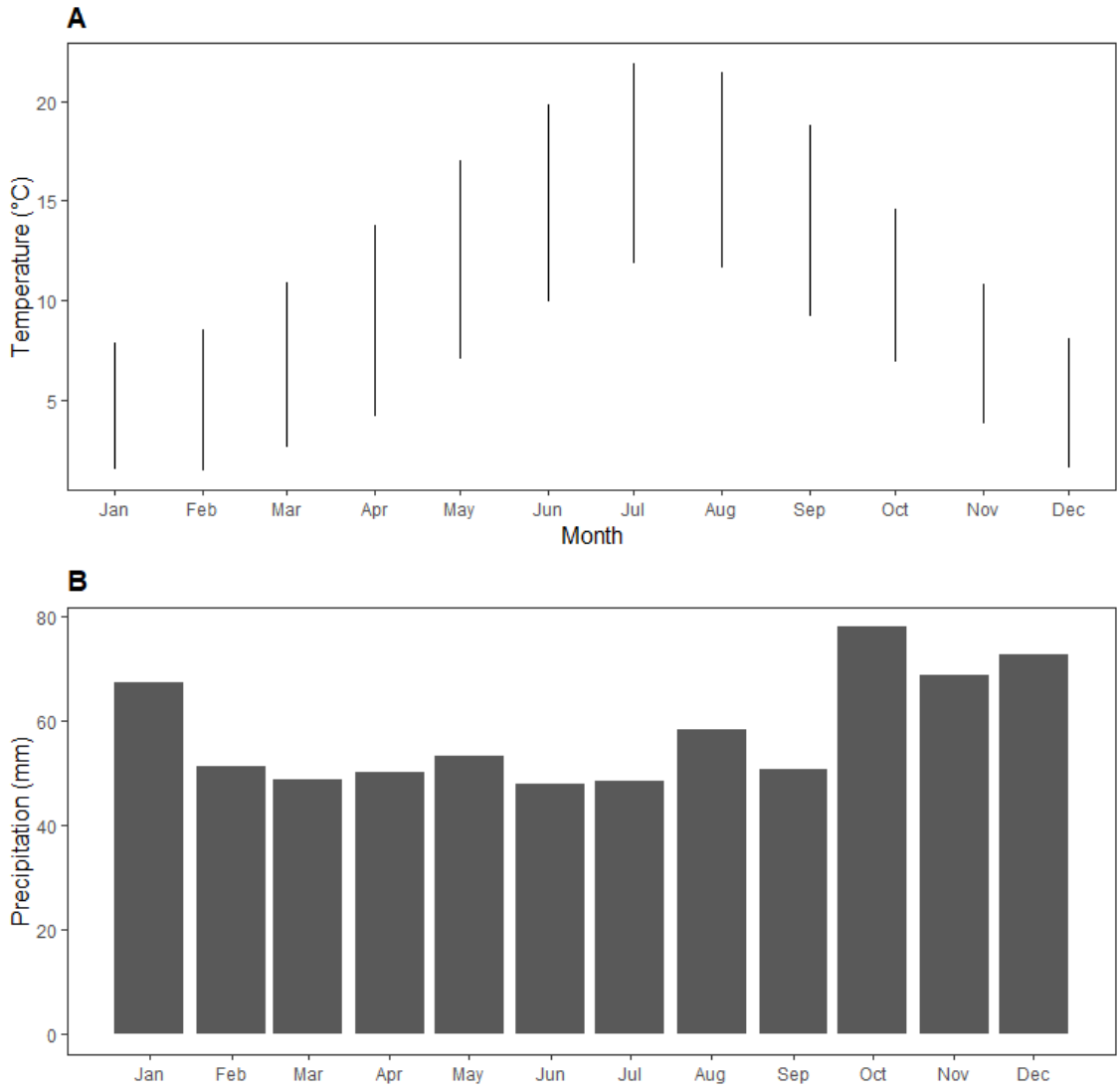

Figure 1.2: Range of daily average temperatures (Figure 2.2A; based on hourly temperature readings) and total rainfall (Figure 2.2B) observed at Credenhill Observing Site between 1991 and 2020 (MET Office, 2022).

1.6.2 UK husbandry standards and biosecurity

Of chicken produced in the UK, approximately 90% conforms to the minimum standards laid out by Red Tractor Assurance (Red Tractor, 2019). Additionally, legislation ensures that housed broiler chicken flocks in the UK are not caged, but are instead permitted the range of large, open-plan chicken houses (DEFRA, 2018; Avara Foods, 2022) and stocking densities are limited to 39 kg/m^2 to prevent overcrowding (DEFRA, 2018; Avara Foods, 2022). Windows providing natural light are recommended for all chicken houses (Red Tractor, 2019).

To protect the birds under a farmer's care from disease, and hence lower mortality and raise expected yields, it is expected that biosecure areas and practices are adopted throughout a poultry farm (Aviagen, 2018; DEFRA, 2018; Red Tractor, 2019). Many of these have been legally mandated specifically to try and reduce *Campylobacter* transmission (European Commission, 2017). Currently recommended practices include removing soiled bedding and disinfecting chicken houses between flocks, ensuring new farms are built at distance from existing ones, limiting site access to only necessary staff and visitors, changing boots when entering biosecure areas, and providing sanitation facilities for hands and boots (Aviagen, 2018; DEFRA, 2018; Red Tractor, 2019). These all attempt to create a disease breaks, both during and between production cycles, to try and minimise the risk of colonisation by *Campylobacter* and other pathogens.

The effects of heat stress are keenly felt by chickens and can impact their welfare severely, weakening their ability to respond to infection challenges and resulting in economic losses from reduced yields (Quinteiro-Filho *et al.*, 2012; Lara and Rostagno, 2013; Rostagno, 2020). For this reason, integrated management of heating and ventilation systems are of critical importance for ensuring optimal broiler chicken husbandry standards (Aviagen, 2010).

1.6.3 *Campylobacter* prevalence in the UK chicken industry

Campylobacter perseveres throughout the poultry meat industry; once infected birds are slaughtered, the carcasses will often still be contaminated with *Campylobacter* by the time they reach supermarket shelves, despite how sensitive *Campylobacter* is to its environment (Gölz *et al.*, 2018). European Union and UK law has specified a mandate for routine *Campylobacter* testing in poultry production environments since 2017, specifying that, as of present, no more than 30% of carcasses tested may contain over 1,000 cfu/g before intervention is recommended. The threshold is due to be reduced to 20% of carcasses as of the beginning of 2025 (European Union, 2017; National Archives, 2017).

Unlike slaughterhouse testing, there is currently no mandated scheme for testing for *Campylobacter* on poultry farms. However, despite this, on-farm testing is widely practiced in the industry, with the most common broiler chicken flock methodology utilising overshoe-style swabs worn over the farmers' boots to collect a community sample of the poultry house interior (Vidal *et al.*, 2013; Madden *et al.*, 2014, 2016).

1.6.3.1 Retail environment prevalence

Studies consistently show a high prevalence of *Campylobacter* on retail chicken products, regardless of geographic location. Reports suggest that contamination reaches almost 65% in the United States (Berrang *et al.*, 2018), up to 90% in Australia (Walker *et al.*, 2019) and 55% in Japan (Ohnishi and Hara-Kudo, 2021). Europe-wide surveillance (n = 13,445) shows a presence on shelves of almost 38% of samples tested (EFSA and ECDC, 2018). A weighted average from studies across Africa suggests a prevalence of around 45% in chicken meat on the continent (Asuming-Bediako *et al.*, 2019).

A comprehensive investigation following slaughterhouses that represent almost 90% of broiler flocks processed in the UK between 2007 and 2009 found that 79% of carcass samples tested were contaminated with *Campylobacter* (Lawes *et al.*, 2012). Testing was conducted by the Food Standards Agency (FSA), the UK's governmental agency responsible for food-related public health, for *Campylobacter* presence on whole chickens sold in UK supermarkets, commencing in 2014, at which point it was found that

83% of supermarket carcasses were positive for *Campylobacter*, of which 22% were contaminated with over 1,000 cfu/g (Food Standards Agency, 2015; Public Health England, 2015). In their report on this, all supermarkets were named, along with the corresponding *Campylobacter* prevalence that was found on their chickens. This spurred action within the industry such that, by the time the 'name and shame' reports by the FSA ceased in 2017-2018, contamination levels had been reduced such that 56% of supermarket chicken carcasses assessed by public health bodies in the UK had some level of *Campylobacter* present, with only 7% being 1,000 cfu/g or higher, well within the limits outlined in government policy (Jorgensen *et al.*, 2019). Jorgensen *et al.* (2019), who reported these findings, could not speculate on what specific changes led to this reduction. Industry publications from the time show that a number of major UK poultry processors adopted the use of SonoSteam, developed by Force Technology, in their production lines, which uses a combination of steam and ultrasound to remove bacterial contamination (Poultry World, 2015; Refrigerated And Frozen Foods, 2015; Food And Drink Business Europe, 2017). However, due to the competitive nature of the industry, many of the precise mechanisms used to reduce Campylobacter contamination remain trade secrets.

An EU report from 2011 stated that that a 50% reduction in *Campylobacter* contamination incidence in UK broiler chicken flocks from the 76% reported at that time would result in a 34% risk reduction for humans (BIOHAZ, 2011) While current carcass contamination levels in the UK represent a distinct improvement compared to previous years, no significant improvements have been made since 2016 (Jorgensen *et al.*, 2021). Considering that 360 cfu is estimated to be the minimum infectious dose for *Campylobacter* in humans (Hara-Kudo and Takatori, 2011), this potentially does still indicate a severe public health burden, despite the progress that has been made.

Control of *Campylobacter* at a processing plant level is implemented in multiple ways. Carcass scalding, rinsing and rapid chilling, routinely used in EU and UK slaughterhouses, all contribute to the removal and inactivation of microorganisms and do give a measurable reduction in *Campylobacter* contamination (Alter, 2016; European Commission, 2017; Umaraw *et al.*, 2017). However, contamination levels at retail are still high enough to warrant public health intervention. Newer, more experimental

systems such as combined ultrasound-heat treatment, while proven to be effective, are expensive to implement and lead to noticeable damage to the product (Alter, 2016).

According to polling, the most acceptable form of *Campylobacter* control on fresh chicken, for UK consumers, was found to be improved farm hygiene practices. Opinions on freezing, vaccination and feed additives are divisive, while extreme interventions such as irradiation or chemical carcass washing are highly unpopular (MacRitchie, Hunter and Strachan, 2014). This is broadly reflective of observed industry practice and public policy, which currently does not allow for irradiation or chemical treatment of carcasses.

Due to the considerable expenditure and limited results obtained from attempting to reduce *Campylobacter* load on carcasses at the processing plant, attention has increasingly been turning to limiting *Campylobacter* infections on farms before the chickens reach the slaughterhouse. The primary defences employed on commercial farms are biosecurity and farm hygiene (Aviagen, 2018). Control measures known to be effective in preventing *Campylobacter* colonisation of a poultry house include boot disinfection, changing boots before entering poultry houses, water treatment and cleaning of the farm environment and inside the poultry house (Gibbens *et al.*, 2001; Newell *et al.*, 2011; Ghareeb *et al.*, 2013; Hansson *et al.*, 2016; Meunier *et al.*, 2016; European Commission, 2017). However, current solutions are not universal, providing more benefit in some farm environments than others, potentially burdening farmers implementing certain control measures with a large cost and inconvenience while providing minimal impact on *Campylobacter* contamination of their flocks (Havelaar *et al.*, 2007; Hansson *et al.*, 2016; Pitter *et al.*, 2018).

1.6.4 Prior findings in *Campylobacter* seasonality in UK flocks

An investigation by Lawes et al. (2012) assessing UK broiler chicken flocks slaughtered between 2007 and 2009 revealed that the prevalence of *Campylobacter* was highly seasonal, with incidence at a peak anywhere between June and November. This is corroborated by other work, such as that of Ellis-Iversen et al. (2009), who found that UK broiler flocks are most likely to be contaminated with *Campylobacter* in July or August.

Given that this seasonal trend is well-documented in the UK, there has been some investigation into its origins presented in published research. Hypotheses of the underlying causes of the mirrored seasonality observed in humans have been put forward by studies, but this has not been the case in chickens, despite them being a primary source of human infections (Hansson *et al.*, 2016). This is true not just in the UK, but elsewhere in the world as well.

1.6.4.1 Key findings of UK-based research into *Campylobacter* in broiler chickens

Reference	**Finding**
Meldrum *et al.* (2005)	Both human and chicken cases of *Campylobacter* in the UK peak in the summer, but are not strictly temporally linked.
Bull *et al.* (2006)	Strains of *Campylobacter* found in the environment around the chicken house have been found to be identical to those colonising the chickens. *Campylobacter* from infected flocks could be found in the air downstream of the chicken house.
Wilson *et al.* (2008)	97% of sporadic human incidence of *Campylobacter* infection in humans can be genotypically traced to farmed animals, such as chickens.
Ellis-Iversen *et al.* (2009)	*Campylobacter* peaks in UK chicken flocks between July and August. Flocks were more likely to be infected if the one that previously occupied the house was also positive.
Lawes *et al.* (2012)	*Campylobacter* amongst commercial chicken flocks in the UK is at its highest in summer or autumn months. Flocks were more likely to be infected if they had been previously thinned.
Williams *et al.* (2013)	Different breeds of chickens lend themselves to different severities of illness when challenged by *Campylobacter.*
Goddard *et al.* (2014)	The majority of *Campylobacter* infections in UK broiler chicken flocks begin towards the end of the production cycle (30-35 days).

1.6.5 Open problems and unanswered questions

At the time of writing, there are substantial knowledge gaps in understanding *Campylobacter* infections amongst broiler chickens. Of primary concern to poultry producers are the lack of knowledge regarding the transmission routes *Campylobacter* takes to cause the initial infection of a flock, and the driving forces behind the seasonality observed in chicken infections, both poorly understood as of the present (Hansson *et al.*, 2016). So far, only suggestions of what drives seasonality of *Campylobacter* has been put forward in the form of associated risk, rather than definitively identified factors which will result in flock infection.

1.7 Thesis Objectives

1.7.1 Working hypotheses

This thesis is guided by the assumption that incidence of campylobacteriosis in broiler chickens normally follows a pronounced seasonal trend, with heightened cases during warmer times of the year. Using this principle, we aim to test the following hypotheses:

- That *Campylobacter* in the environment around chicken farms is capable of transmission to broiler chickens, and this environmental *Campylobacter* is more numerous or prevalent over warmer months.
- That chickens become more susceptible to *Campylobacter* during summer months, through the impact of stressors less prevalent at other times of year.

1.7.2 Statement of objectives

The aim of the research detailed in this thesis is to elucidate the underlying reasons behind the seasonal incidence of *Campylobacter* infection in commercial broiler chicken flocks. It shall:

- Assess whether flocks become *Campylobacter* positive earlier in the production cycle during summer months, and whether this impacts on the rate at which flocks are infected with *Campylobacter.*

- Monitor environmental levels of *Campylobacter* and compare with the *Campylobacter* status of concurrent broiler chicken flocks, thereby assessing any potential impact on infection levels.
- Determine whether any seasonal events (temperature and humidity fluctuations, farming practices, etc.) or parameters pertaining to each farm (ventilation system, house construction materials, etc.) can be associated with more frequent *Campylobacter* infections.
- Determine whether proportional changes in the chicken gut microbiota composition can be correlated with *Campylobacter* infections, and whether there are seasonal fluctuations in its composition.
- Assess whether damage to the chicken gut as a result of *Campylobacter* infection, or extraintestinal spread of *Campylobacter*, is more severe or prevalent during certain seasons.

From this, a more complete picture of *Campylobacter* epidemiology in chicken flocks shall be constructed. Through this, it is hoped that this research shall provide an indication of where the industry can find better control solutions.

2 General Methods

2.1 Statement of Ethics

2.1.1 Human Participants

This study was performed in accordance with the Declaration of Helsinki (World Medical Association, 2013). Ethical approval was not sought from Swansea University Research Integrity, Ethics and Governance Committee because humans were not the subjects of the study. However, human volunteers did assist or facilitate sample collection. All farm staff gave verbal consent before any sampling occurred on either premises owned by the volunteers, or owned by Avara Foods Ltd.

2.1.2 Funding

The study was funded by Avara Foods Ltd, and conducted in association with Avara. Avara approved the study before volunteers were sought amongst their contracted farms. All farms were volunteered by their managers, who were informed before and throughout the study of all procedures conducted on their farms and were given copies of their corresponding results afterwards. No farmer was given identifiable information from any farms other than their own.

2.1.3 Animals

All samples were collected by trained, approved, qualified staff, and were collected in the course of routine duties and practices on farms and at the slaughterhouse. Staff, both from the farms and from Avara Foods Ltd, did not interact directly with the chickens for the specific purposes of the present study and did not collect samples from live animals. As no samples were collected from live animals and no experiments were conducted on live animals, approval was not required under the Animal Scientific Procedures Act (1986) (National Archives, 2022). Furthermore, as no samples were collected by Swansea University students or staff, approval was not required by Swansea University Animal Welfare and Ethical Review Body.

2.2 Farm descriptions and production routine

Farms were recruited through an email sent to the owners of all farms supplying Avara Foods Ltd. Eleven responses were received and all were included in the subsequent study. A survey was sent to each farm to gather information on the construction and surroundings of each farm, the results of which are presented in Table 2.1.

All volunteer farms were located within an area of around 2,000 km^2 across the counties of Herefordshire, Monmouthshire and Worcestershire in the United Kingdom (Figure 2.1). Farms varied from between three and fifteen dedicated chicken houses, with site capacities ranging from 78,000 to 327,000 birds per placement. One house on each site was utilised for the study, selected to be most representative of the typical house on that farm. Farms were assigned a code number to anonymise them in any reports published; these codes shall henceforth be used to refer to individual farms.

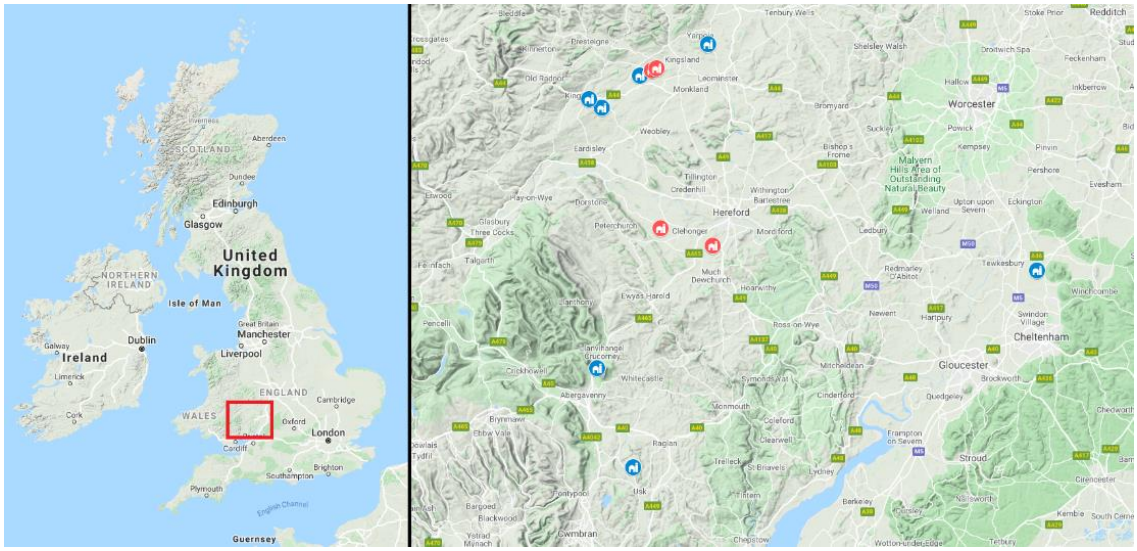

Figure 2.1: Map of the United Kingdom with highlighted area showing the location of farms used for the study. Red farms are directly owned by the company, blue are contracted farms. Images from Google Maps (Google, 2020).

Table 2.1: Summary of details collected from surveys of the eleven farms observed throughout the study period.

Farm Number	**1A**	**1B**	**1C**	**1D**	**2A**	**2B**	**2C**	**3A**	**3B**	**3C**	**3D**
Year of House Construction	2015	2000	2014	1990	1987	2000	2014	2005	1989	1984	1980
House Area (m^2)	2208	2231	2380	1333	2007	1673	2510	1314	1635	1636	948
Construction	Steel Clearspan	Steel Clearspan	Steel Clearspan	Wooden Posted	Steel Posted	Steel Posted	Steel Clearspan	Steel Posted	Wooden Posted	Wooden Posted	Wooden Posted
Heating System	LPG Overhead	Biomass Overhead	Biomass Underfloor	LPG Overhead	Biomass Overhead	LPG Overhead	Biomass Overhead	LPG Overhead	LPG Overhead	LPG Overhead	LPG Overhead
Ventilation System	Side Inlet, Summer Vents	Ridge Inlet, Summer Vents, Gable End Fans	Side Inlet	Side Inlet, Summer Vents, Gable End Fans	Side Inlet	Ridge Inlet	Side Inlet, Summer Vents	Ridge Inlet	Side Inlet, Summer Vents	Side Inlet, Summer Vents	Crossflow
Total Houses on Site	4	6	4	3	5	4	4	8	6	7	15
Primary Water Source	Borehole	Borehole	Borehole	Borehole	Borehole	Borehole	Mains	Borehole	Borehole	Mains	Mains

The eleven farms were observed for ten production cycles, hereby termed 'Phase One'. The duration of a production cycle is from when newly hatched chicks are first placed in the chicken house, to when the last grown birds are taken for slaughter. This is usually between 36 and 40 days, depending on how quickly the birds grow to a target weight. Birds are removed on two occasions: A primary clearance, known as 'thinning', at around 30 to 32 days of bird age whereupon approximately one third of birds are taken for slaughter, and a final clearance at the end of the cycle, during which the remaining birds are taken for slaughter. Upon completion of a production cycle, a period of 8 to 10 days, known as 'turnaround', is taken to disinfect the house and replace the bedding in preparation for the next production cycle. Table 2.2 gives the placement dates associated with each production cycle.

Table 2.2: Median dates on which each production cycle commenced for chicken flocks observed through Phase One of the project.

Production cycle number	Median placement date
1	20th September 2018
2	8th November 2018
3	27th December 2018
4	13th February 2019
5	3rd April 2019
6	20th May 2019
7	4th July 2019
8	20th August 2019
9	5th October 2019
10	21st November 2019

At the conclusion of Phase One, a subset of farms was selected for continued monitoring for a further seven production cycles, hereby termed 'Phase Two'. These farms were 1A, 1B, 2A and 3A. Table 2.3 gives the placement dates associated with each production cycle.

Table 2.3: Median dates on which each production cycle commenced for chicken flocks observed through Phase Two of the project.

Production cycle number	Median placement date
11	15th April 2020
12	3rd June 2020
13	21st July 2020
14	5th September 2020
15	22nd October 2020
16	8th December 2020
17	24th January 2021

2.3 Air Sample Preparation

Air samples were collected once per cycle on each farm using a Sartorius AirPort MD8 (Sartorius AG, Göttingen, Germany) as close to day five of the production cycle as possible (range = 3 to 10 days since start of production cycle).

The AirPort MD8 was placed in an upright position within an area between the chicken house incorporated in the study and a house immediately adjacent. The device was loaded with one 80 mm gelatine membrane (Sartorius product no. 17528-80-ACD), removed from its sterile packaging, using nitrile gloves to handle it, and one cubic metre of air (1,000 litres) was passed through at a rate of 50 litres per minute. Upon completion, the filter was detached and placed back into its original sterile packaging and returned to the laboratory.

Upon sample return, the gelatine filter of the air sample was removed from its plastic housing and manually broken up into small pieces, then fitted inside a 2 ml microcentrifuge tube. To this, 1,350 µl of nuclease-free water and 150 µl of 1,000 mg/l Proteinase K One (Thermo Scientific, Waltham, MA, USA) stock solution were added, and the microcentrifuge tube shaken at room temperature until the filter had completely degraded. The microcentrifuge tube was then centrifuged at 4,500 x g for five minutes and the supernatant removed. The DNA was extracted from the resulting pelleted material as per Section 2.6.

2.4 Bootsock Swab Preparation

Disposable overshoes made from 40 g non-woven polypropylene (Tunika Safety Products, Bolton, UK), henceforth referred to as bootsocks, were individually placed inside resealable bags (Minigrip LLC, Alpharetta, GA, USA) and distributed to the managers of each site in the study. One of these was used per day for every day in which live chickens were within the house under observation. Site personnel carried out all bootsock sampling duties.

2.4.1 Interior Swabs

For swabs taken within the poultry house, the bootsock was removed from its bag only once on the inner side of the double-barrier biosecurity system in the control room of the poultry house. From there, it was placed over one Wellington boot designated for use within the house and worn for the duration of the first welfare check made of the birds each day. Upon exiting the house on completion of duties, the bootsock was immediately removed from the Wellington boot and returned to its corresponding bag before leaving the inner double-barrier. The bag was then labelled with the date of collection and returned to the laboratory.

2.4.2 Exterior Swabs

For swabs taken outside the poultry house, the bootsock was removed from its bag at the site manager's office. From there, it was placed over one Wellington boot used for general farm purposes and worn for the walk from the site office, across the farm's yard, to the front door of the poultry house involved in the project. Before entering, the bootsock was immediately removed from the Wellington boot and returned to its corresponding bag. The bag was then labelled with the date of collection and returned to the laboratory.

2.4.3 Processing

Upon sample return, the sole of the bootsock with visible biological material adhered to it was excised with sterile scissors, placed back in its resealable bag and stored at 4°C until required for DNA extraction. When needed, the sample was placed in a stomacher bag (Seward Ltd., Worthing, UK) and 25 ml of sterile isotonic saline solution (Oxoid Ltd., Basingstoke, UK) poured upon it. The bag was then manually kneaded for one minute to

disperse any biological material collected into solution. The bag was left to stand for a further ten minutes to allow inorganic solids to sediment, then 5 ml of supernatant transferred to a bijou and stored at -20°C for future DNA re-extraction if needed. 1 ml of supernatant was also transferred directly to a 1.5 ml microcentrifuge tube, centrifuged at 8,000 x g for five minutes and the supernatant removed. DNA was extracted from the resulting pelleted material as per Section 2.6.

2.5 Viscera Collection

Viscera were only collected over Phase Two of the project. Sampling was conducted by factory quality assurance and laboratory staff at the Avara Foods Ltd. chicken processing plant at Grandstand Road, Hereford.

For every 'clearance' arriving from a production cycle under observation as part of the project, ten sets of viscera were collected from the FSA veterinary inspection point and transported to Avara's laboratory within the same site. From the viscera of each of the ten chickens, the following were prepared:

- The contents of the ileum, placed in a 7 ml bijou (Thermo Scientific, Waltham, MA, USA) and stored at -20°C.
- The contents of one caecum, placed in a 7 ml bijou and stored at -20°C.
- A section of ileum, approximately 1 cm in length, excised with sterile scissors, placed in a 7 ml bijou containing 2 ml of formalin and stored at room temperature.

Additionally, the liver was removed from the associated viscera, dipped in 70% ethanol, and flamed to sterilise the surface. From this, a small cube of approximately 0.5 cm^2 was excised using sterile scissors, placed in a 2 ml microcentrifuge tube containing 1 ml RNAlater (Invitrogen, Waltham, MA, USA) and stored at -20°C. Where possible, collected viscera samples were taken from the same chicken and identified together.

All samples stored at -20°C were transported to the laboratory at Swansea University for testing monthly, shipped on ice packs. These were immediately returned to a -20°C environment upon arrival.

2.6 DNA Isolation

Total DNA was isolated from processed air samples, liquid bootsock extracts, initial sample extracts and liver sections using a QIAGEN QIAamp DNA Mini Kit and from ileal or caecal contents using a QIAGEN QIAamp Fast Stool Mini Kit (QIAGEN, Hilden, Germany), both according to the manufacturers' instructions. Total DNA concentration for each sample was then determined using a NanoDrop One (Thermo Scientific, Waltham, MA, USA).

2.7 qPCR for *Campylobacter* Detection and Speciation

A quantitative real-time PCR using dual-labelled fluorescent probes was used to test for presence of *Campylobacter* spp. as a whole and provide a speciation if *C. jejuni* or *C. coli* were present.

For each sample under test, a reaction was prepared (25 µl) containing 12.5 µl Agilent Brilliant II qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), 1 µl of each primer (Table 2.4), 0.25 µl of each probe (Table 2.5), 3.75 µl of nuclease-free water and 2 µl of eluate from the DNA isolation detailed in Section 2.6. These were tested, alongside reference DNA of known concentration extracted from control strains (NCTC 11168 or M1 for *C. jejuni,* RM2223 or NCTC 13366 for *C. coli*) and purified water blanks, in an Agilent AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). The qPCR conditions consisted of 10 minutes at 95°C, then 40 cycles of 1 minute at 95°C then 1 minute at 55°C. Fluorescence results were analysed with Agilent AriaMx software v1.8 (Agilent Technologies, Santa Clara, CA, USA). Any samples which produced only a response in the *Campylobacter* spp. 16S probe and no response for either of the species-specific probes were tested again through the PCR described in Section 2.8.

Table 2.4: Primers used for quantitative PCR for Campylobacter detection and speciation.

Species	Target Gene	Primer	Sequence (5'-3')	Reference
Campylobacter jejuni	*HipO*	Forward	GTT ATT GGA AGG GGT GGT CA	Lisa K. Williams (personal correspondence, 2018)
		Reverse	GCC ACA ATA AGC AAA GAA GCA	
Campylobacter coli	*GlyA*	Forward	GCG TGA ATT TAG CGG AAA AG	
		Reverse	TAA GGG CAG GCG TTC CTA AT	
Campylobacter spp.	16S rRNA gene	Forward	CGT GCT ACA ATG GCA TAT ACA ATG A	Lund et al. (2004)
		Reverse	CGA TTC CGG CTT CAT GCT C	

Table 2.5: Fluorescent probes used for quantitative PCR for Campylobacter detection and speciation.

Species	Target Gene	Sequence (5'-3')	Reference
Campylobacter jejuni	*HipO*	[ROX] AGT GCT CCA GAA AAG GCA AA [BHQ2]	Lisa K. Williams (personal correspondence, 2018)
Campylobacter coli	*GlyA*	[FAM] GTG CCT GGC GAA ACT AGA AG [BHQ1]	
Campylobacter spp.	16S rRNA gene	[HEX] CAG AGA ACA ATC CGA ACT G [Eclip]	Lund et al. (2004)

2.7.1 qPCR Standard Curves

Four reference strains of *Campylobacter* spp. (NCTC 11168 and M1 for *C. jejuni,* RM2223 and NCTC 13366 for *C. coli*) were cultured on Columbia Blood Agar (Oxoid Ltd., Basingstoke, UK) and the DNA isolated and quantified as stated in Section 2.6. Using the known concentration of DNA, a copy number per microlitre was calculated for each strain by assuming an average genome size of 1.7 Mbp and a base pair molecular weight of 650 Daltons. Serial dilutions of each known quantity of DNA were analysed by quantitative PCR, as detailed in Section 2.7, producing the standard curves provided in Figure 2.2.

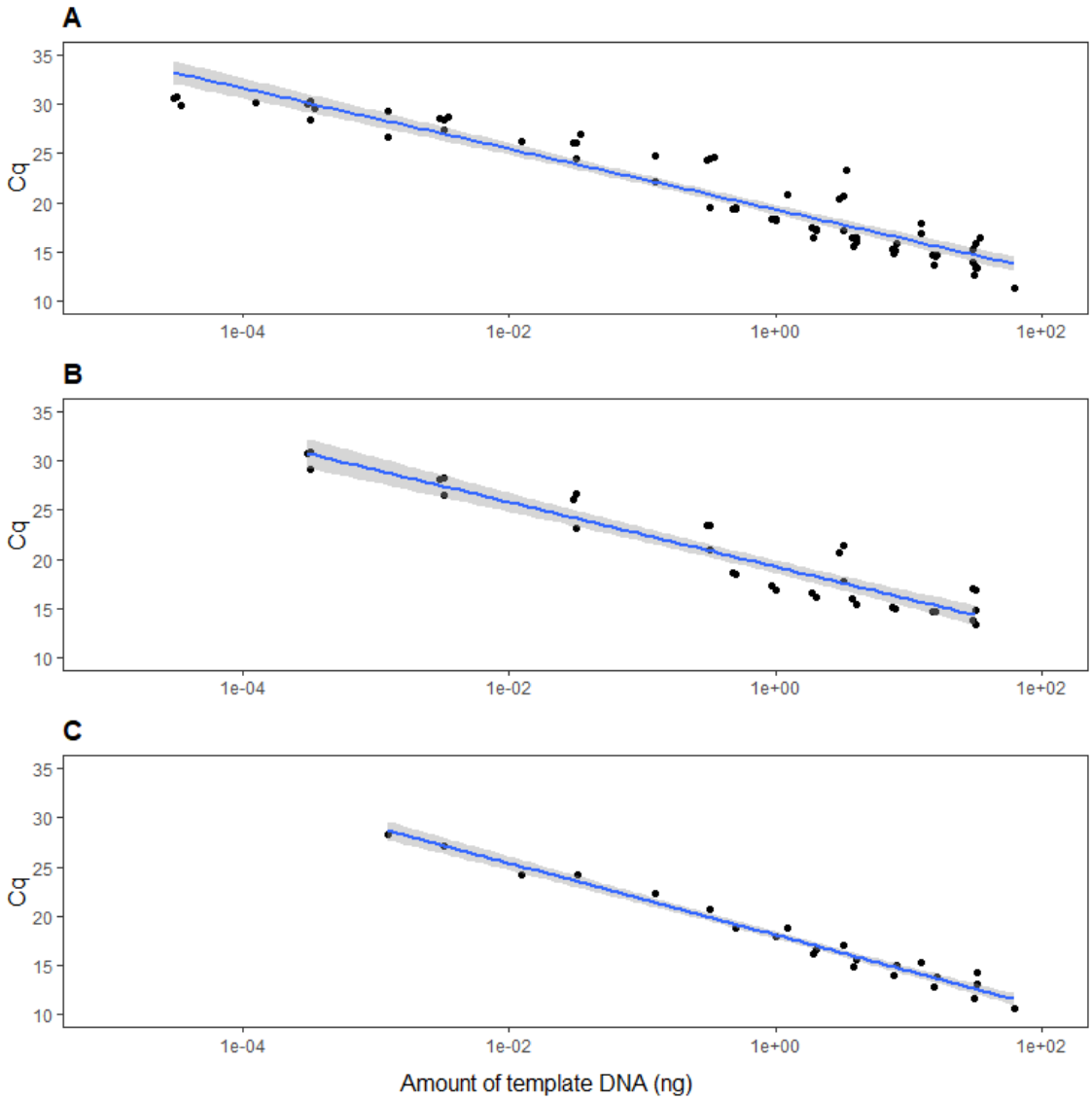

Figure 2.2: Standard curves used in calculations of DNA concentration from C_q values obtained at qPCR using the primers detailed in Table 2.4 and probes detailed in Table 2.5. Demonstrated curves are for Campylobacter 16S (Figure 2.2A, top), Campylobacter jejuni (Figure 2.2B, middle) and Campylobacter coli (Figure 2.2C, bottom).

2.7.2 qPCR Copy Number Calculations

The calculations used in this project to quantify the number of *Campylobacter* detected in each sample are derived primarily from the established $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The derivation begins with the following known equations:

$$X_t = X_0 \times (1 + E_X)^{C_{t,X}}$$
$$R_t = R_0 \times (1 + E_R)^{C_{t,R}}$$

X_t / R_t = Copies of target gene at threshold (test/reference)
X_0 / R_0= Copies at start (test/reference)
E_X E_R = PCR efficiency (test/reference)
$C_{t,X}$ / $C_{t,R}$ = Threshold cycle no. (test/reference)

In which 'test' refers to the isolated reference *Campylobacter* strain DNA, of known concentration, used in a given PCR, and 'reference' refers to the idealised version of the same experiment calculated from the known concentration of reference *Campylobacter* DNA and standard curves described in Section 2.7.1. Since DNA copy number is directly proportionate to the nanograms of DNA present, assuming approximately the same genome size for all species of *Campylobacter*, the two can be used interchangeably in equivalent calculations.

First, the positive control and standard curve are used to calculate $C_{t,R}$, the theoretical ideal C_t for the given initial copy number. It is assumed that the DNA copy number required to cross the C_t threshold is same for both. Since a ratio of C_t values is required, E_R is always given as 1.

$$X_t = X_0 \times (1 + E_x)^{C_{t,x}} = R_t = R_0 \times 2^{C_{t,r}}$$

Since the initial copy numbers, X_t and R_t, are the same, any difference between $C_{t,X}$ and $C_{t,R}$ should be entirely due to the difference in efficiency between the observed experiment and the theoretical ideal. Because of this, we can describe E_X in terms of only $C_{t,X}$ and $C_{t,R}$:

$$(1 + E_x)^{C_{t,x}} = 2^{C_{t,r}}$$
$$E_X = (2^{C_{t,R}})^{\left(\frac{1}{C_{t,X}}\right)} - 1$$

The equations described above can now be modified to calculate the initial copy number S_0 for an unknown sample S, the qPCR of which was conducted under identical circumstances to the 'test' reference (ie. the PCR efficiency of S is known to be E_X), which has a C_q of $C_{t,S}$ and where $R_{0,S}$ is the theoretical ideal copy number from the standard curve given $C_{t,S}$.

$$S_0 \times (1 + E_x)^{C_{t,s}} = R_{0,s} \times 2^{C_{t,s}}$$

$$S_0 \times \left((2^{C_{t,R}})^{(\frac{1}{C_{t,X}})} \right)^{C_{t,S}} = R_{0,S} \times 2^{C_{t,S}}$$

$$S_0 = \frac{R_{0,S} \times 2^{C_{t,S}}}{\left((2^{C_{t,R}})^{(\frac{1}{C_{t,X}})} \right)^{C_{t,S}}}$$

In this way, an equation can be created which uses a positive control and standard curves to calculate the number of copies of target DNA present a sample of unknown composition, provided that the positive control was tested under identical conditions to the unknown sample.

2.8 PCR for Species Identity Confirmation

This PCR was used in the event of a 16S response only being present following qPCR as described in Section 2.7. For each sample, two PCRs were conducted; one using a generic *Campylobacter* spp. reverse primer and two forward primers, one specific to *C. jejuni* and one specific to *C. coli*, and another PCR with *Campylobacter* spp. 16S primers. Primer details are provided in Table 2.6. Each PCR reaction consisted of 12.5 µl HotStarTaq Master Mix (QIAGEN, Hilden, Germany), 1 µl of each primer used, 2µl of template DNA extracted from the sample to be tested, and nuclease-free water to make up the remaining volume to 25 µl. For all PCRs, conditions consisted of 15 minutes at 95°C, followed by 35 cycles of one minute each at 94°C, 58°C and 72°C in order, followed by a final extension of ten minutes at 72°C. Each sample was then combined with 5 µl loading dye (Invitrogen, Waltham, MA, USA) and bands examined by gel electrophoresis. Gels were made of TAE buffer, SybrSafe (Invitrogen, Waltham, MA, USA) and 2% agarose. Samples were run alongside a 1 kb ladder (Promega, Madison, WI, USA).

Electrophoresis was conducted at 70 volts for 30 minutes, extended from that point in 15-minute increments until dye had migrated to approximately three-quarters of the distance of the gel. Bands were observed using the Bio-Rad Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA) to confirm presence or absence.

Table 2.6: Primers used for PCR for Campylobacter detection and speciation.

Species	Target Gene	Primer	Sequence (5'-3')	Reference
Campylobacter jejuni	*LpxA*	Forward	ACA ACT TGG TGA CGA TGT TGT A	Klena et al. (2004)
Campylobacter coli	*LpxA*	Forward	AGA CAA ATA AGA GAG AAT CAG	
Campylobacter spp.	*LpxA*	Reverse	CAA TCA TGD GCD ATA TGA SAA TAH GCC AT	
Campylobacter spp.	16S rRNA gene	Forward	CTC AGT AAT GCA GCT AAC G	
		Reverse	ACT AGT TTA GTA TTC CGG C	

The *Campylobacter* spp. 16S rRNA primers specified in Table 2.6 were designed in Mega X (Kumar *et al.*, 2018) through manual comparison of the genomes of *C. jejuni* (NCTC 11168 and M1), *C. coli* (NCTC RM2223 and NCTC 13366) and *Brachyspira* (GenBank accession number JX232353.1).

2.9 Testing for *Campylobacter* by Avara Foods Ltd.

Avara Foods Ltd. tests for *Campylobacter* on bootsocks collected from their farms twice per production cycle, once approximately three days before thinning and once between thinning and clear. These are primarily used to aid in the scheduling of flocks to be delivered to factory, to minimise cross-contamination on the production line between *Campylobacter* positive and negative flocks.

Testing is based around a Loop-Mediated Isothermal Amplification (LAMP) reaction, using the Genie II detection system and reagents (OptiGene Ltd., Horsham, England) and using a Standard Operating Procedure provided by the manufacturer (OptiGene, 2016).

This provides a positive or negative result, with no speciation. The primer/probe sequences used are not available to the public.

Bootsock swabs, provided by Avara Foods Ltd., are taken by farm staff walking the full length of the inside the poultry house to be tested on a day of the farm manager's choosing. This is sent to Avara's internal laboratories via conventional postal services. On arrival, the swab is placed inside a sample pot with a ball bearing and 100 ml of water. The lid is fixed on and the sample pot is shaken for 15 seconds to loosen the biological matter affixed to the bootsock swab. 3 ml of the resulting liquid is then removed with a syringe and dispensed into a tube containing a lysis buffer, which is shaken to mix the buffer with the bootsock extract and heated for 5 minutes at 80°C, then left to cool to room temperature. 5 µl of the resulting lysate is then mixed with 20 µl of *Campylobacter* reaction mix (OptiGene Ltd., Horsham, England) in a PCR reaction strip and a lid affixed. The completed strip is then placed inside a Genie II isothermal amplification platform (OptiGene Ltd., Horsham, England), which will give a presence/absence reading based on the detection of fluorescence produced by the LAMP reaction.

2.10 Statistical methods and data visualisation

A result of p = 0.05 was used as the cutoff value for statistical significance in all tests. All tests were carried out using the R statistical computing environment (R Core Team, 2020), using reproducible add-on functions added on in the form of R packages, obtained from the Comprehensive R Archive Network (CRAN) (Hornik, 2012). All graphic representations of data were created with the R packages ggplot2 (Valero-Mora, 2010; Wickham, 2016) and vcd (Meyer, Zeileis and Hornik, 2006).

2.10.1 Generalised Additive Models

Generalised Additive Models (GAMs) were used as described by Hastie and Tibshirani (1986) through the R package mgcv (Wood, 2011). This process utilises the sum of fitted smooth, non-parametric functions to capture the impact of each predictive variable on the observed result. In this way, a GAM can incorporate a greater proportion of the observed data into its model than linear or parametric regression models, as it is more able to compensate for complex, nonlinear associations between variables.

GAMs are initially derived from linear regressions. Linear regression can be summarised as:

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_p x_p + \epsilon$$

Where y is the dependent variable to be explained by the model, x is each known value to be used as a regressor, β is the weighting vector applied to each regressor and ε is the error of the model. This model will provide the best possible linear association between variables x and y, provided that an appropriate calculation for each β is used, such as Least Squares. In the case of additive models, the simple linear model is expanded upon by substituting β for a scatterplot smoothing function and using this to describe the function linking the predictor variables to expected dependent variable values, calculated from a description of the distribution of the data. Scatterplot smoothers are nonlinear regressions which provide a best fit between two variables and can be accomplished through several different techniques, some of which suit some data better than others and should be chosen based on which provides the best fit for the two parameters specified for the regression. Examples of smoothers include simple linear regression, best-fit polynomial curves and moving averages.

The overall equation underlying a GAM can therefore be expressed as:

$$g\big(E(y)\big) = \beta_0 + s_1(x_1) + \cdots + s_p(x_p)$$

Where E(y) is the expected value of the dependent variable, g denotes the link function, β_0 is the offset and s is the smooth function applied to each known regressor variable x. Each smooth function provides an estimation of the relationship to dependent variable and magnitude of impact for each predictor variable, and the sum of these functions provides an estimation of the dependent variable for the complete model accounting for all predictors.

2.10.2 Chi-Squared Test

Chi-Squared Tests are goodness-of-fit tests which determine the significance and magnitude of any difference between expected and observed frequencies within a contingency table. In this study, Chi-Squared tests were conducted as described by Pearson (1900) using the functionality of base R (R Core Team, 2020). The Chi-Squared

statistic is defined as the normalised sum of squared deviations between expected and observed values.

The formula for calculating the Chi-Squared statistic can be given as:

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

In which X^2 is the Chi-Squared statistic, O is the observed frequency and E is the expected frequency. This provides an indication of the likelihood that observed and expected frequencies are independent of one another.

For contingency tables containing counts of five or less, the corrective factor described by Yates (1934) was applied to alleviate the error of small sample sizes. This corrective factor is an alteration of the Chi-Squared statistic, in which the difference between observed (O_i) and expected (E_i) results is diminished by 0.5. This can be represented as:

$$X^2_{Yates} = \sum_{i=1}^{n} \frac{(|O_i - E_i| - 0.5)^2}{E_i}$$

2.10.3 Wilcoxon Rank-Sum Test

The Wilcoxon Rank-Sum Test is a non-parametric test used to compare two independent sample groups, the null hypothesis for which is that the medians of the two sample groups are identical. In this study, Wilcoxon Rank-Sum Tests were used as described by Mann and Whitney (1947) using the functionality of base R (R Core Team, 2020).

The U statistic, which serves as the basis for this test, is calculated with the following equations:

$$U_1 = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$

$$U_2 = n_1 n_2 + \frac{n_2(n_2 + 1)}{2} - R_2$$

In which U is the U statistic, n is the sample number and R is the sum of ranks. The smallest U statistic out of these two formulae should be taken as the correct one for the test. The U statistic is an estimator of difference between medians and hence provides an indication of whether there is a difference between sample groups.

2.10.4 Kruskal-Wallis Rank-Sum Test

The Kruskal-Wallis Rank-Sum Test is a non-parametric test for assessing whether multiple sample groups belong to the same underlying distribution, through a comparison of the means of ranks. It effectively provides a similar function to the Wilcoxon Rank-Sum Test, described in Section 2.10.3, but allows for the simultaneous comparison of an arbitrary number of groups. In this study, Kruskal-Wallis Rank-Sum Tests were carried out as described by Kruskal and Wallis (1952) using the functionality of base R (R Core Team, 2020).

The basis for the Kruskal-Wallis Rank-Sum Test is the H statistic, which is given as:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{c} \frac{{R_i}^2}{n_i} - 3(N+1)$$

In which c is the number of samples, n_i is the size of the i^{th} sample, N is the total sum of all sample sizes and R_i is the sum of ranks in the i^{th} sample. The H statistic provides an indication of the variance of ranks across sample groups and thus informs whether the underlying distributions are comparable.

2.10.5 Wilcoxon Matched-Pairs Signed Rank Test

The Wilcoxon Signed Rank Test provides a non-parametric assessment of differences in means between paired observations. In this study, these tests were carried out as described by Wilcoxon (1945) using the functionality of base R (R Core Team, 2020).

For all pairs of observations, the sign (positive or negative) of the difference is calculated. These are then ranked, with a rank of 1 assigned to the smallest difference, 2 for the second smallest and so on, and the sum of positive ranks and negative ranks

(both denoted as R) is calculated: $S^+ = \Sigma R_i$ for positive ranks, $S^- = \Sigma R_i$ for negative ranks. The smaller of these two rank-sum values is taken as the test statistic. For this, a probability of difference can be calculated:

$$P = 2\left[1 + \sum_{n}\left(\sum_{i=n}^{1=r-(\frac{n}{2})} \Pi_n^i\right)\right]$$

In which P is the probability of the observed rank-sum occurring under the null hypothesis that there is no difference between groups, n is the total number of observations, q is the number of paired differences, and r is the rank number under consideration.

2.10.6 Welch Two-Sample t-Test

The Welch Two-Sample t-Test is an improved version of the canonical Student's t-Test which does not need to assume that the variances of the two sample groups to be compared are equal. It provides a test of whether the means of two sample groups are equal. In this study, these tests were carried out as described by Ruxton (2006) using the functionality of base R (R Core Team, 2020).

The t- statistic is given as:

$$t = \frac{\mu_1 - \mu_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_1^2}{n_2}}}$$

In which μ is the sample mean, s^2 is the sample variance and n is the sample size. By comparing the calculated t-statistic, along with the degrees of freedom for the sample groups, against the t-distribution, a statistical relevancy can be calculated in the form of a p-value.

2.10.7 Kolmogorov-Smirnov Test

The Kolmogorov-Smirnov Test provides a non-parametric means of testing whether two probability distributions are the different. In this study, these tests were carried out as described by Massey (1951) using the functionality of base R (R Core Team, 2020).

The test statistic D is defined as:

$$D = maximum|F(x) - G(x)|$$

In which F(x) is the observed cumulative distribution function of the first sample and G(x) is the cumulative distribution function of the second sample. This statistic must be compared to a critical value, defined as:

$$D_{crit} = c(\alpha)\sqrt{\frac{m+n}{mn}}$$

In which c(α) is the inverse of the Kolmogorov distribution at α, m is the sample size of the first distribution and n is the sample size of the second distribution. If the test statistic is greater than the critical value, then there is a significant difference between the two distributions.

2.10.8 Cox Proportional Hazards Model

Proportional Hazards Models are useful for Survival Analysis in which the time elapsed until an event occurs is the primary parameter of interest which may be associated with cofactors. In this study, these tests were carried out as described by Cox (1972) and Breslow (1975) using the R package survival (Therneau and Grambsch, 2000).

The initial basis of Cox Proportional Hazards is the Hazard Rate, defined as:

$$\lambda(t) = \frac{P(T = t)}{S(t)}$$

In which λ(t) is the Hazard Rate, P represents probability, T is the failure time of the parameter under observation when treated as a random variable, t is the observed

failure time, and S(t) is the proportion of proportion of observations still surviving at time t. This statistic provides the probability of failure at a given time t. This is modified under Cox Proportional Hazards to become:

$$\lambda(t) = \lambda_0(t)e^{(x^t\beta)}$$

In which $\lambda_0(t)$ is the baseline hazard at time t, x is a covariate vector and β is a coefficient vector. Through this, calculation of β is possible and hence estimation of the contribution from each covariate to the Hazard Rate. Cox (1972) demonstrated that an estimation of β is possible without prior knowledge of the baseline hazard:

$$P\left(T_j = t_j \middle| R(t_j)\right) = \frac{\exp\ x_j^T\beta}{\sum_{i:t_i \geq t_j} \exp x_j^T\beta}$$

In which R(t) is the population at risk of failure at time t, and j is the subject under observation. The equation provides the probability that observation j fails at time i, rather than any other observation in the set. From this, a partial likelihood can be calculated, focusing the contribution of covariate x to the survival model:

$$\ell(\beta|X) = \sum_j ln\frac{\exp x_j^T\beta}{\sum_{i:t_i \geq t_j} \exp x_j^T\beta}$$

In which ℓ denotes the log-likelihood function. This is directly analogous to the weighting that covariate x has on the survival probability for sample j, and hence provides an estimate of the size of x's impact on survival rate.

2.10.9 Gaussian Process Regression

The Gaussian Process, also known as 'kriging', is a means of interpolating a regression fit to a set of given observations. In this study, these tests were carried out as described by Rogers and Sedda (2012) using the functionality of base R (R Core Team, 2020).

The formula for calculating the value to be interpolated at a given point is given as:

$$y_i = \sum w_d y_d + e$$

In which y_i is the value to be interpolated at position i, y_d is any known observed value a distance of d from point i, w_d is the weight given for the point a distance of d away from i, and e is the error.

The weighting applied to each point is dependant on a model fitted to the data. In the case of Gaussian processes performed with a computer, such as those employed in the presented work of this thesis, this will be based upon the best fitting model out of many different ones that are tried by software in turn.

2.10.10 Analysis of Variance (ANOVA) and confirmation with Tukey's Honestly Significant Difference (HSD)

Analysis of Variance (ANOVA) is a method of assessing the influence of categorical variables on a single continuous, dependent variable. In this study, these tests were carried out as described by Fujikoshi (1993) using the functionality of base R (R Core Team, 2020).

ANOVA is performed by comparing the variation between sample group means against the variation within each sample group. The variation between groups is summarised using the Mean Squares, described as:

$$MSB = \frac{\sum_{j=1}^{k}(\bar{x}_j - \bar{x})^2}{n-k}$$

In which $\bar{x}$ is the mean, n is the number of samples, and j is one of k sample groups.

The variation within each group is also summarised using the Mean Squares, described as:

$$MSW = \frac{\sum_{j=1}^{k}\sum_{j=1}^{l}(x - \bar{x}_j)^2}{k-1}$$

In which $\bar{x}$ is the mean, and x is a sample within group j of k sample groups. From these two values, the ANOVA statistic F can be calculated:

$$F = \frac{MSB}{MSW}$$

If the result of an ANOVA analysis was found to be statistically significant, confirmation as to which groups were statistically significant from one another was performed using Tukey's HSD Test. This was carried out as described by Tukey (1949).

The test is defined as:

$$q_s = \frac{Y_A - Y_B}{SE}$$

In which Y_A is the larger of two means being compared, Y_B is the smaller, SE is the standard error of the sum of means and q_s is the test statistic. This statistic is compared to a corresponding value from a continuous probability distribution normalised against the standard deviation of the original sample, and if q_s is larger then the two means under test are considered significantly different.

2.10.11 Similarity Percentages (SIMPER) Analysis

Similarity Percentages (SIMPER) analysis provides a means of assessing the magnitude of contribution provided by each component species to the dissimilarity between two sample groups. In this study, it was conducted as described by Clarke (1993) using the R package vegan (Dixon, 2003).

The analysis is performed using the contribution of each species to the Bray-Curtis dissimilarity, which is defined as:

$$\delta_{ijk} = \frac{|x_{ij} - x_{ik}|}{\sum x_{ij} + x_{ik}}$$

In which δ_{ijk} is the contribution of species i to the difference between sample groups j and k, and x is the abundance of species i in sample groups j and k. Through this pairwise comparison, the contribution of each species to the dissimilarity between sample groups j and k can be demonstrated.

2.10.12 Spearman's Rho

Spearman's Rho is a means of assessing the correlation between two variables by measuring the similarity in the rank order of each variable. In this study, it was conducted as described by Puth, Neuhäuser and Ruxton (2015) using the functionality of base R (R Core Team, 2020).

Between two paired groups of samples x and y, Spearman's Rho is defined as:

$$\rho = \frac{\sum_{i=1}^{n}\{(x_i - \overline{x})(y_i - \overline{y})\}}{\sqrt{\sum_{i=1}^{n}(x_i - \overline{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \overline{y})^2}}$$

In which ρ is Spearman's Rho, x_i is the *i*th value of x, y_i is the *i*th value of y, and $\bar{x}$ and $\bar{y}$ are the means of x and y respectively. Rho will be positive for positive correlations and negative for negative correlations, with magnitude providing an indication of the strength of the correlation.

2.10.13 Shapiro-Wilk Test

For some of the analyses performed, such as ANOVA and t-tests, an assumption of the data being well-modelled by the normal distribution is required for the test to be at its most accurate. In these instances, the normality of the dataset was first confirmed with the Shapiro-Wilk test for normality, carried out as described by Shapiro and Wilk (1965) using the functionality of base R (R Core Team, 2020).

The test statistic, W, is defined as:

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

In which x_i is the ith sample in the dataset, $x_{(i)}$ is the ith smallest number in the dataset, $\bar{x}$ is the mean and a_i is a coefficient based on expected values from a perfectly normally distributed dataset.

2.10.14 Logarithmic transformation of data

In some of the analyses conducted in this thesis, some data required a logarithmic transformation to approximate a normal distribution more closely. In these instances, the natural logarithm of each value in the dataset was taken as a substitute in analyses. The natural logarithm is defined as the logarithm to the base of Euler's Number (e).

3 The impact of weather, husbandry and disease on seasonal variation of *Campylobacter* in commercial broiler chicken flocks and on the timing of infection onset.

3.1 Introduction

The gastrointestinal tracts of newly hatched commercial chicks are generally colonised with low, often undetectable, levels of bacteria, due to sanitised hatchery conditions and the need for external sources of bacteria to colonise the juvenile gastrointestinal tract (Kubasova *et al.,* 2019a). Vertical transmission from breeder flocks is considered by most research to be of negligible impact on subsequent infections on broiler farms (Bull *et al.*, 2006; Callicott *et al.*, 2006; Battersby, Whyte and Bolton, 2016b). Most flock infections are thought to arise from horizontal transmission from the farm site and its immediate surroundings (Hansson *et al.*, 2007). Chicks are normally placed on farm within 24 hours post-hatch and, indeed, *Campylobacter* spp. can be isolated from the farm environment prior to the placement of any birds and the same subtype can be shown to have infected the flock by the end of the production cycle (Bull *et al.*, 2006). However, the precise risk factors, vectors and sources of colonisation are broad and poorly understood (Newell and Fearnley, 2003; Hansson *et al.*, 2016).

Other farm animals, stagnant water, farm equipment and certain parts of the chicken house, such as the anteroom and drinker lines, have all been implicated as potential persistent reservoirs of *Campylobacter* (Newell and Fearnley, 2003; Bull *et al.*, 2006; Ridley *et al.*, 2011; Ellis-Iversen *et al.*, 2012; Robyn *et al.*, 2015; Battersby, Whyte and Bolton, 2016b; Frosth *et al.*, 2020). Sequence types observed in nearby animals and ponds, or in previous positive flocks from the same chicken house, have been detected in subsequent *Campylobacter*-positive flocks (Frosth *et al.*, 2020), although the farm environment can also become contaminated following on from *Campylobacter* spp. detection in a chicken flock (Bull *et al.*, 2006).

Current best farming practice in the UK is designed to mitigate risk of *Campylobacter* spp. crossing the threshold of the chicken house through various biosecurity practices,

such as the implementation of a double-barrier entry system (Hansson *et al.*, 2007; European Commission, 2017). There are also restrictions on movements between chicken houses, interspecies proximity, site visitors and an 'all in, all out' policy to create disease breaks between flocks (DEFRA, 2018). Nonetheless, some farming practices will pose an inherent risk of flock infection through a necessary break of biosecurity. Processes such as thinning require that biosecurity is broken so birds can be caught and involve bringing vehicles and equipment on site that has previously been on other farms (Allen *et al.*, 2008; Ellis-Iversen *et al.*, 2012). Other factors under the farmer's control, such as the cleaning regime of the chicken house in between flocks, the chicken house construction or even the general tidiness of the farm can be linked to the likelihood of *Campylobacter* infections occurring (Hansson *et al.*, 2010). However, while biosecurity defences are in place, there must be an inevitable balance between measures taken and what is practical for the industry (Millman *et al.*, 2017).

Annual patterns in *Campylobacter* infection of poultry flocks are observed globally (Nylen *et al.*, 2002; Jore *et al.*, 2010; Jorgensen *et al.*, 2011; Kalupahana *et al.*, 2018). A well-established link with temperature is noted, with flocks reared during months with higher average temperatures being more likely to be *Campylobacter* positive by slaughter (Nylen *et al.*, 2002; Jore *et al.*, 2010). The underlying causation behind these observed seasonal trends, however, remains largely unknown at present (Hansson *et al.*, 2016; Sibanda *et al.*, 2018), despite the efforts of published research to explore which factors are likely to be contributory.

There is limited evidence to suggest that some animals whose presence on poultry farms may occur in seasonal patterns, such as insects (Hald *et al.*, 2004; Royden *et al.*, 2016), wild birds and other animals (Newell and Fearnley, 2003; Agunos *et al.*, 2014; Hald *et al.*, 2016) may play some role in *Campylobacter* transmission to housed broilers by moving through poultry house ventilation systems (Figure 3.1), though any impact these make do not explain the bulk of observed infection data (Newell and Fearnley, 2003; Agunos *et al.*, 2014; Sibanda *et al.*, 2018).

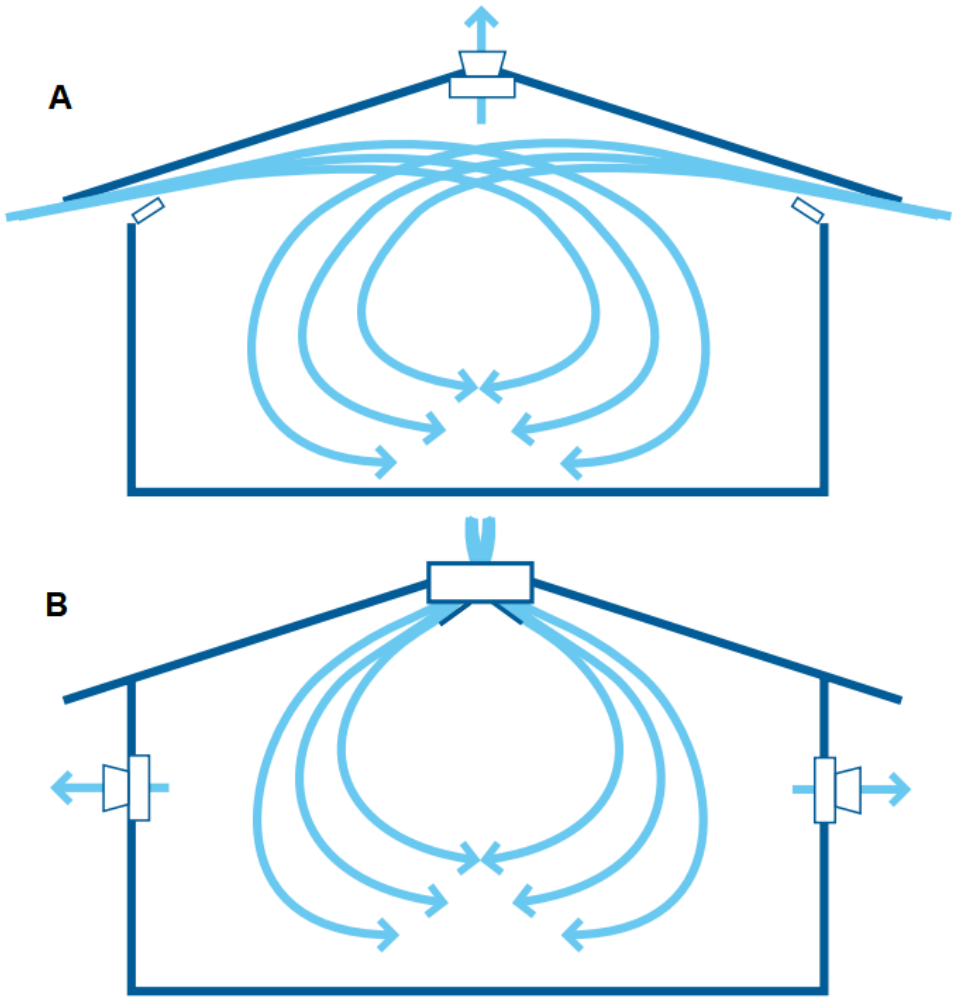

Figure 3.1: Diagrams illustrating broiler chicken house air flow in a side inlet (Figure 3.1A, top) and ridge inlet ventilation system (Figure 3.1, bottom). (Aviagen, 2010)

To date, two studies attempted to explore differences in seasonal acquisition of *Campylobacter* among broiler flocks by examining when the infection began in the production cycle (Evans and Sayers, 2000; Weber *et al.*, 2014). However, these conflicted; Evans and Sayers (2000) found no difference in age of infection onset across seasons, while Weber *et al.* (2014) suggested an earlier acquisition of *Campylobacter* occurring at the time when infections are at their peak. However, both studies had limitations. Evans and Sayers (2000) only began measuring *Campylobacter* at an age of 28 days, while infections can begin much earlier, and Weber *et al.* (2014) tested a limited number of caeca for *Campylobacter* spp. per day through necropsy of already deceased

birds, rather than testing the environment or live birds that would go on to slaughter, and did not measure any farm or environmental statistics beyond the season the sample was collected in. No published research has, so far, attempted to utilise the onset of a *Campylobacter* infection in a broiler flock as a statistical factor in broader explanations tying together climate, management, and welfare parameters to show what influences lead to *Campylobacter* infections occurring in the first instance.

3.1.1 Chapter Aims

The aim of experiments detailed in this chapter was to ascertain if *Campylobacter* infections among commercial broiler chicken farms could be linked to weather, or to farm management practices which may be changed from season to season, or to geographic factors that vary between farms. In this way, potentially causative parameters could be linked to *Campylobacter* infection seasonality.

Specifically, objectives were to:

- Assess whether flocks become *Campylobacter* positive earlier in the production cycle during summer months, and whether this impacts on the rate at which flocks are infected with *Campylobacter.*
- Determine whether seasonally variable parameters, such as temperature, farming practices or humidity, can be associated with increased *Campylobacter* infections.

To achieve this, eleven farms were monitored daily for 16 months for the presence of *Campylobacter* on their broiler chicken flocks, recorded alongside measured weather and farm parameters.

3.2 Methods

3.2.1 Data collection

All eleven farms described in Section 2.2 were observed for ten production cycles commencing on or after the 20th August 2018. The final flock clearance in the study took place on the 7th January 2020. Due to flocks that had to be excluded when farmers could not collect samples, a total of 103 production cycles were observed overall.

A summary of what parameters were recorded, on both a daily and per-cycle basis, is presented in Table 3.1. Farm staff were provided with a daily diary sheet for each production cycle to fill in with results.

Table 3.1: Table of recorded parameters used in the present study.

Parameters with daily results		**Parameters with results once per cycle**
Recorded by project researchers	Recorded by farm staff	Recorded by Avara Foods Ltd. staff
Campylobacter spp. presence or absence Environmental temperature (°C, maximum, minimum, average) Environmental humidity (RH%, maximum, minimum, average) Precipitation (mm)	Mortality Culls (runt, leg, other) Bird average weight (kg, recorded by automatic weighers) Water consumption (litres per 1,000 birds) House temperature (°C, maximum and minimum House humidity (RH%, maximum and minimum) Feed type	*Campylobacter* spp. presence or absence (once before thinning, once before clearance) Feed conversion ratio (farm-wide only) European Performance Efficiency Factor (EPEF; farm-wide only) Wheat in feed (%, farm-wide only) Factory rejects (septicaemia, runts, ascites, cellulitis, hepatitis, pericarditis, perihepatitis) Parent flock age (weeks)

Weather data was downloaded weekly from Meteoblue.com. This website provides modelled hourly climate and weather data for any arbitrary set of map co-ordinates (Meteoblue, 2022). At the time that research was conducted, Meteoblue offered up to two weeks of historic data available for download from any given location, with an explicit clause in their user licence permitting free use for educational and academic purposes, although at time of publication of this thesis that particular service appears to have been discontinued.

3.2.2 Bootsock Collection and Testing

Bootsocks were collected daily by farmers as per Section 2.4.1, as part of the first welfare checks of each day. Bootsocks were stored on farm at ambient temperature and collections of bootsocks were made at the end of every cycle for return to the laboratory. Any bootsocks to be tested were processed by suspending collected biological debris in isotonic saline, as per Section 2.4.3. DNA was isolated using a QIAGEN QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per Section 2.6, then tested for *Campylobacter* presence using a multiplex qPCR as detailed in Section 2.7, with subsequent confirmatory PCR and gel electrophoresis as per Section 2.8 if species identity could not be ascertained by the primary qPCR.

For the sake of efficiency, not every collected bootsock was tested. Instead, one bootsock was tested every five days, from day ten until the final day of the production cycle. Of these, if one was positive and one was negative, all bootsocks in between these two points were also tested to isolate the first day of infection. This is exemplified in Figure 3.2.

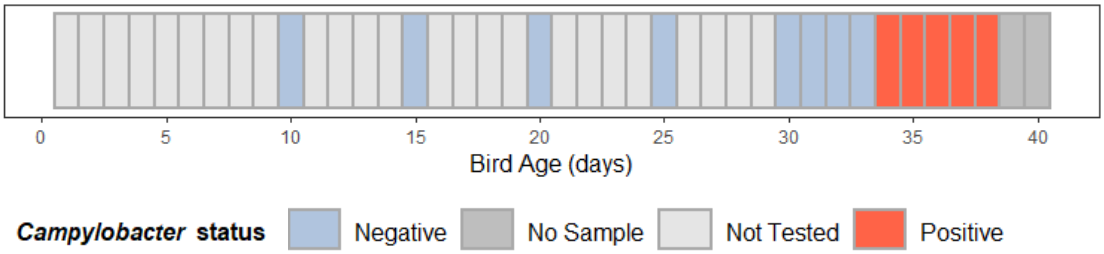

Figure 3.2: Example data illustrating bootsock testing methodology. Presented testing is from Farm 2A, cycle 5. Borders have been emphasised to show distinct days.

A flock was designated as 'positive' if *Campylobacter* was detected on two days within any consecutive three-day window. If only the final day of the flock was found to be *Campylobacter* positive, the flock was also deemed 'positive'. The first positive day out of these was deemed the day of transition, and hence the first day of the infection. This first positive day, combined with the immediately preceding day with no detection of *Campylobacter*, were deemed 'transition days', in which the infection was acquired between the two samples being taken.

3.2.3 Standards

Information on temperature and weight standards were provided by Avara Foods (Figure 3.3). Bird growth (%) is calculated by expressing the actual measured bird weight as a percentage of the idealised bird weight described in the standards, for a given age.

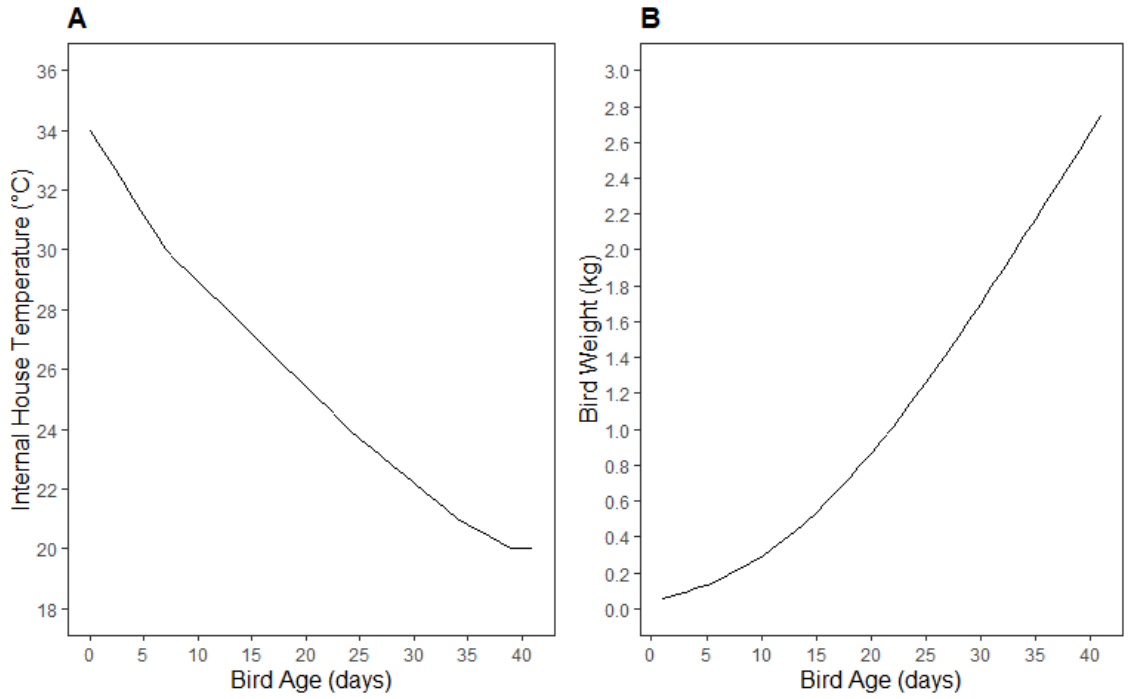

Figure 3.3: Daily internal house temperature (Figure 3.3A, left) and bird weight (Figure 3.3B, right) standards, as used by Avara Foods Ltd.

These standards are initially derived from the Ross 308 Broiler Manual (Aviagen, 2018), smoothed and adjusted by Avara according to observed best management practice and expected performance on company farms.

3.2.4 Statistical methods

All analyses were constructed in R (R Core Team, 2020) as described in Section 2.10.

The impact of observed parameters (Table 3.1) and categorical parameters recorded in the site survey (Table 2.1) on the likelihood of *Campylobacter* being present in a chicken house was determined through a series of GAMs as described in Section 2.10.1. Survival analysis was conducted as described in Section 2.10.8.

Graphs and visualisations were constructed out using ggplot2 (Valero-Mora, 2010; Wickham, 2016). For all plots describing the constituent model partials of GAMs, model partial values are centred with a Y-axis value of zero representing the mean value for the variable modelled in the graph. Therefore, positive values indicate when an explanatory variable, on the X-axis, has affected the model in a way that moves the modelled variable in a positive direction away from the mean, and vice-versa, with Y-axis magnitude showing any change in the variable predicted by the model away from the mean. For purposes of graphing each model partial, all other covariates not represented by the Y-axis but incorporated in the model are held at their mean value. For GAM model partial graphs describing categorical variables, such as farm, the X and Y axes are reversed.

European Performance Efficiency Factor (EPEF) was calculated as per the equations defined in Kryeziu et al. (2018), with the formula given as:

$$EPEF = \frac{(100 - Mortality) \times Body\ Weight}{Age \times (Feed\ Used/Weight\ Gained)}$$

In which Mortality is the percentage of birds which died before slaughter, Body Weight is the average bird weight in kilograms, Age is the final bird age at slaughter in days, Feed Used is the cumulative kilograms of feed used by all birds, and Weight Gained is the total accumulative weight gained by the flock over the entire production cycle.

3.3 Results

3.3.1 Historic Data

Campylobacter spp. infection data from Avara Foods Ltd., from both prior to and over the course of the study, was made available for all 168 farms owned or contracted by the company at the time of the study. This was collated internally using Avara's internal *Campylobacter* testing regime, as per Section 2.9. Plotting the percentage of flocks entering the factory each week that were identified by Avara as *Campylobacter* positive (Figure 3.4) shows a clear and distinct seasonal trend. The trendline indicates summer peaks in infections occurring anywhere between May and September, lasting

three to four months at a time and ranging between 70% and 95% of farms becoming positive, depending on the year. Conversely, the winter dip in infections occurred anywhere between December and May, lasting three to four months with between 20% and 35% of farms becoming positive, depending on the year.

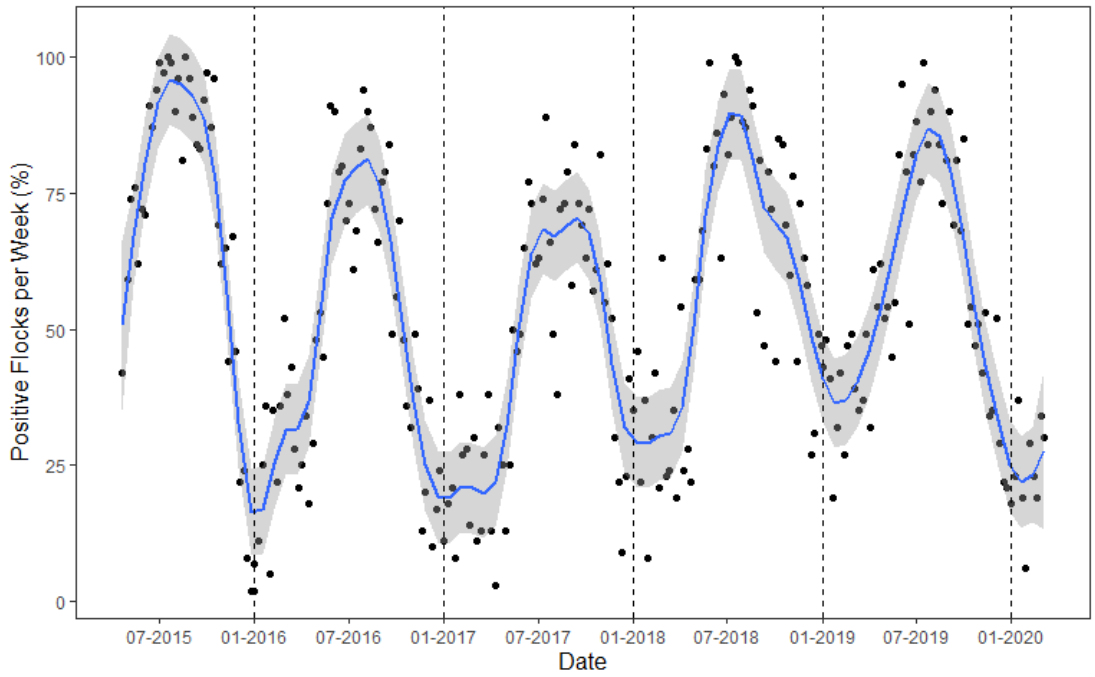

Figure 3.4: Graph of weekly percentage of flocks contaminated with Campylobacter spp. *tested prior to slaughter by Avara Foods Ltd. against time. Trendline is a fitted additive model. Shaded grey area shows 95% confidence interval. Dotted lines indicate year start/end.*

3.3.2 Bootsock Testing Overview and Descriptive Statistics

Over the course of the study, approximately 3,900 daily bootsocks were collected, of which 988 were tested and 203 were positive for *C. jejuni* or *C. coli* (21%). The bootsocks were collected for ten production cycles over eleven farms, for a total of 103 tested production cycles, of which 51 never contained a positive sample (50%). Cycles varied in length from 36 to 41 days. The basic statistics for each farm are summarised in Table 3.2 and full results for testing is visualised in Figure 3.5.

Table 3.2: Summary statistics for farms analysed in Chapter 3.

Farm	1A	1B	1C	1D	2A	2B	2C	3A	3B	3C	3D
Cycles Tested	10	9	8	10	10	10	10	10	10	9	7
Positive Cycles	5	2	2	7	6	2	7	5	3	5	3
Mean Age of Infection Onset	31.4	26.0	29.5	22.3	30.8	34.0	33.9	24.4	28.3	27.8	28.3
Standard Deviation	5.9	10.0	4.5	10.0	7.5	3.0	6.4	6.4	2.5	4.9	7.0

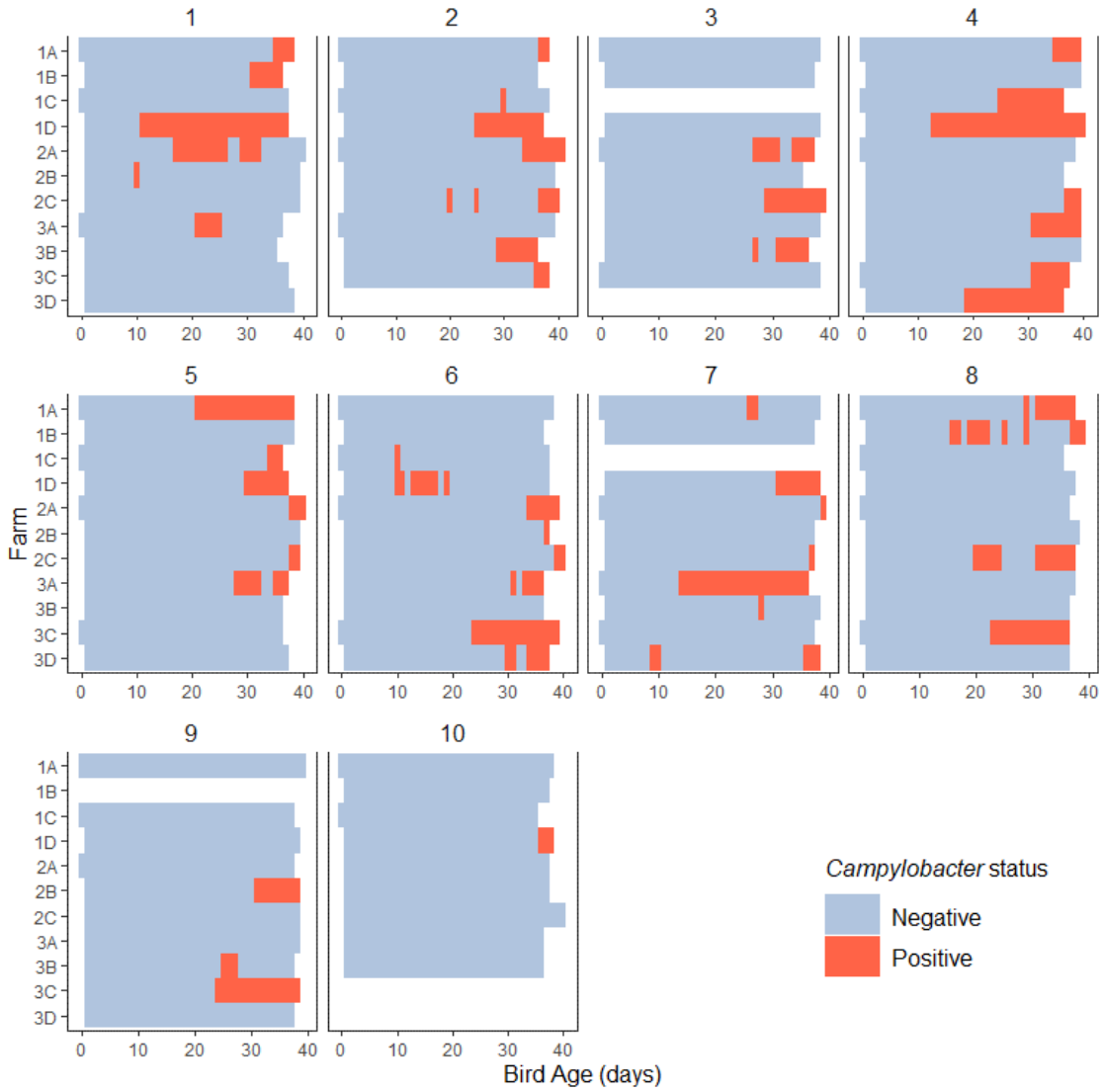

Figure 3.5: Daily Campylobacter testing results for eleven broiler flocks, over ten production cycles.

According to daily bootsock tests, the probability of a flock being contaminated with *Campylobacter* increased with bird age, within a production cycle. A fitted GAM of bird age against a binomial distribution of *Campylobacter* status (1 for positive, 0 for negative; Figure 3.6A) gave a statistically significant nonlinear fit but does not explain most of the observed variance ($p < 0.001$, adj. $R^2 = 0.135$). The mean age of infection onset was 28.6 days (mode = 36, SEM = 1.07), and 64.7% of positive flocks remained consistently positive after contracting an infection. Including farm as a categorical variable that can affect *Campylobacter* status in the additive model (Figure 3.6B) shows that there is a farm-specific effect on the probability of a flock becoming *Campylobacter* positive.

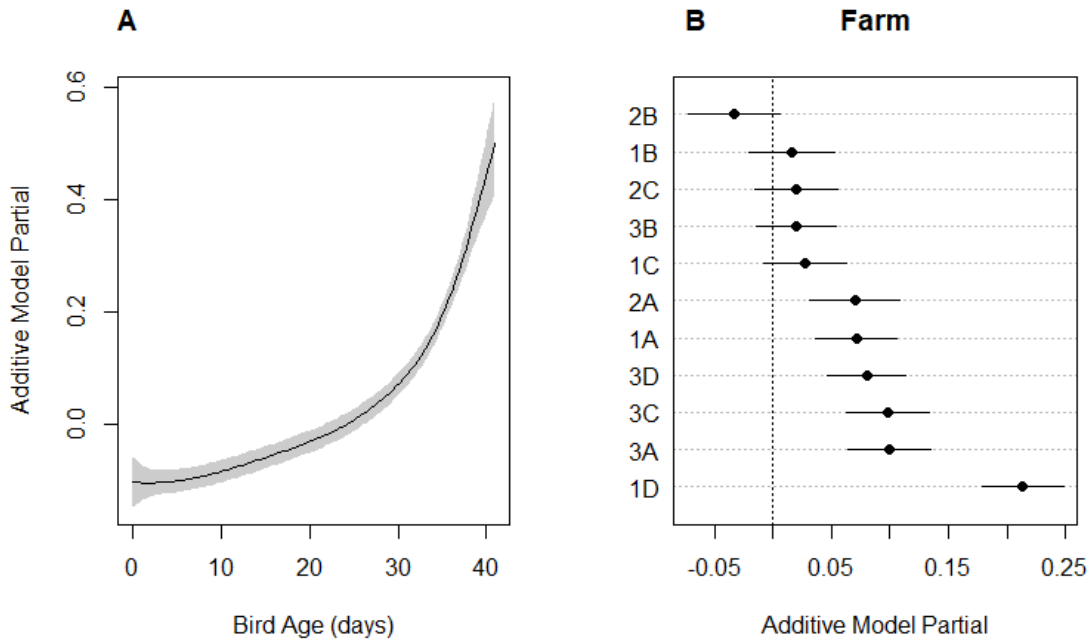

Figure 3.6: Details from a Generalised Additive Model output, predicting the impact of bird age (Figure 3.6A, left; p < 0.001) and farm (Figure 3.6B, right) on the probability of a flock being Campylobacter positive.

3.3.3 Comparison of *Campylobacter* Testing Methodologies

A comparison was made between Avara's internally reported *Campylobacter* testing regime and the detection of *Campylobacter* at the end of each production cycle within this study. A Chi-Squared test comparing the frequencies of positive and negative detections within each group (Table 3.3) does not suggest a statistically significant association ($p = 0.059$).

Table 3.3: Contingency table comparing Campylobacter detections at final clearance as reported by Avara and by the testing methodology used in this study.

	Reported Negative at Laboratory	**Reported Positive at Laboratory**
Reported Negative by Avara	33	21
Reported Positive by Avara	18	25

To further investigate the differences between *Campylobacter* detections by Avara and the present study, a time-series graph was constructed by representing *Campylobacter* detection as a binary variable and plotting Gaussian Process regressions of each dataset against the date of clearance (Figure 3.7). This revealed a notable difference between the two testing methodologies. Avara's internal testing showed a seasonal trend, with a reduction in detections around April 2019 and January 2020, while no comparable reduction in detections is noted for laboratory testing in 2019.

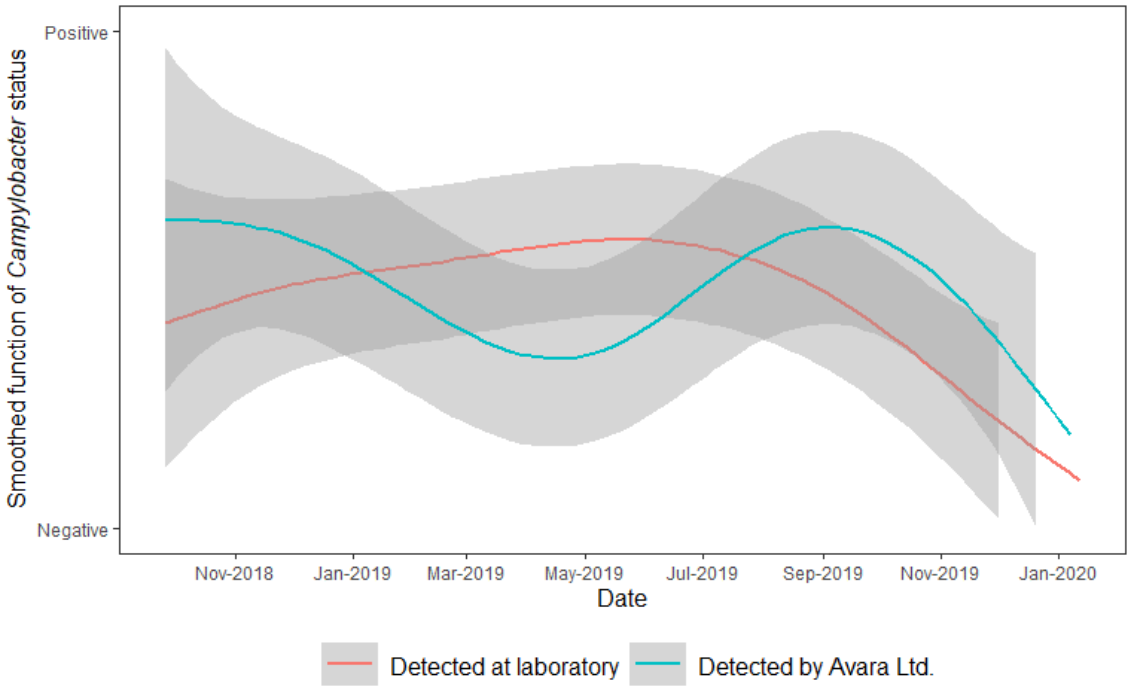

Figure 3.7: Graph showing Gaussian Process regressions of binomial Campylobacter data, based on detection at final clearance, from testing using Avara's internal Campylobacter detection methodology and the PCR described in Chapter 2.7. Shaded areas represent 95% confidence intervals.

3.3.4 Climate and *Campylobacter* Incidence

Environmental temperature observed across the observation period ranged from 33.4°C to -5.7°C. Humidity tended to decrease when temperatures were at their highest. Rainfall was sporadic and year-round, with the driest period occurring around January and February each year. Graphs describing the weather observed over the course of this study are presented in Figure 3.8

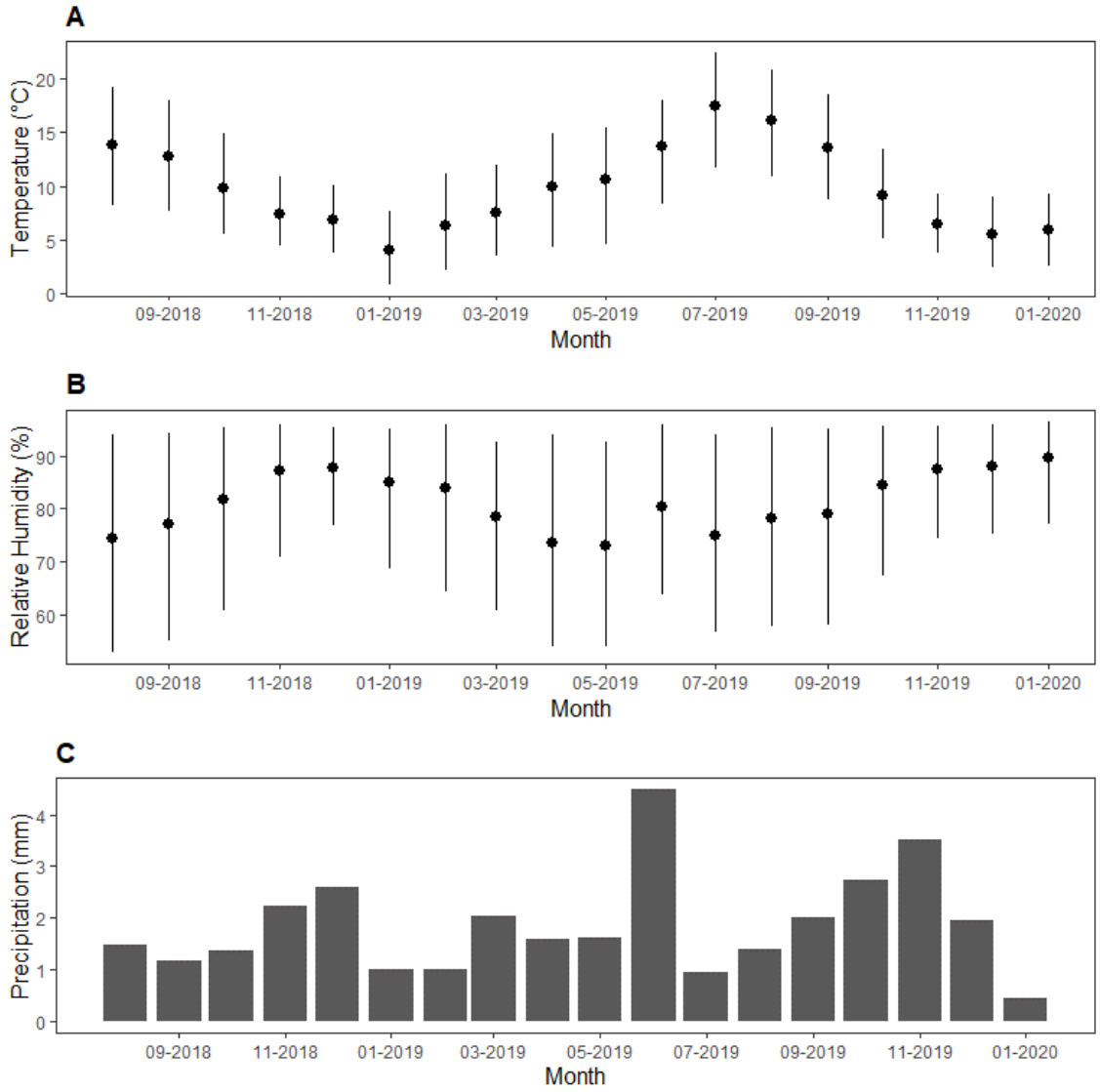

Figure 3.8: Charts describing weather parameters observed across the farms studied. Graphs for temperature (Figure 3.8A, top) and relative humidity (Figure 3.8B, centre) display mean daily reading, along with average daily maximum and minimum for each month. Precipitation (mm per day, average) is presented in Figure 3.8C (bottom).

A fitted GAM of binomial data derived from daily *Campylobacter* status (1 for positive, 0 for negative) using the daily maximum environmental temperature, the daily average environmental relative humidity, bird age and farm as predictor variables (Figure 3.9) demonstrates that, along with the farm- and age-dependent effect observed previously in section 3.3.2, there is also a significant nonlinear effect from the external temperature of the environment (Figure 3.9A; $p = 0.012$) and significant negative correlation from relative humidity (Figure 3.9C; $p < 0.001$). However, since this does not explain most of the observed *Campylobacter* (adj. $R^2 = 0.189$), the absolute temperature or humidity around the poultry house had only a slight bearing on the overall picture of whether a flock is positive or not. This was confirmed by conducting a Welch Two-Sample t-Test to assess difference in the means of maximum temperature values observed on *Campylobacter* positive and negative flocks; no significant difference was found ($t = -0.457$, $p = 0.648$, $df = 443.96$).

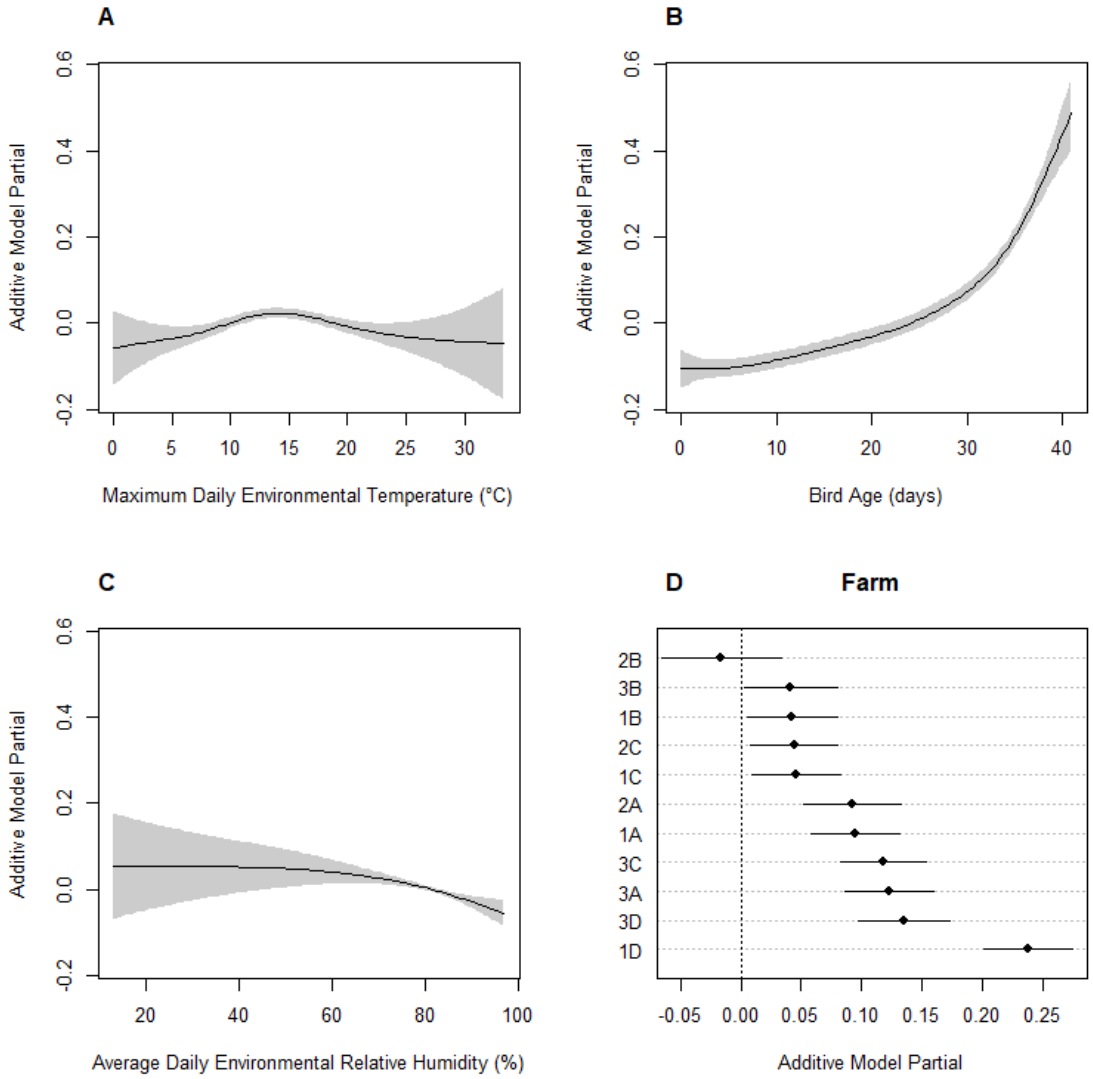

Figure 3.9: Details from a Generalised Additive Model output, predicting the impact of maximum environmental temperature (Figure 3.9A, top left; p = 0.012), bird age (Figure 3.9B, top right; p < 0.001), environmental relative humidity (Figure 3.9C, bottom left; p < 0.001) and farm (Figure 3.9D, bottom right) on the probability of a flock being Campylobacter positive.

To assess whether sudden increases in environmental temperature preceded the onset of *Campylobacter* infection, the day preceding infection onset and first day of infection for each positive flock (n = 54) were labelled as 'transition days' (detailed in Section 3.2.2) so they could be isolated for analysis. The daily mean of hourly temperature readings for the first transition day of a positive flock was 1.40°C warmer than the same measurement for that flock (SD = 3.94) and the preceding week was, on average, 1.38°C warmer than the average for that flock (daily mean of hourly readings; SD = 4.49). However, both these differences are not significant according to Wilcoxon

Matched-Pairs Signed Rank test (p = 0.091 and p = 0.085 respectively). When these differences are visualised (Figure 3.10), there is no observable difference between transition day temperatures, or those of the preceding week, and the flock average temperature when it is above approximately 9°C, but a divergence in the trendline occurs below this point.

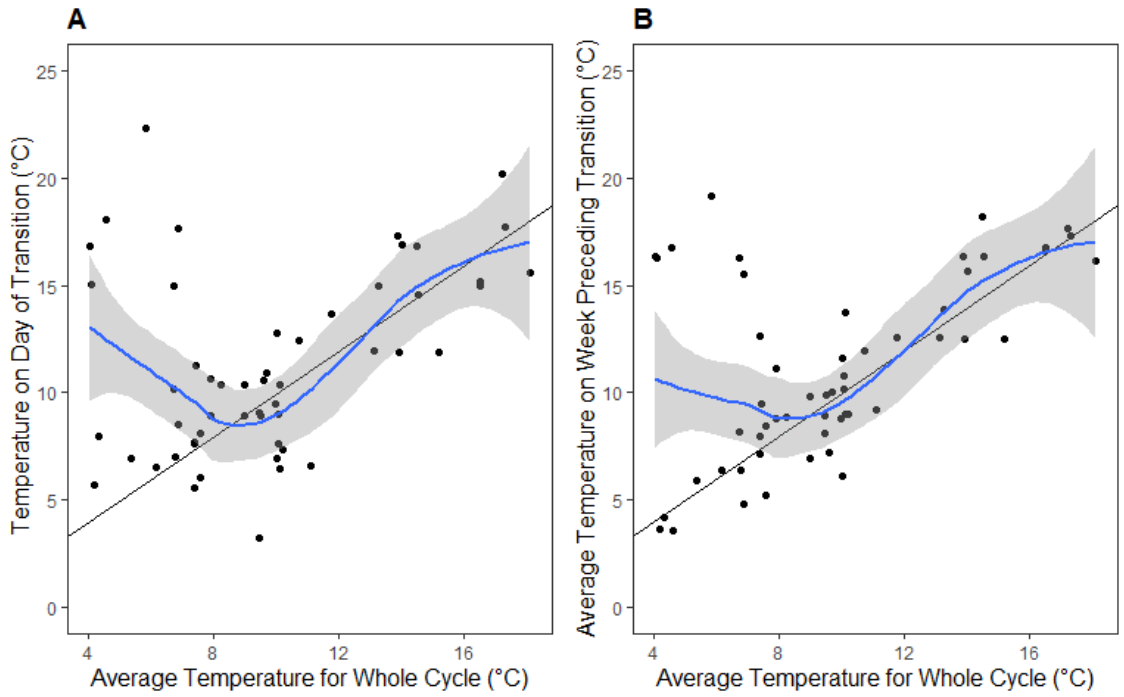

Figure 3.10: Scatter plots demonstrating temperature differences between the mean daily environmental temperature, temperature observed on the day of transition to Campylobacter positive (Figure 3.10A, left) and the week preceding Campylobacter infection (Figure 3.10B, right). Isocline (black; indicates where parameters are equal) and Loess regression (blue) are plotted. Shaded area represents 95% confidence interval.

A one-way ANOVA analysis comparing all transition days (n = 110), days on which any flock is positive (n = 288) and days on which any flock is negative (n = 2981) revealed no significant difference between the means of each group (F = 1.864, p = 0.166; Figure 3.11).

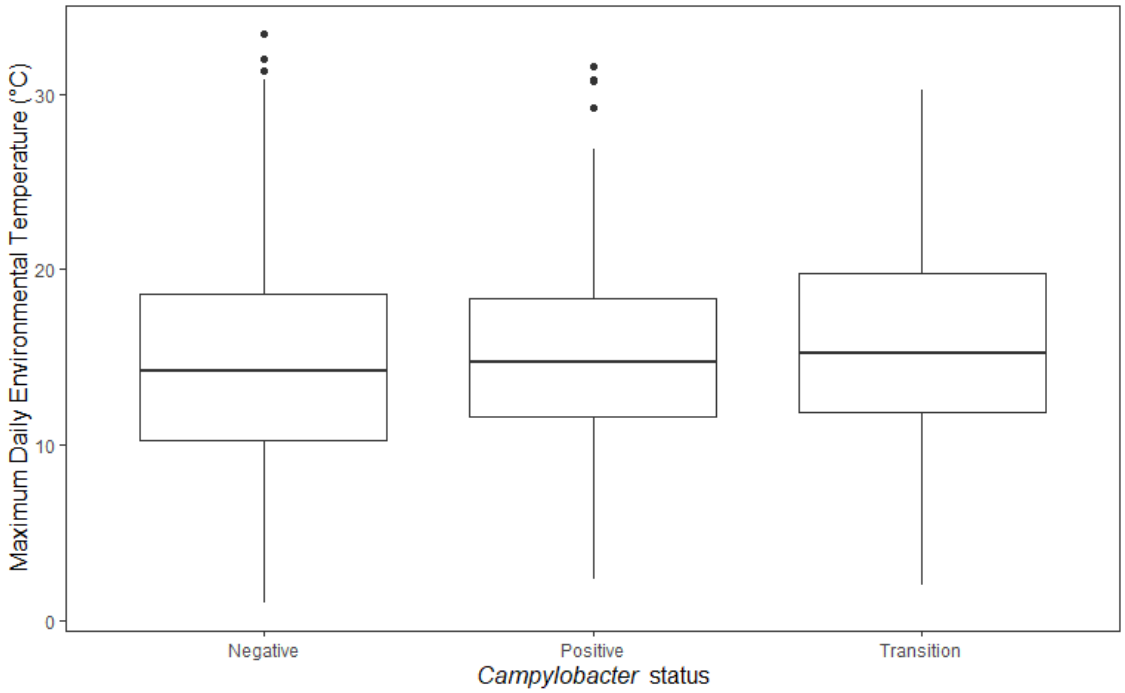

Figure 3.11: Boxplot of maximum daily environmental temperature observed on poultry farms, according to Campylobacter status. Dots represent outliers, lines represent maximum/minimum values, boxes represent 25th-75th percentiles, horizontal lines represent medians.

A Wilcoxon Rank-Sum test comparing the daily precipitation levels during the week preceding infection onset and the precipitation measured for all other observations revealed that precipitation during the week preceding a *Campylobacter* infection was not significantly different to precipitation levels recorded at other times (p = 0.105).

3.3.5 Husbandry Practices and *Campylobacter* Incidence

The internal temperature and humidity of the chicken house is controlled by the farmer throughout the birds' lives, through a combination of heating and ventilation systems. Other parameters, too, such as changes in feeding regime and lighting, are altered frequently in response to bird requirements. The control of these parameters broadly follows guidance laid out by the parent company that the farms supply, but

there is flexibility permitted and it is reasonably expected that the farmer will deviate from strict adherence to company standards in response to the needs of the birds. It is therefore important to explore differences in management style and how they might impact on the likelihood of *Campylobacter* infection of the flock.

To assess whether the internal house temperature was regulated well across all seasons and bird temperature requirements, a fitted GAM was constructed of the difference between observed internal house temperature on all eleven farms sampled over Phase One of the project and the equivalent temperature on Avara's standard (as described in Section 3.2.3), using external temperature, bird age and farm as predictor variables (Figure 3.12). External temperature was observed to be highly positively correlated with positive deviation in house internal temperature from the Avara standard ($p < 0.001$). The week of bird age also correlated positively, with weeks further along in the birds' life corresponding with positive deviation from the Avara standard ($p < 0.001$), and all farms are distinct, with some being hotter or cooler than others ($p < 0.001$). This model explains over half of the observed deviation in house internal temperature (adj. R^2=0.634).

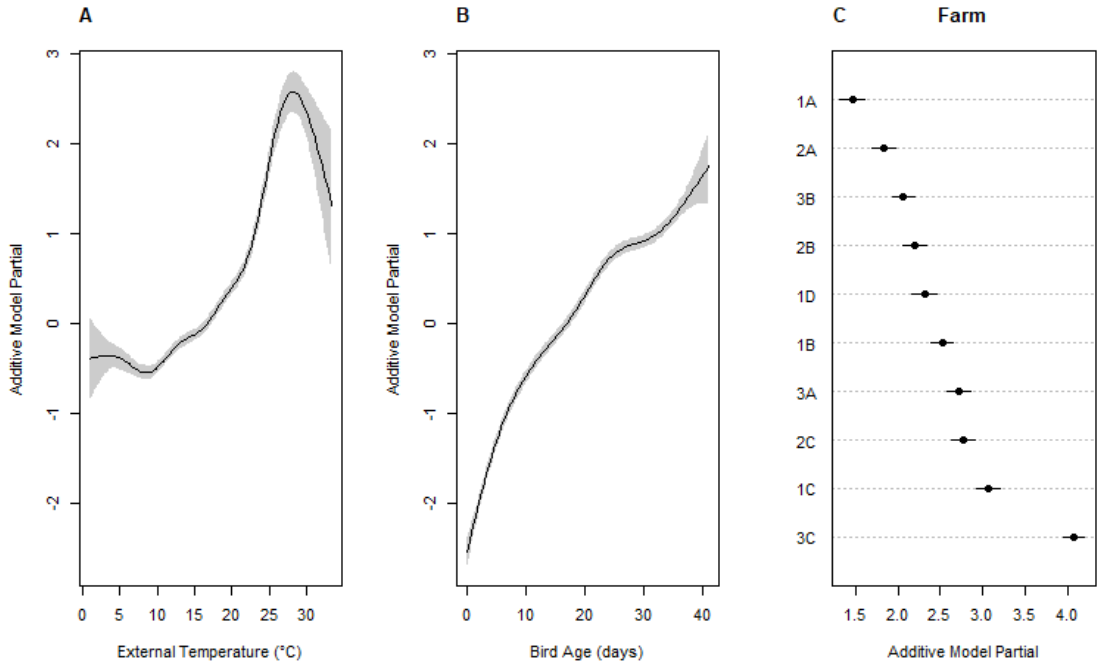

Figure 3.12: Details from a Generalised Additive Model output, predicting the impact of average daily external temperature (Figure 3.12A, left; $p < 0.001$), bird age (Figure 3.12B, centre; $p < 0.001$) and farm (Figure 3.12C, right) on deviation of internal house temperature away from the company standard temperature profile. This graph shows only farms for which the internal house temperature information was made available by the farmer.

When visualised against date (Figure 3.13A), there are apparent spikes in internal house temperature during summer months away from Avara's temperature standard, with peaks increasing in magnitude with bird age. This difference is also apparent when plotted against maximum daily external temperature (Figure 3.13B), implying that temperature control is considerably harder for the farmer in warmer months and at more advanced bird ages.

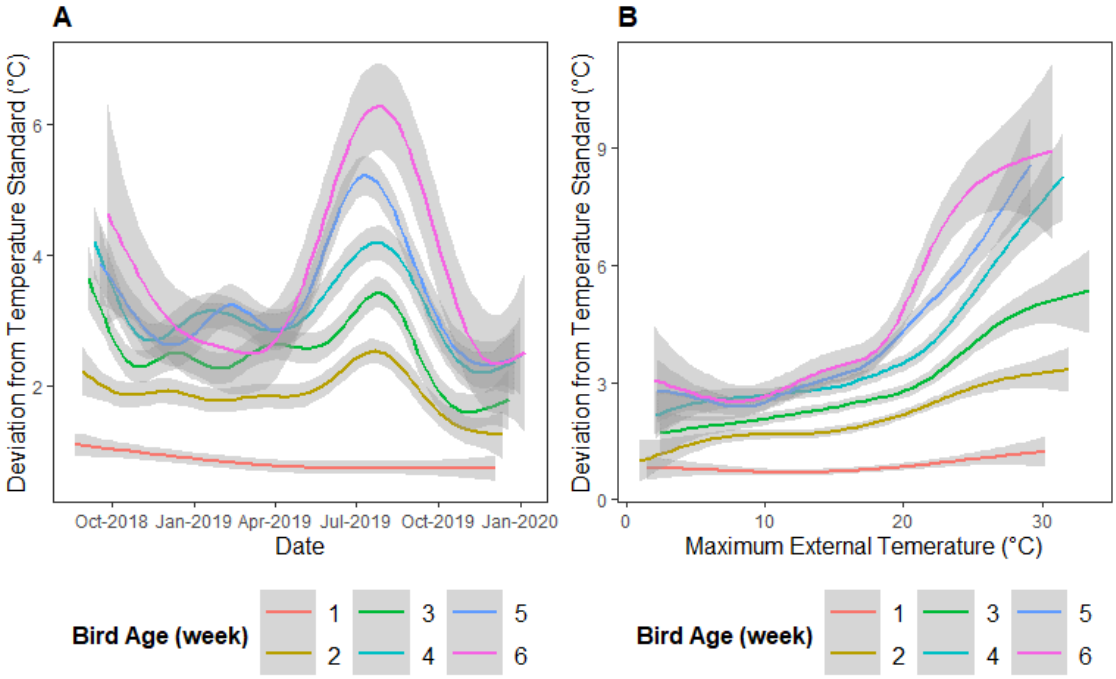

Figure 3.13: Graph showing the relationship between date (Figure 3.13A, left) and maximum daily external temperature (Figure 3.13B, left) on deviation of internal house temperature away from the company standard temperature profile. Shaded area represents 95% confidence interval. All lines represent Gaussian Process smoothing.

3.3.6 Disease, Performance and *Campylobacter* Incidence

European Production Efficiency Factor (EPEF) is a standardised poultry performance metric, derived from feed conversion ratio, daily weight gain and mortality rates and is defined in Section 3.2.4. This figure is calculated and reported by Avara for each farm at the end of each production cycle, across an entire farm rather than for individual chicken houses. A Welch Two-Sample t-Test comparing EPEF on farms where the house examined in this project cleared as *Campylobacter* positive versus *Campylobacter* negative revealed no significant difference between groups (t = 0.509, p = 0.613, df = 76.365).

Table 3.4 gives results of the statistical analysis of *Campylobacter* positive and negative farms at clearance. No statistically significant difference can be seen for any tested parameter.

Table 3.4: Results of Wilcoxon Rank-Sum tests, comparing Campylobacter positive (n=29) and negative (n=54) farms at final clearance.

Parameter	**Mean Value for Negative Flocks (%)**	**Mean Value for Positive Flocks (%)**	**Wilcoxon Rank-Sum P value**
Mortality	2.232	2.269	0.761
Culls	1.949	1.957	0.278
Septicaemia	0.265	0.264	0.667
Runts	0.013	0.014	0.861
Ascites	0.158	0.157	0.767
Cellulitis	0.182	0.185	0.924
Hepatitis	0.059	0.059	0.372
Pericarditis	0.011	0.012	0.648
Perihepatitis	0.063	0.064	0.437

To assess whether *Campylobacter* had an impact on bird weight, a fitted GAM was constructed of mean bird weight reported by the Avara factory at final house clearance (n = 83 production cycles), using age of birds at clearance, the age in weeks of the parent flock that initially supplied chicks to the farm, percentage of feed made up of raw wheat, *Campylobacter* status at clearance and farm (Figure 3.14; adj. R^2 = 0.748; mean bird weight = 2.344 kg). From this, it can be inferred that there is no statistical impact from *Campylobacter* status at clearance on final bird weight. All other parameters did show a significant impact on final weight, with bird age having a linear correlation, and wheat inclusion in the feed and parent flock age having nonlinear associations.

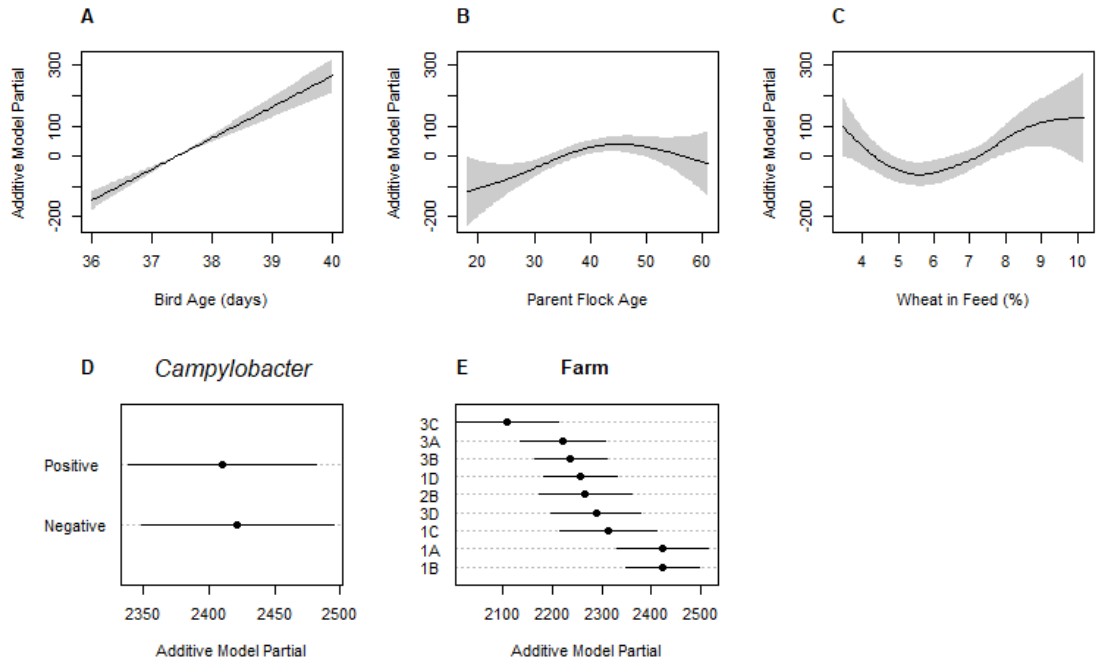

Figure 3.14: Details from a Generalised Additive Model output, predicting the impact of bird age at clearance (Figure 3.14A, top left; p < 0.0001), average age of parent flock (Figure 3.14B, top centre; p = 0.0065), percentage of wheat incorporated in feed (Figure 3.14C, top right; p = 0.0049) Campylobacter status at final clearance (Figure 3.14D, bottom left; p = 0.625) and farm (Figure 3.14E, bottom centre) on average bird weight at clearance.

The feed supplied to the chickens is changed throughout their lives to meet their dietary needs. These changes are discrete and are at defined points to coincide with growth, with four to five different feeds used in succession. To assess the impact of changes in diet on likelihood of a flock becoming *Campylobacter* positive, a Kolmogorov-Smirnov test was conducted to compare the frequency distribution of the number of days since a feed change that a flock became *Campylobacter* positive and the frequency distribution of days since a feed change for all negative days observed (Figure 3.15). This revealed that there was no difference between the distributions (D = 0.110, p = 0.505), and hence showing a flock is not more or less likely to become infected immediately following a diet change.

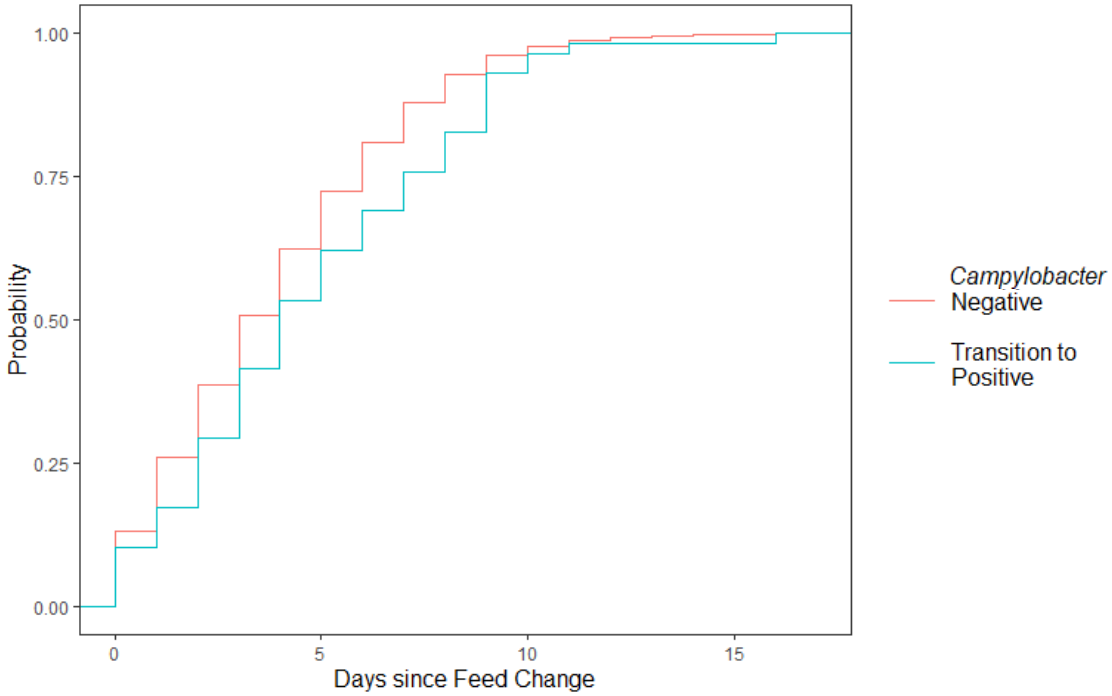

Figure 3.15: Cumulative probability plots of the number of days since a flock changed diet for all observed Campylobacter negative days (n = 2471) and days on which a flock transitioned to Campylobacter positive (n = 29).

A fitted GAM of water consumption, using bird age, average external temperature, farm, and *Campylobacter* status as predictor variable (Figure 3.16) revealed that all the parameters included in the model had a statistically significant impact on the amount of water consumed by a flock. Water demand increased linearly with age over most of the birds' life. Consumption also increased linearly with temperature, although the impact of temperature is slight, with a 25°C temperature difference only representing an increase of under 10 litres per 1,000 birds within the additive model. Water consumption varied by farm and by *Campylobacter* status, with positive flocks drinking less than negative flocks, with the impact of *Campylobacter* being more minor than that of farm.

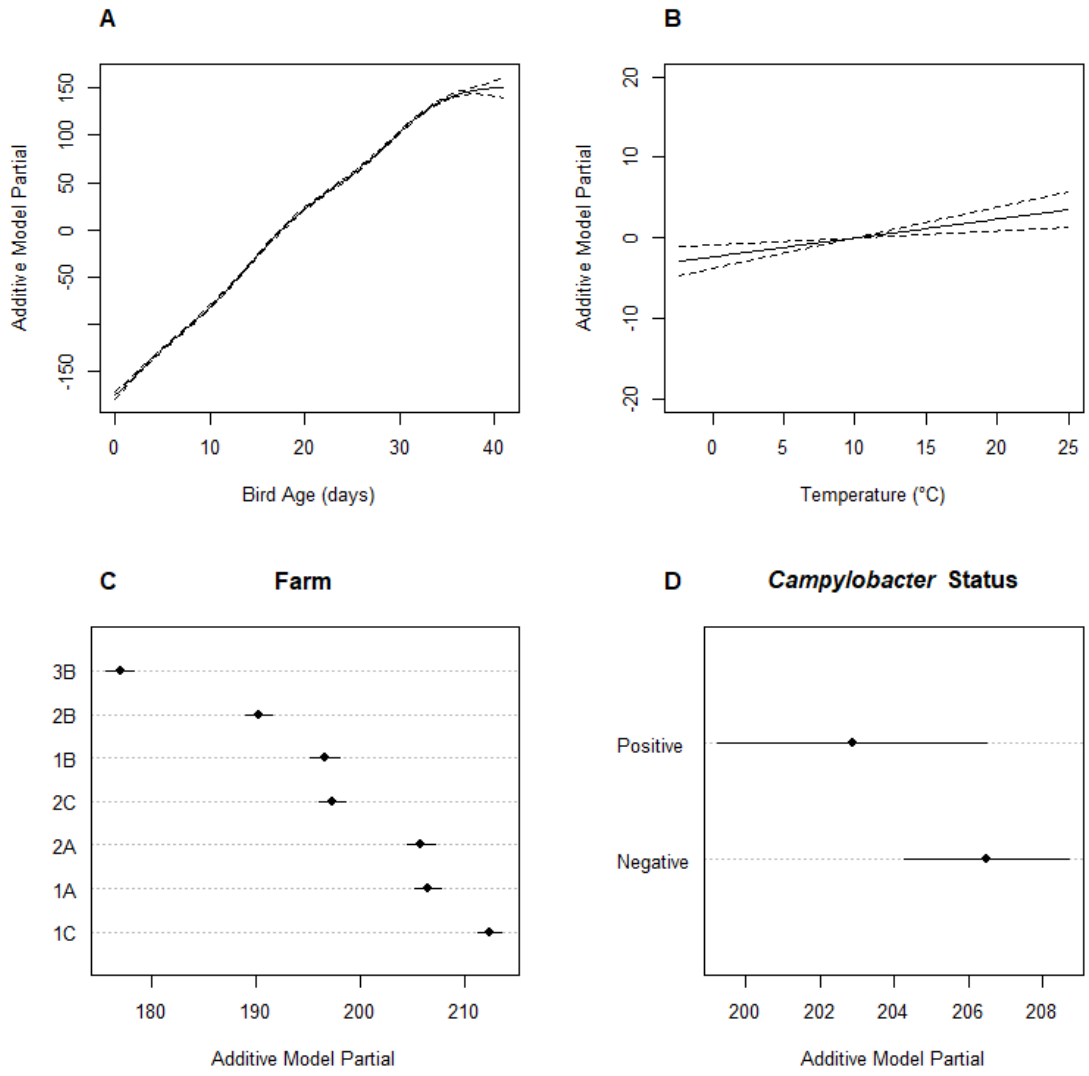

Figure 3.16: Details from a Generalised Additive Model output, predicting the impact of bird age system (Figure 3.16A, top left; p < 0.001), mean daily external temperature (Figure 3.16B, top right; p < 0.001), farm (Figure 3.16C, bottom left) and Campylobacter status (Figure 3.16D, bottom right; p = 0.021) on daily water consumption (litres per 1,000 birds; n = 2664). This graph shows only farms for which the water consumption information was made available by the farmer.

3.3.7 Farm Construction and *Campylobacter* Incidence

Since different farms have distinct rates of *Campylobacter* infection incidence, it is important to consider the physical differences between farms and assess the impact that each variable could potentially have on the risk of *Campylobacter* infection.

A fitted GAM of binomial data derived from daily *Campylobacter* status (1 for positive, 0 for negative) using maximum observed daily external temperature, bird age, ventilation system, house construction as predictor variables (Figure 3.17) revealed that side inlet ventilation (Figure 3.17B; p = 0.015) and wood posted house construction

(Figure 3.17C; $p < 0.001$) were significantly linked to an increased probability of *Campylobacter* acquisition. This implies that flocks housed in structures with these attributes were more likely to develop an infection.

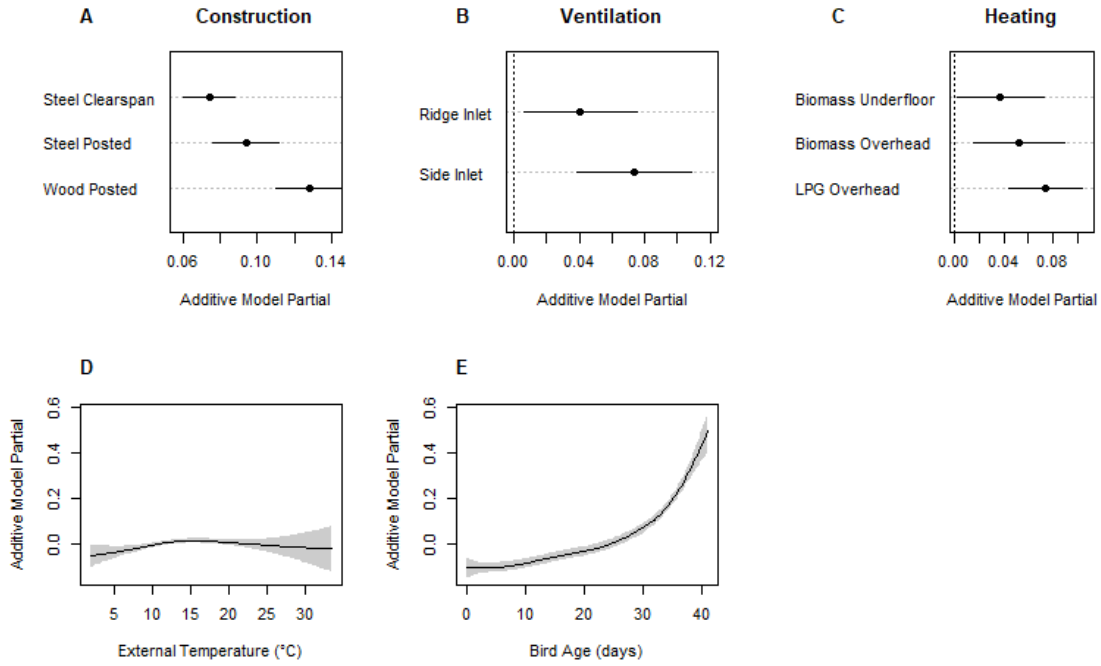

Figure 3.17: Details from a Generalised Additive Model output, predicting the impact of ventilation system (Figure 3.17A, top left), house construction (Figure 3.17B, top centre) heating system (Figure 3.17C, top right), external daily average temperature (Figure 3.17D, bottom left; $p = 0.0136$) and bird age (Figure 3.17E, bottom centre; $p < 0.001$) on the probability of a flock being Campylobacter positive.

To assess the impact of house construction parameters on flock infection onset timing, a Survival Analysis was conducted using Cox Proportional-Hazards, assessing the impact of ventilation, construction, and heating types on the duration a flock remains *Campylobacter* negative (Figure 3.18). There are no statistically significant differences between each group. Underfloor biomass heating does initially appear to have a protective effect against *Campylobacter*, but this is confounded by the fact that there was only a single farm with this attribute, hence there was no statistical significance ($p = 0.200$).

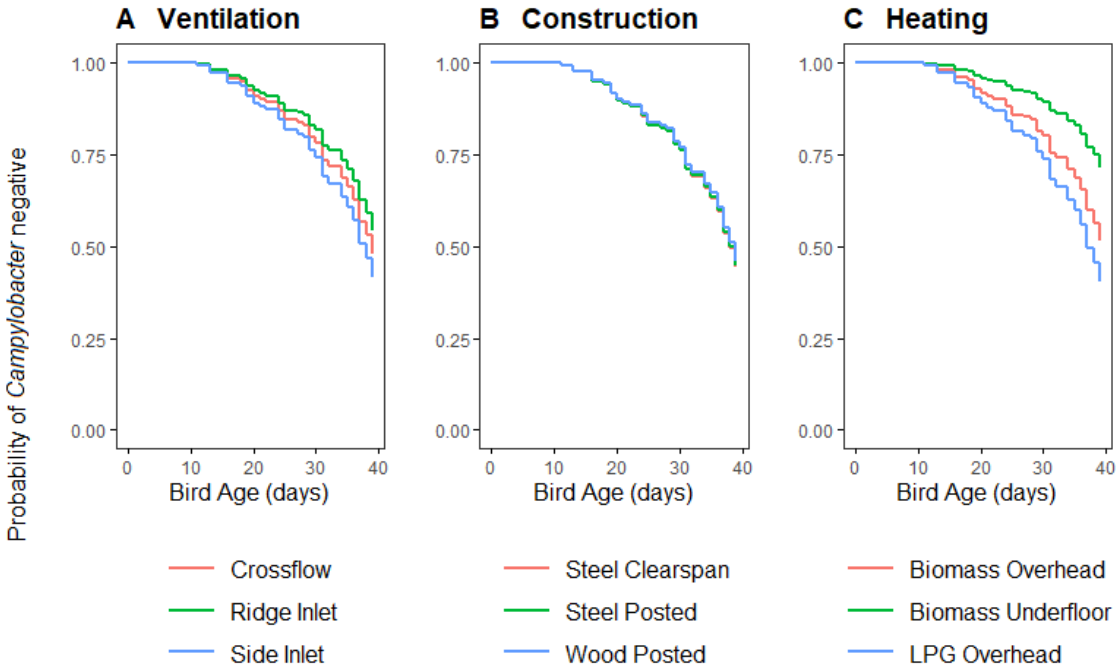

Figure 3.18: Cox Proportional-Hazards Model adjusted survival analysis curves showing probability of a flock being Campylobacter positive against bird age, separated by chicken house ventilation type (Figure 3.18A, left; p = 0.170), construction style (Figure 3.18B, centre; p = 0.720) and heating system (Figure 3.18C, right; p = 0.200).

Based on the findings of the impact of weather (Section 3.3.4), an analysis was conducted comparing environmental temperature difference between production cycle daily mean and the mean observed during the week prior to *Campylobacter* infection, separated by house construction parameters, for *Campylobacter* positive production cycles on which the average temperature was below 9°C (Figure 3.19). A Wilcoxon Rank-Sum test, comparing the temperature difference between cycle average and the week preceding infection onset between ventilation systems, suggests that there is not significant difference, but due to the low sample size statistical power is not strong enough to provide conclusive evidence ($p = 0.053$, $1-\beta = 0.253$). Kruskal-Wallis Rank-Sum tests, comparing the temperature difference between cycle average and the week preceding infection onset between construction and heating systems, gives no statistically significant difference between each group.

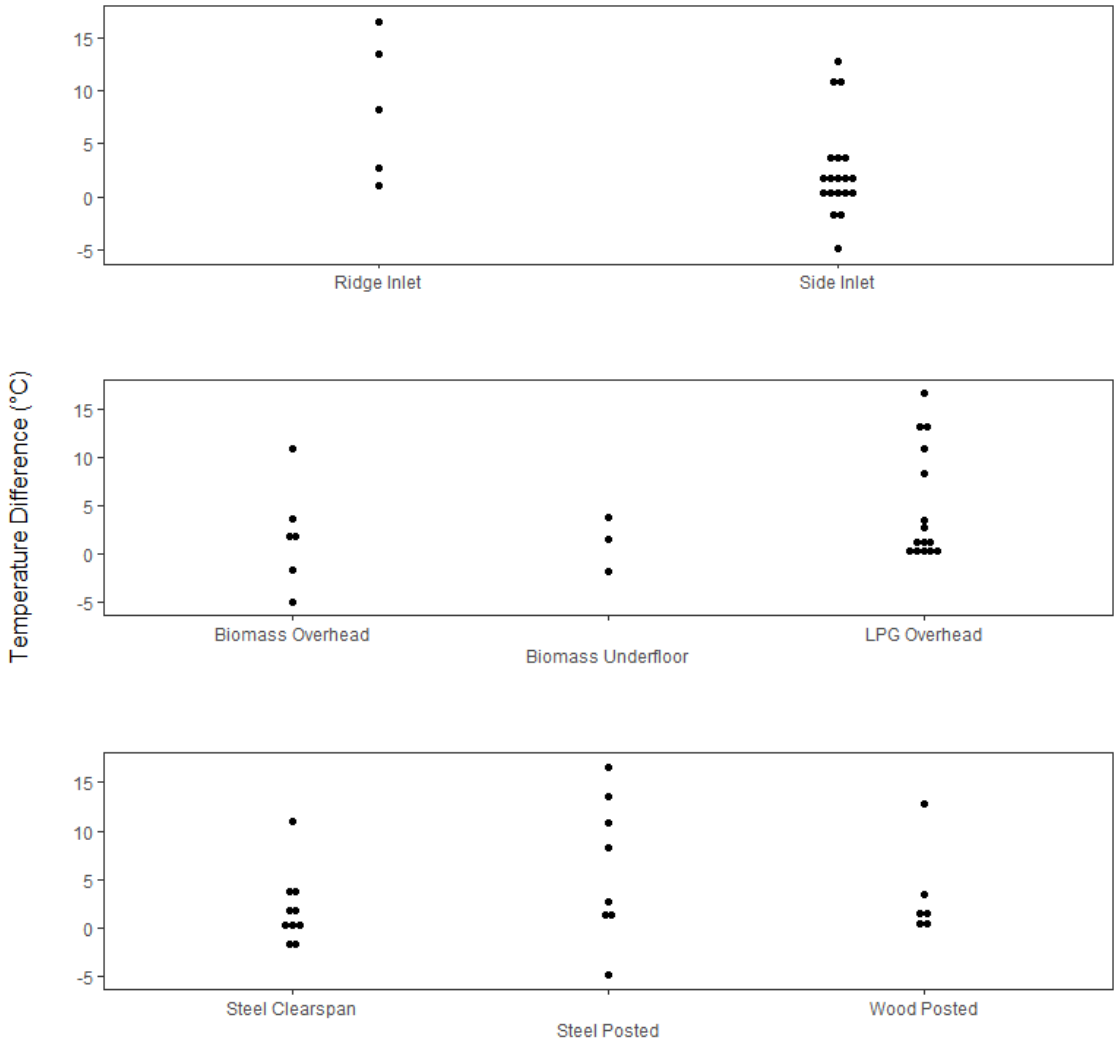

Figure 3.19: Dot plots showing the temperature difference observed between the cycle average temperature and temperature observed the week preceding Campylobacter infection, broken down by ventilation system, heating system and house construction, for cycles on which the average temperature was below 9°C.

3.3.8 Infection Timing

To assess the impact of time of year on the age at which *Campylobacter* infects chicken flocks, a fitted GAM of bird age at the onset of infection was created, using a subset of data consisting only of production cycles which acquired a *Campylobacter* infection, using month of the year and farm as predictor variables (Figure 3.20). From

this, no statistically significant impact on flock infection timing was found between different times of year (Figure 3.20A, p = 0.178).

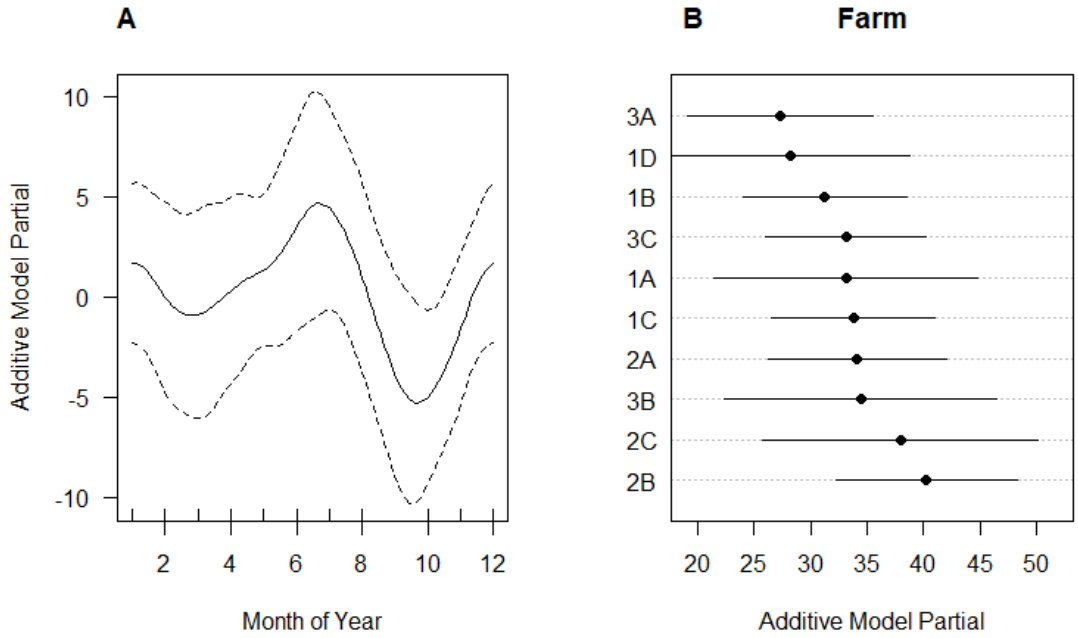

Figure 3.20: Details from a Generalised Additive Model output, predicting the impact of month of the year (Figure 3.20A, left; numbered according to order, from January to December; p = 0.178) and farm (Figure 3.20B, right) on the bird age at which Campylobacter infection began.

To further elaborate on the observed lack of statistical impact of month of the year on the age of the bird at the onset of *Campylobacter* infection shown in Figure 3.20, a Survival Analysis was conducted using Cox Proportional-Hazards, assessing the impact of season on the duration a flock remains *Campylobacter* negative (Figure 3.21). This showed no difference between seasons (p = 0.695). A Chi-Squared analysis of whether a flock was more or less likely to be infected before or after thinning in different seasons similarly showed no significant difference (Table 3.5, p = 0.238).

Table 3.5: Contingency table comparing whether a flock acquired a Campylobacter infection before or after thinning, and what season the flock was cleared in (summer defined as April to September, winter as October to March).

	Infection Onset Pre-Thin	**Infection Onset Post-Thin**
Flock Cleared in Summer	11	11
Flock Cleared in Winter	18	9

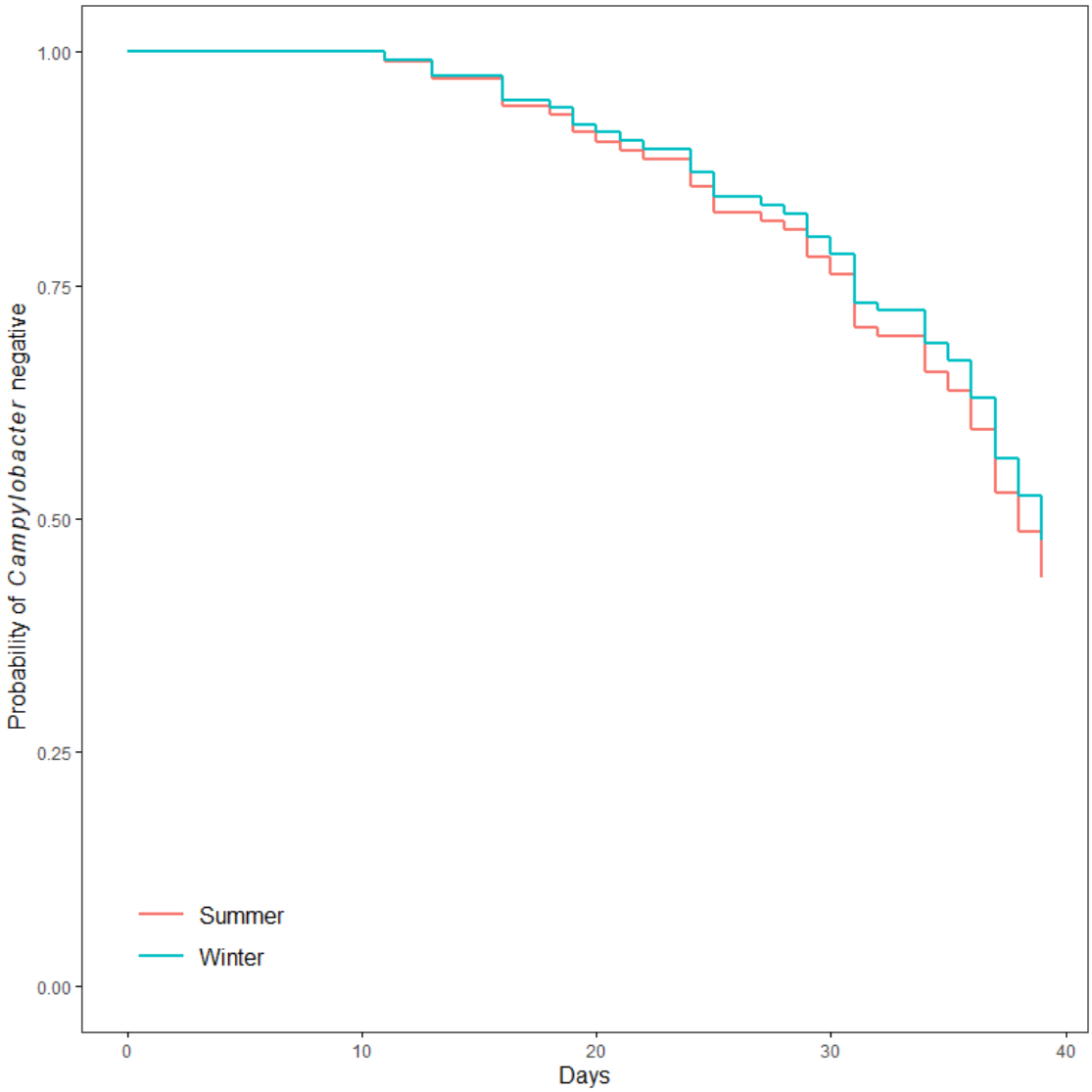

Figure 3.21: Cox Proportional-Hazards Model adjusted survival analysis curves showing probability of a flock being Campylobacter positive against bird age, separated by season (summer defined as April to September, winter as October to March).

3.4 Discussion

In this study, a set of continuous and discrete parameters, including weather and season information, dietary information, house internal temperature and house construction types were recorded across eleven broiler chicken houses on different farms, and compared with presence of *Campylobacter* spp. within the chicken flocks housed within to find statistical associations. Of these, the most significant connections were seen in the type of house construction and ventilation system in use, with wooden posted houses and side inlet ventilation both being associated with a higher risk of *Campylobacter* infection. This study also aimed to test whether there was any seasonal variation in the age of onset of *Campylobacter* infection in these flocks, and none was apparent in the data.

One of the largest noted co-factors corresponding with *Campylobacter* acquisition amongst broiler chickens in the work presented here is the way the farm is constructed. Out of overall construction styles, poultry houses with a wooden posted frame were found to be the most at-risk for flock contamination. This is an older construction method that is not commonly implemented (Morspan Ltd., 2021), but still available from some contractors. The findings of research into risk factors associated with flock *Campylobacter* colonisation by Sommer, Nauta and Rosenquist (2016) suggest that upgrading to more modern poultry houses can reduce risk of flock infection. Whilst wood may have putative contact antimicrobial effects in a poultry production environment (Munir *et al.*, 2019), the increased risk of infection observed here may be due to factors intrinsic to the material itself as wood is considerably harder to disinfect than other materials such as plastics (Welker *et al.*, 1997). All the wooden houses observed in this study are over three decades old (Table 2.1), and while retrofits and refurbishments of the houses have been conducted by the farms' owners over the years, they are still forced to design the ventilation and heating systems around the constraints of an increasingly antiquated design. This is reflected in the fact that none of the wooden houses had any heating system other than the more outdated LPG overhead heaters. Since, in the presented work, these legacy systems were associated with an increased risk of *Campylobacter* infection among the birds contained within these houses, it is possible that these heating and ventilation systems may predispose the chickens to heat

stress, which is known to be conducive to infection of the gut by organisms such as *Campylobacter* (Humphrey, 2006; Rostagno, 2009; Quinteiro-Filho *et al.*, 2012; Verbrugghe *et al.*, 2012; Lara and Rostagno, 2013).

Ventilation system was also implicated in the presented study as being closely linked to the probability of a flock becoming *Campylobacter* positive. The two main systems in current production, side inlet and ridge inlet, each make up around half of UK broiler chicken houses in 2012 (Pollard, 2012). However, the data presented here indicates that ridge inlet houses are associated with a lower risk of *Campylobacter* infection, a conclusion supported by Hansson et al. (2010), with tentative evidence also presented here that *Campylobacter* infections among chickens in ridge inlet houses begin at a more advanced bird age than comparable infections among chickens in side inlet ventilated houses. Since air is drawn in from a higher point with ridge inlet ventilation, it may be protective against airborne debris and insects. Insects tend to fly close to the ground, with greatest density occurring between 0.2 and 5.4 metres above ground, dependent on species and local geography and climate (Byers, 2011).This may mean that the bulk of flying insects do not get exposed to the air intake of ridge inlet houses but could enter a side inlet house, due to its air intakes being physically lower. Insects, particularly flies, have been implicated as a potential source of *Campylobacter* for broiler flocks (Hald *et al.*, 2004; Royden *et al.*, 2016) and so excluding them from ventilation inlets may be conducive to preventing infection. Different ventilation techniques may also represent different levels of thermal performance, and thus the observed distinction in *Campylobacter* infection rates between ventilation system could be due to heat stress events. Figure 3.19 demonstrates that a higher environmental temperature differential between cycle average and the week preceding onset correlated with positive flocks in ridge inlet houses during colder months, when compared to side inlet houses, which were more likely to be positive at less extreme environmental temperature swings. While no thermal or insect-excluding abilities can be inferred from the data presented here, it suggests that research into ventilation implementation in broiler houses may prove a promising route for developing new *Campylobacter*-excluding biosecurity measures.

The age of onset of *Campylobacter* infection in broiler chickens, according to the research presented in this chapter, does not vary with season. This is in keeping with the findings of Evans and Sayers (2000), although there are differences between this chapter and that study. The collection time of Evans and Sayers (2000) was weekly, commencing from 28 days to 56 days, and is based on cloacal swabs of 16 birds per assessment, while the presented work in this chapter is based on daily bootsocks of the broiler house bedding, with a maximum age of 41 days observed through the sampling period. The median bird age on which *Campylobacter* infection began in the present chapter was 30 days, while the finding of Evans and Sayers (2000) was that the 50th percentile for infections fell between 36 and 42 days of bird age. While cloacal swabs tend to be preferable to bootsocks in strictly culture-based studies due to improved detection rates (Vidal *et al.*, 2013; Ingresa-Capaccioni *et al.*, 2014), PCR analysis of bootsocks has proven to be more sensitive when assessing the *Campylobacter* status of a flock than culture of caecal swabs from the same flock (Matt *et al.*, 2016).

A noted discrepancy was found between paired *Campylobacter* results between Avara's internal testing regime and the methodology used in this study. Results at clearance disagreed approximately 40% of the time, representing a substantial conflict in findings. Avara's testing is based around a Loop-Mediated Isothermal Amplification (LAMP) reaction, using the Genie II detection system and reagents (OptiGene Ltd., Horsham, England), and using a protocol written by the manufacturer (OptiGene, 2016). However, recent field trials of the system (Llarena *et al.*, 2022) comparing against the 'gold standard' qPCR for the *Campylobacter* spp. 16S rRNA gene, as used in the study presented here and first described by Lund et al. (2004), found that the OptiGene system only had a sensitivity of 65%, in keeping with findings presented here. Further indication of the robustness of the data collected for this study is evidenced by the detection of subsequent positives on almost every day after the first detection on positive farms, as demonstrated in Figure 3.2 and Figure 3.5, giving credence to the acquisition and persistence of *Campylobacter* in a flock being readily detected by the methodology described here.

What is perhaps more confounding is the absence of any 'winter dip' away from the summer peak in infections, over the winter of 2018-2019, seen outside of Avara's

testing. Whilst positive flocks did decrease in numbers as expected over the winter of 2019-2020, the previous winter unexpectedly breaks the trends observed in historic data from Avara and from general trends seen in broader literature (Patrick *et al.*, 2004; Hartnack *et al.*, 2009; Jonsson *et al.*, 2010, 2012; Jore *et al.*, 2010; Chowdhury *et al.*, 2013; Weber *et al.*, 2014). Historic data from Avara's previous years of *Campylobacter* records show a predictable, repeating pattern in infections, demonstrated in Section 3.3.1, with a peak in infections occurring anywhere between May and September every year. Public health records indicate that there was a peak in human campylobacteriosis in England and Wales of 1764 cases in week 23 of 2018 (Public Health England, 2018), followed by a dip to 530 cases in week 52 (Public Health England, 2019b), followed by a return to a peak of 1645 cases in week 23 of 2019 (Public Health England, 2019a), and so the lack of seasonality observed in chickens in this project was not reciprocated in contemporaneous human cases. A late summer peak in infections was seen over the course of the project presented here in Avara's testing data, however this too seemed incongruous with the historic record. Figure 3.4 demonstrates that the dip in infections across all of Avara's farms was at its lowest in January 2019, but Figure 3.7 reveals that the lowest rate of detection amongst Avara's tests for this project occurred in April 2020, four months after the dip observed across all other farms. This discrepancy from the historic trend and that seen across all of Avara's farms is, in part, due to the small sample size and limited timeframe for seasonal observations, but additionally it was revealed in Figure 3.9 that environmental temperature and humidity only have a weak effect on *Campylobacter* incidence among the flocks observed in this project.

Taking into consideration the other notable findings from the work put forward in the present study, such as the impact of poultry house ventilation and construction materials, it is likely that atmospheric temperature and humidity contribute towards the likelihood of *Campylobacter* acquisition of a flock by a more indirect means than anticipated. Ishihara et al. (2017) demonstrate the secondary nature of this relationship by demonstrating that *Campylobacter* acquisition by a broiler chicken flock displays different levels of correlation with temperature and humidity across different seasons, a trend observed in the presented study to a degree in Figure 3.10, in which a stronger influence of temperature fluctuation on *Campylobacter* acquisition becomes apparent

below a threshold of approximately 9°C. In addition, Line (2006) found that low humidity was able to delay *Campylobacter* acquisition by a newly placed broiler chicken flock from a contaminated poultry house. Given the findings presented here regarding the link between house ventilation and construction and the likelihood of an infection occurring (Figure 3.17) and negative association between humidity and *Campylobacter* incidence (Figure 3.9), there is a possibility that *Campylobacter* seasonality observed on some farms is driven by the house construction materials allowing some pathogens to evade the house disinfection process between poultry flocks, and subsequent high humidity predisposing the new flock to acquisition of *Campylobacter*.

The regulation of internal house temperature is noted as being one of the most intensely controlled parameters under the farmer's control in broiler chicken production (Aviagen, 2010; Aviagen, 2018; DEFRA, 2018). This is due to the risk of thermal stress on the birds, a condition noted as having severe impacts on bird welfare (Humphrey, 2006; Lara and Rostagno, 2013) including the induction of gut inflammation under the pressure of pathogens (Quinteiro-Filho *et al.*, 2012). For this reason, deviation in internal house temperature away from the standards laid down by Avara was measured as a potential risk factor in flock acquisition of *Campylobacter.* While no direct correlation could be shown in the data between *Campylobacter* infection and deviation from temperature standards, external temperature and bird age were intrinsically linked to deviation away from the temperature standard, with advanced bird ages and higher external temperatures contributing to often severe deviation in internal house temperature away from ideal conditions (Figure 3.13). Heat stress events in warmer months may therefore be a contributing factor to *Campylobacter* seasonality observed in broiler chicken flocks.

3.5 Conclusions

This work demonstrates that the age of onset of *Campylobacter* spp. infections among broiler chickens does not vary with season, and there is no impact on rate of detection of *Campylobacter* arising from differences in infection timing. Temperature alone is not responsible for most of the seasonal variation in *Campylobacter* infection

rates. Several important risk factors were identified as associated with increased *Campylobacter* infections, such as ventilation system and house construction form, which point towards prospective interventions to exclude *Campylobacter* from poultry houses, predominantly in the design considerations of new poultry house constructions.

4 *Campylobacter* detection on broiler chicken farms, its impact on incidence of broiler *Campylobacter* infection and variation across season.

4.1 Introduction

Persistence of *Campylobacter* within a poultry house, from one production cycle to the next, has been examined by previous studies for its likelihood as a source of flock infection in UK broiler chicken production, with some studies supporting the continuation of *Campylobacter* within a chicken house environment (Battersby *et al.*, 2017; de Castro Burbarelli *et al.*, 2018; Frosth *et al.*, 2020) and some suggesting that it is not a likely origin of new infections (Evans and Sayers, 2000; Shreeve *et al.*, 2002; Rushton *et al.*, 2009). Attention has also been placed on environmental sources of *Campylobacter* spp., such as wild animals or bodies of water, as reservoirs for flock infection. *Campylobacter* spp. are readily detected in soils and other outdoor surfaces across the countryside of the UK (Jones *et al.*, 2017) and commercial chicken flock infection has been shown to be influenced by *Campylobacter* infections on nearby chicken farms (Chowdhury *et al.*, 2012). Persistent detection of *Campylobacter* spp. from multiple points across a farm site has been repeatedly demonstrated as being a risk factor in flock infection (Newell and Fearnley, 2003; Bull *et al.*, 2006; Ellis-Iversen *et al.*, 2012; Robyn *et al.*, 2015; Battersby, Whyte and Bolton, 2016b; Frosth *et al.*, 2020).

Chicken houses are not sealed environments and there are multiple necessary breaches in biosecurity, both continuous and intermittent. Farm staff must enter the chicken house environment multiple times per day (DEFRA, 2018). The risk of transmission of pathogens by staff entry is mitigated through biosecurity protocols (European Commission, 2017), as specified both in codes of practice (DEFRA, 2018) and company policy. Many commonly implemented biosecurity measures, such as establishing hygiene barriers at the entrances to chicken houses or mandating the use of disinfectant footbaths, have been shown to reduce the risk of *Campylobacter* contamination of a chicken flock (Adkin *et al.*, 2006; Newell *et al.*, 2011; Sibanda *et al.*, 2018). However, these measures are not infallible and contact between staff and birds

can be linked to *Campylobacter* acquisition (Battersby, Whyte and Bolton, 2016a). Catching of birds to be taken away for slaughter is a considerable interaction between staff and birds, resulting in inevitable reductions in biosecurity (Millman *et al.*, 2017), with some studies suggesting that catches which partially depopulate a flock increase the incidence of *Campylobacter* infection in remaining birds (Ellis-Iversen *et al.*, 2012; Koolman, Whyte and Bolton, 2014; Higham *et al.*, 2018).

Mechanical ventilation of broiler chicken houses has been postulated as a route for infection (Hald *et al.*, 2004; Hansson *et al.*, 2007; Royden *et al.*, 2016). The possibility of insect-borne transmission of *Campylobacter* to broiler flocks has been suggested (Hald *et al.*, 2004; Royden *et al.*, 2016), but other materials capable of passing through ventilation systems, such as dust and aerosols, can also carry airborne *Campylobacter* within the farm environment (Chinivasagam *et al.*, 2009; Olsen *et al.*, 2009; Søndergaard *et al.*, 2014). This is further evidenced through detection of culturable airborne *Campylobacter* downwind of contaminated poultry houses by Bull et al. (2006), indicating that contaminated particulates can carry viable organisms through chicken house ventilation systems.

Given the broad variety of environmental reservoirs that have been posited as risk factors in chicken flock *Campylobacter* infection, a substantial body of explorative studies are attested to in modern literature. However, reports conflict as to the degree to which each potential source of infection contributes to flock contamination (Sibanda *et al.*, 2018), and none have been posited as having a substantive impact on the seasonal pattern seen in *Campylobacter* infection in commercial broiler chickens (Robyn *et al.*, 2015; Hansson *et al.*, 2016; Sibanda *et al.*, 2018).

4.1.1 Chapter Aims

The aim of experiments detailed in this chapter is to ascertain whether the detection of *Campylobacter* spp. in the immediate farm environment can be correlated with the detection of *Campylobacter* spp. in the broiler chicken flocks grown on the same farm. In this way, the risk posed by horizontal transfer from the farm environment can be assessed for its role in flock infection and hence corresponded with the seasonal incidence of *Campylobacter* infection in broiler chickens.

Specifically, objectives were to:

- Monitor the air around poultry houses for aerosolised *Campylobacter* and assess its likelihood as a source of infection.
- Evaluate the presence of *Campylobacter* in the farm environment and assess the likelihood of transmission from the surroundings to interior of the poultry house by personnel.
- Assess the prevalence of *Campylobacter* on materials and consumables being brought into the poultry house for routine husbandry activities.

This was achieved by monitoring eleven farms over a period of 16 months, with four monitored for an additional 12 months afterwards, and recording the *Campylobacter* status of the flocks present daily, along with that of environmental samples collected simultaneously.

4.2 Methods

For the first ten cycles of the project detailed in this chapter, the flocks observed were the same as those described as observed in Chapter 3. Of these, farms 1A, 1B, 2A and 3A were observed for a further seven production cycles (cycles 11 to 17). These additional cycles were observed between April 2020 and March 2021. Cycles 11 to 17 shall henceforth be referred to as 'Phase Two' of the project, and all previous cycles as 'Phase One', as attested to in Section 2.2.

4.2.1 Air Sample Collection

Air samples were collected using a commercial Sartorius AirPort MD8 air sampler (Sartorius AG, Göttingen, Germany) once per production cycle. This device has been proven as effective in this specific role in prior published research (Søndergaard *et al.*, 2014; Hoorfar *et al.*, 2020; Johannessen *et al.*, 2020). The age of the birds at sample collection was chosen to represent a timepoint where chickens were present in the house, but very unlikely to be *Campylobacter* positive, and hence maximising the chance that detected *Campylobacter* originated from environmental sources. The full methodology is detailed in Section 2.3.

4.2.2 DNA Extraction from Air Samples

The DNA extraction protocol used for processing air samples was based on the findings of Chen et al. (2003), which showed that a gelatine cell entrapment matrix could be removed without affecting any entrapped cells through enzymatic processing, and was verified with a pilot study using spiked air filters. The pilot study and the results from it are detailed in Section 4.3.1, and the procedure itself is detailed in Section 2.3.

4.2.3 Bootsock Collection

Paired internal/external bootsocks were only taken for Phase Two of the project.

Bootsock swabs of the inside of the poultry house were collected daily by farmers as per Section 2.4.1, as part of the first welfare checks of each day. Farmers were also instructed to take an additional environmental bootsock swab each day, as part of the first daily welfare check of the birds, as detailed in Section 2.4.2. The route walked was between the site management office and the front door of the poultry house under study.

Collections of bootsocks were made at the end of every cycle for return to the laboratory. Any bootsocks to be tested were processed by suspending collected biological debris in isotonic saline, as per Section 2.4.3. DNA was isolated using a QIAGEN QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per Section 2.6, then tested for *Campylobacter* presence using a multiplex qPCR as detailed in Section 2.7, with subsequent confirmatory PCR and gel electrophoresis as per Section 2.8 if species identity could not be ascertained by the primary qPCR.

For the sake of efficiency, not every house interior collected bootsock was tested. Instead, one bootsock was tested every five days, from day ten until the final day of the production cycle. Of these, if one was positive and one was negative, all bootsocks in between these two points were also tested to isolate the first day of infection. All environmental bootsocks were tested (n = 655).

4.2.4 Testing of Initial Conditions prior to Chick Placement

4.2.4.1 Sample Collection

At the beginning of every crop cycle, on the day of arrival of chicks for the production process, farmers were instructed to collect six materials utilised in the placement of new chicks, henceforth referred to as 'initial samples'. These consisted of:

- One resealable bag (Minigrip LLC, Alpharetta, GA, USA) filled with bedding or litter material.
- One resealable bag filled with the contents of an enrichment bale.
- One 25 ml universal tube (Starstedt AG & Co. KG, Nümbrecht, Germany) filled with water from drinker lines.
- One 25 ml universal tube filled with starter crumb feed.
- One resealable bag containing an approximately 10 cm x 10 cm square of the paper liner used in chick delivery boxes.
- One resealable bag containing an approximately 10 cm x 10 cm square of cardboard sheet used to corral chicks into an area.

Farmers were instructed to take all samples while wearing nitrile gloves and store the samples in a refrigerated environment until they could be returned to the laboratory.

4.2.4.2 DNA Extraction and PCR Testing

Upon sample return, all initial samples were stored at 4°C until required for DNA extraction. When needed, all samples other than water were placed in individual stomacher bags (Seward Ltd., Worthing, UK) and 25 ml of sterile isotonic saline solution poured upon each. The bags were then manually kneaded for one minute to disperse any biological material collected into solution. The bags were left to stand for a further ten minutes to allow inorganic solids to sediment, then 5 ml of supernatant transferred to bijous and stored at -20°C for future DNA re-extraction if needed. 1 ml of supernatant was also transferred directly to a 1.5 ml microcentrifuge tube, centrifuged at 8,000 x g for five minutes and the supernatant removed. DNA was extracted from the resulting

pelleted material using a QIAGEN QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per Section 2.6.

Water samples were processed by placing 1 ml of the sample in a 1.5 ml microcentrifuge tube, centrifuging at 8,000 x g for five minutes and removing the supernatant. This process was repeated sequentially a further five times, so the total particulate matter of 5ml of water was pelleted. DNA was extracted from the resulting pelleted material using a QIAGEN QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per Section 2.6.

4.2.5 Statistical Methods

All analyses were constructed in R (R Core Team, 2020) as detailed in Section 2.10.

For testing the independence of categorical variables, the Chi-Squared Test was used (Section 2.10.2). Graphing and visualisation were conducted using ggplot2 (Valero-Mora, 2010; Wickham, 2016) and vcd (Meyer, Zeileis and Hornik, 2006).

4.3 Results

4.3.1 Extraction of DNA from Air Samples – Pilot Study Findings

A pilot study was conducted to ensure the efficacy of techniques used to separate cells for DNA extraction from the gelatine matrix that they were adhered to. The pilot study was based off the findings of Chen et al. (2003), regarding enzymatic treatment of gelatine matrices to release entrapped cells.

Five gelatine membranes were streaked with colonies from a stock culture of *C. jejuni* (NCTC 11168), manually broken up into small pieces and placed inside individual 2ml microcentrifuge tubes containing 1,350 µl of nuclease-free water. To each, 150 µl of a range of five different dilutions of Proteinase K was added (0 mg/l, 1 mg/l, 10 mg/l, 100 mg/l, 1,000 mg/l). The microcentrifuge tube lids were closed and incubated at room temperature with vigorous shaking by affixing the microcentrifuge tube to a vortex mixer for one hour. After this, each microcentrifuge tube was centrifuged at 4,500 x g for five minutes and the supernatant removed. DNA was extracted from the resulting pelleted material in each microcentrifuge tube as per Section 2.6, and the concentration

of *C. jejuni* DNA present in the final sample assessed using a NanoDrop One (Thermo Scientific, Waltham, MA, USA). The results of this experiment are detailed in Table 4.1. Based on these findings, it was determined that a concentration of 100 mg/l of Proteinase K would be used in the final protocol, to maximise potential recovery of *Campylobacter* cells from the entrapment matrix.

Table 4.1: Results from a pilot study determining the efficacy of DNA recovery of Campylobacter jejuni from gelatine membranes used in air sampling.

Concentration of Proteinase K in Final Solution (mg/l)	**DNA Concentration Detected at NanoDrop (ng/µl)**
0	8.9
0.1	14.4
1	17.9
10	6.3
100	13.3

4.3.2 Detection of Airborne *Campylobacter*

Due to logistical issues, air samples could only be taken from cycles 4 to 10. Of the 53 air samples collected, five were found to be *Campylobacter* positive (9.4%). Three of these occurred during cycle four. The full testing results are presented in Figure 4.1.

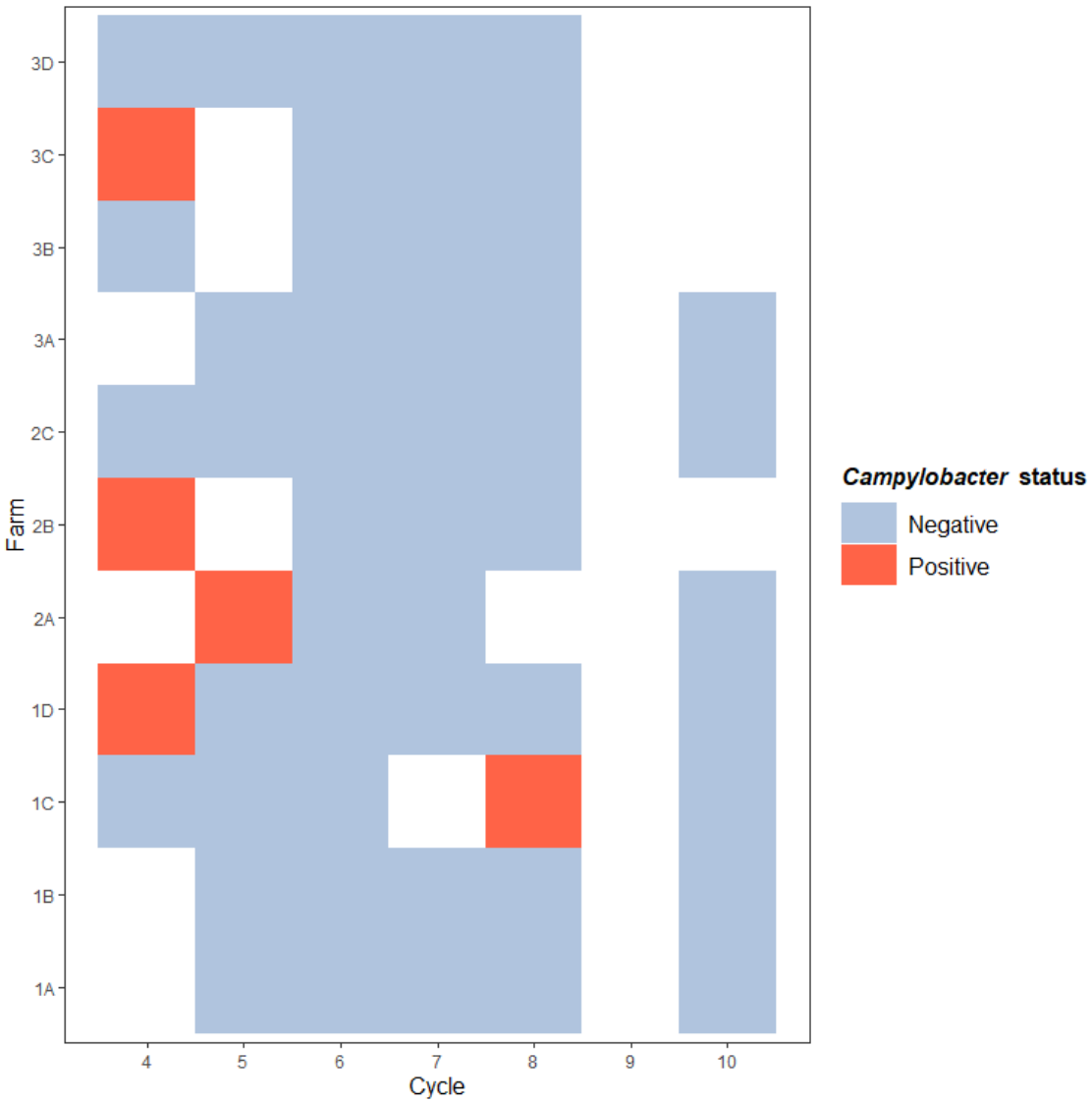

Figure 4.1: Testing results of air samples for Campylobacter spp.*, collected across eleven broiler chicken farms. White space indicates that no sample was collected.*

To assess whether the detected positive air samples were associated with the flock subsequently becoming *Campylobacter* positive, a Chi-Squared Test with Yates Correction was conducted (Table 4.2). No significant impact was found (p = 1.00).

Table 4.2: Contingency table comparing whether an air sample taken in the farm environment at a bird age of five days is Campylobacter positive, against whether the flock subsequently becomes Campylobacter positive at final clearance (n = 53 production cycles).

	Flock positive at clearance	**Flock negative at clearance**
Air sample positive	3	2
Air sample negative	26	22

4.3.3 Detection of *Campylobacter* within the Farm Environment

Campylobacter detection in paired bootsocks, collected over Phase Two, is shown in Figure 4.2. In total, 652 bootsocks were collected from the inside of poultry houses, of which 233 were tested. Environmental bootsocks (n = 655) were collected and all were tested. A summary of descriptive statistics for bootsocks collected from the house interior can be found in Table 4.3.

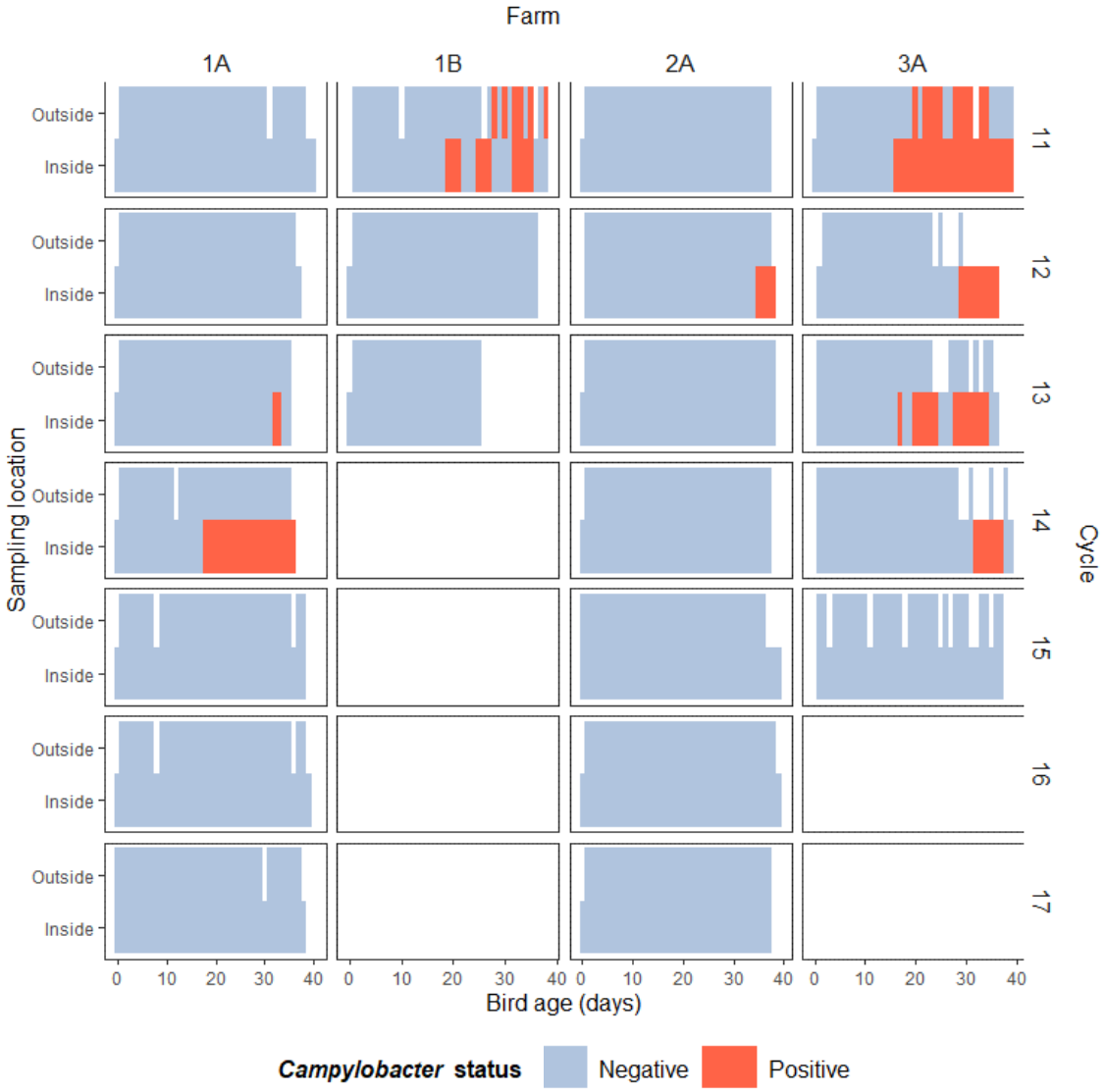

Figure 4.2: Daily testing results for Campylobacter detections inside and outside a poultry house across four broiler chicken farms, observed over seven production cycles.

Table 4.3: Summary statistics for positive bootsocks collected inside poultry houses on farms analysed in Section 4.3.3.

Farm	**1A**	**1B**	**2A**	**2B**
Number of Cycles Tested	7	3	7	5
Number of Positive Cycles	2	1	1	4
Mean Age of Infection Onset (± SD)	25.0 ± 7	19.0	35.0	24.3 ± 6.5

Campylobacter was only detected on bootsocks taken in the external farm environment for two continuous periods. Positive environmental samples began appearing on farms 1B and 3A for cycle 11 only, nine days and four days after a *Campylobacter* infection had established inside the chicken house respectively. No *Campylobacter* was ever found to be present in the farm environment without the chicken house under study first being *Campylobacter* positive. These positive external bootsocks occurred between 3rd June and 21st June 2020.

To assess whether the pattern of *Campylobacter* detection observed in environmental bootsocks could have arisen by chance, a Chi-Squared Test was conducted (Figure 4.3). A statistically significant result was produced ($p < 0.001$), indicating that there is a link between *Campylobacter* detection on the inside and outside of a poultry house ($X^2 = 107.81$). The Pearson residuals of the test indicate that positive indoor and positive outdoor bootsocks, occurring on the same farm on the same day, came about at above the expected rate.

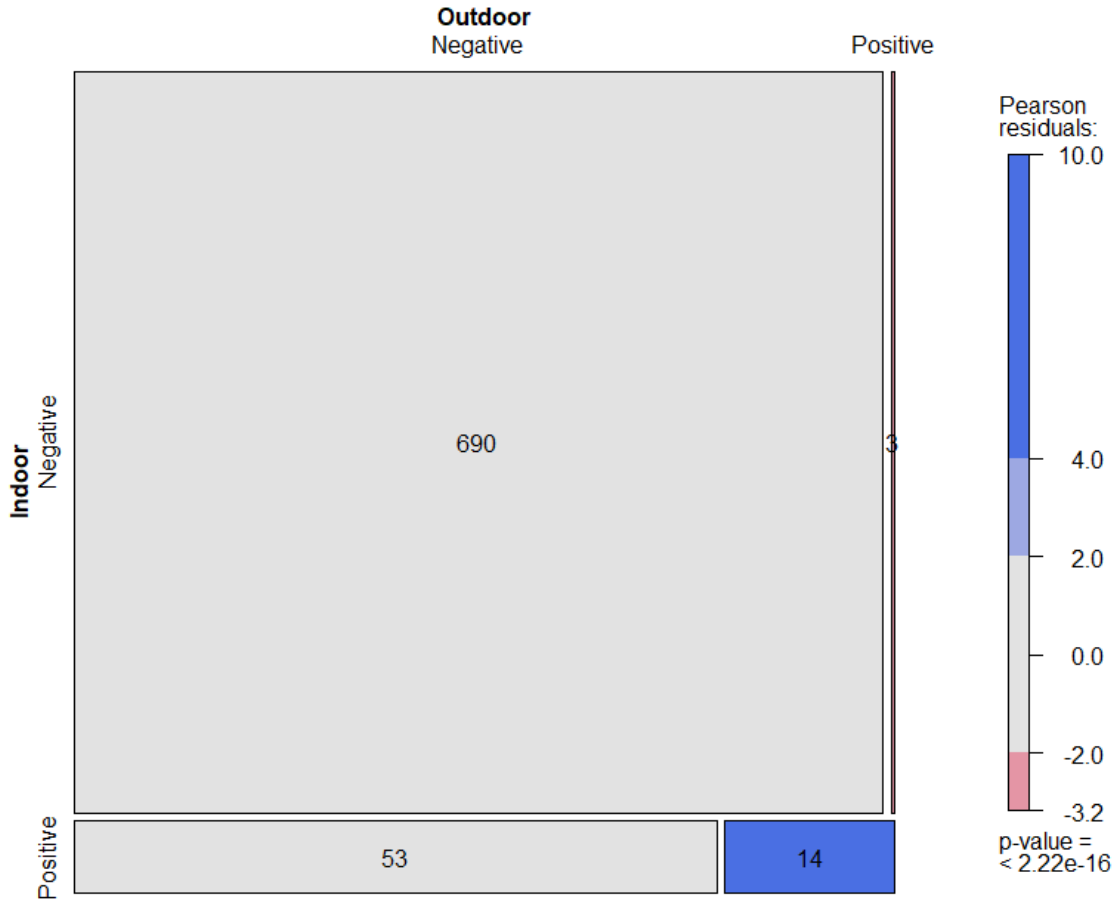

Figure 4.3: Mosaic plot displaying frequencies of bootsocks taken from the interior and exterior of four poultry farms being Campylobacter positive, along with Pearson residuals and Chi-Squared Test p-value.

4.3.4 Detection of *Campylobacter* in Initial Samples

Of the 516 initial samples collected, *Campylobacter* was only detected in five (0.97%). Two were samples of feed, two were cardboard intended for corralling chicks, and one was drinking water. The full testing results are presented in Figure 4.4. Table 4.4 presents the contingency table of *Campylobacter* detections on any of the initial materials and the subsequently placed flock becoming *Campylobacter* positive.

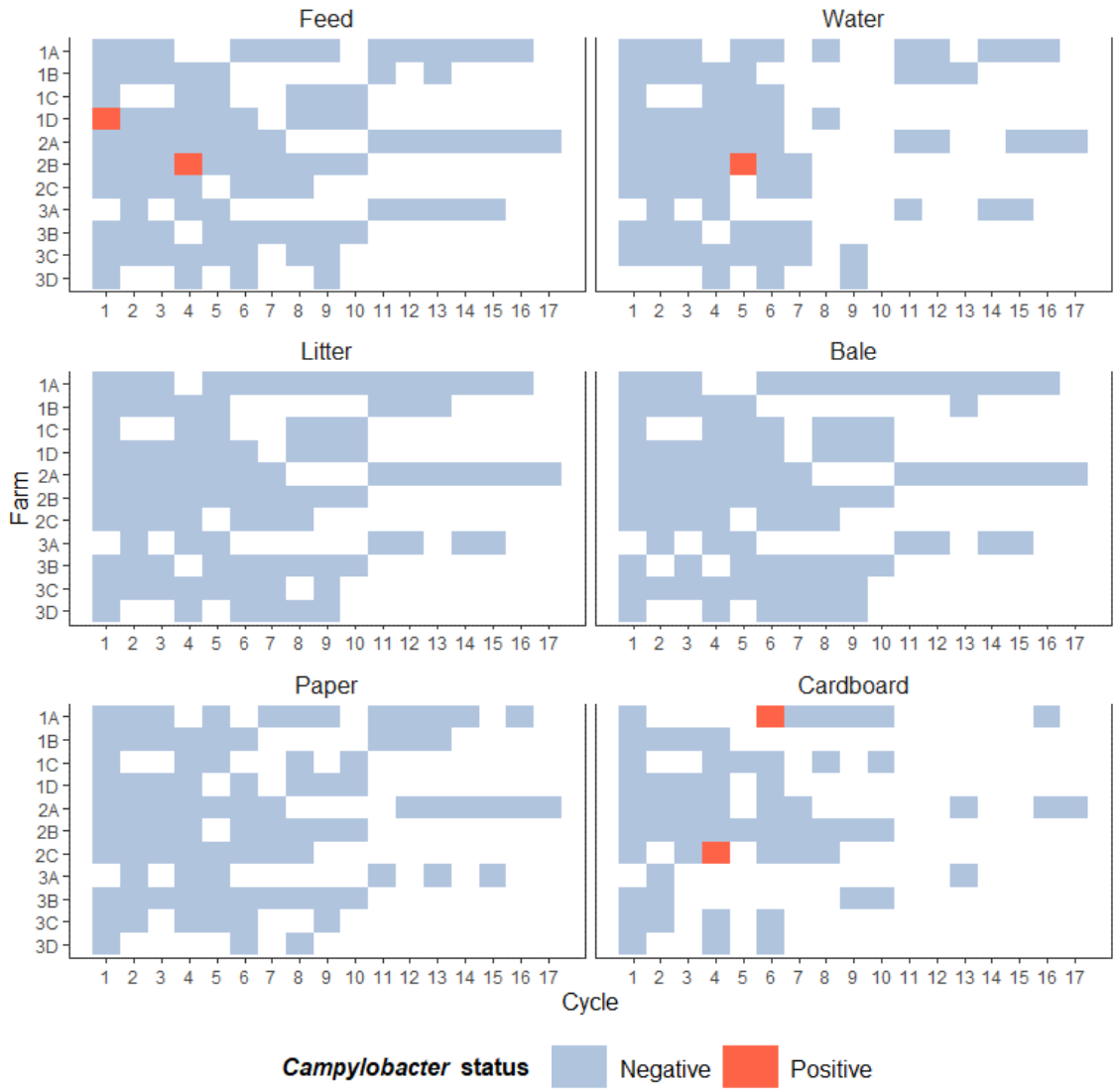

Figure 4.4: Campylobacter detection in six different sample types, taken from chick placement across eleven broiler chicken farms.

Table 4.4: Contingency table giving frequencies of chicken flocks acquiring a Campylobacter infection and the presence of Campylobacter on any of the initial materials utilised at the beginning of the production cycle.

	Flock positive at clearance	**Flock negative at clearance**
***Campylobacter* detected on initial materials**	2	3
***Campylobacter* not detected on initial materials**	47	53

On the two occasions for which *Campylobacter* was detected both on initial materials and at final flock clearance, one was a positive cardboard sample, and one was a positive feed sample.

For all feed samples besides the single confirmed *Campylobacter* positive sample, a strong 16S presence was detected by the initial PCR for *Campylobacter* detection (Section 2.7), but no response was detected on any of the species-specific primers. A subsequent enquiry utilising the primers used, as described by Lund et al. (2004), was conducted using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST) service (NCBI, 1988). Through this, it was revealed that, while the primers are indeed highly selective for *Campylobacter* spp., there are other organisms that have sequences that match, including the common agricultural gastrointestinal pathogen *Brachyspira* (GenBank accession number JX232353.1), which is capable of infecting swine, birds, dogs and humans. Using the *Brachyspira* 16S rRNA gene sequence as a comparative template, new primers were designed in MEGA7 (Kumar, Stecher and Tamura, 2016) specifically to exclude *Brachyspira* but include *Campylobacter* spp. 16S rRNA genes. These are described in the PCR for species confirmation (Section 2.8). All feed samples were further tested according to this method and, for all samples besides the singular sample previously identified as *Campylobacter* positive, no *Campylobacter* 16S rRNA genes were detected.

4.3.5 Thinning, Previous Flocks and *Campylobacter* incidence

Full testing results for collected pairs of bootsocks is presented in Figure 4.5. Of the 119 thin/clear sample pairs collected, 44 were found to be positive at clearance (37%), of which 24 were already positive at thinning and 20 were found to have acquired their infection between thinning and clearance.

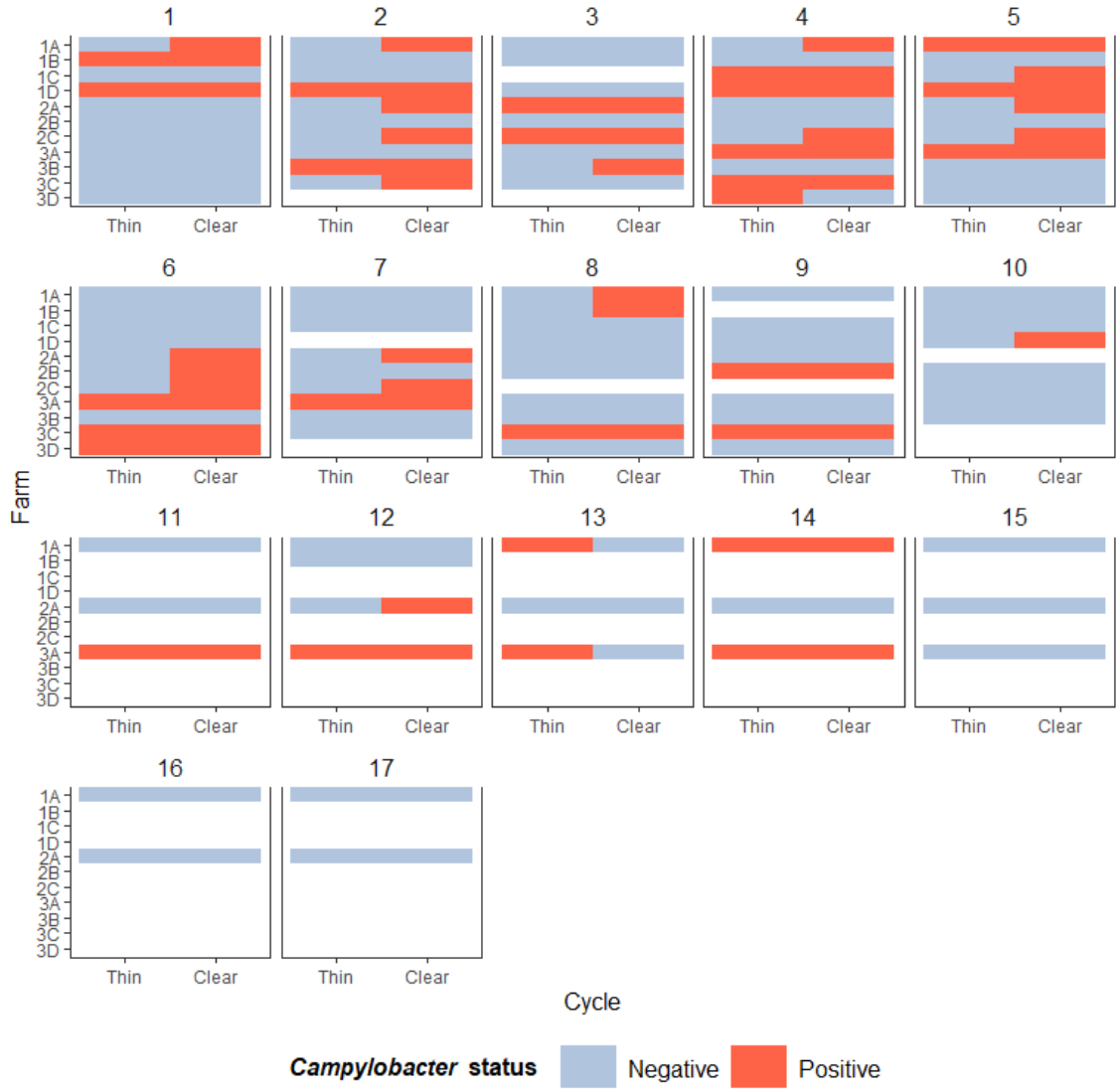

Figure 4.5: Testing results for Campylobacter presence on bootsocks collected from thinning and clearance across eleven chicken houses.

To assess whether flock acquisition of *Campylobacter* between thinning and clearance varied seasonally, a Chi-Square analysis with Yates' Correction was performed (Figure 4.6; $p < 0.001$). It was found that there was a statistically significant association between a flock being *Campylobacter* positive at thinning and subsequently being positive at clearance, regardless of season. For production cycles presenting as *Campylobacter* negative at thinning, fewer became subsequently positive in the winter than summer (17% and 28% respectively).

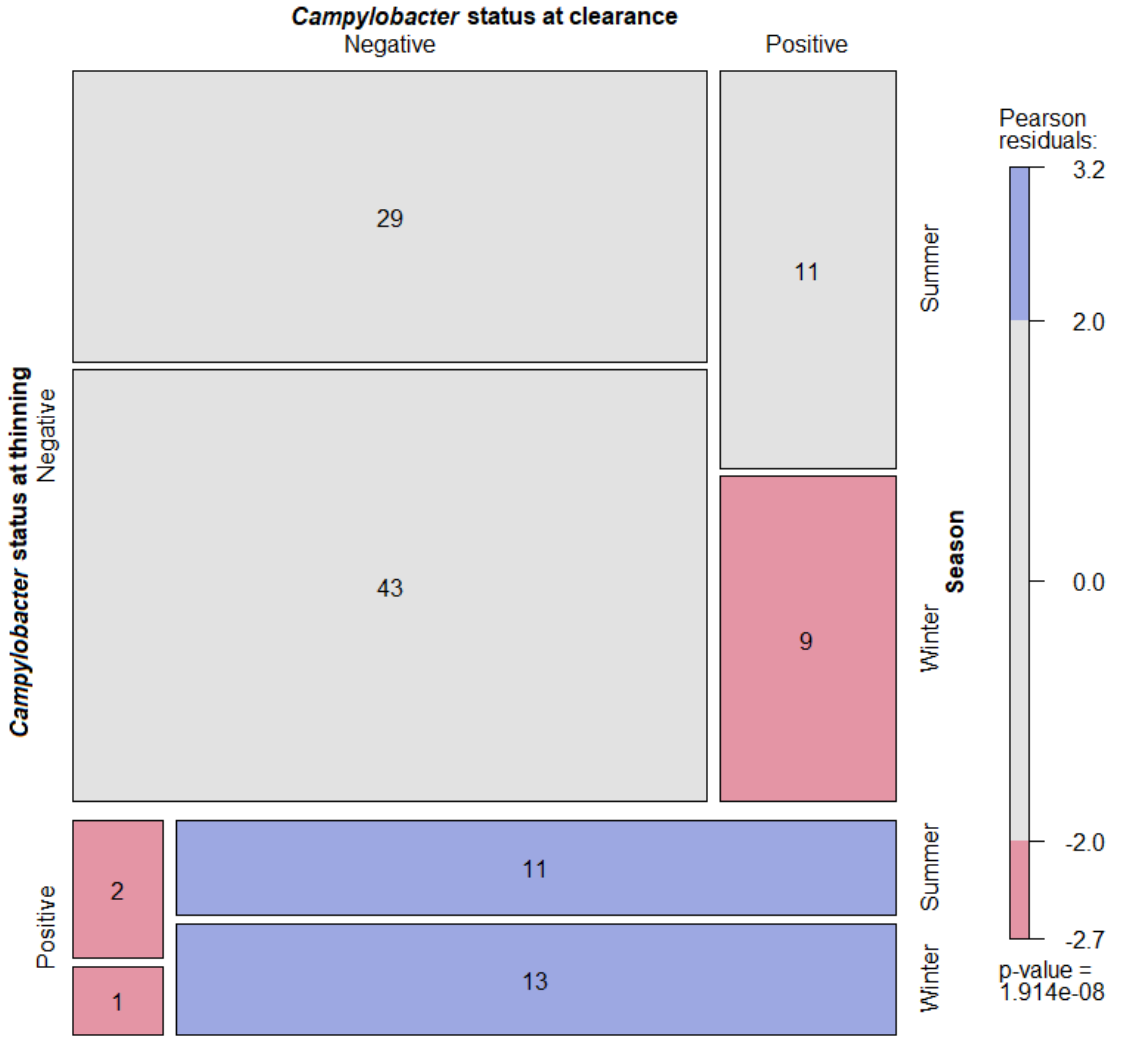

Figure 4.6: Mosaic plots displaying frequencies of positive and negative Campylobacter detections at thinning and clearance amongst poultry flocks, along with Pearson residuals and Chi-Squared Test p-value, by season (summer defined as April to September, winter as October to March).

To assess whether the *Campylobacter* status of the preceding flock impacted the status of the succeeding one, a Chi-Square analysis was performed (Figure 4.7; p = 0.187). No significant difference was observed, both for whether the *Campylobacter* status of a previous flock influences the next, or whether this varies with season.

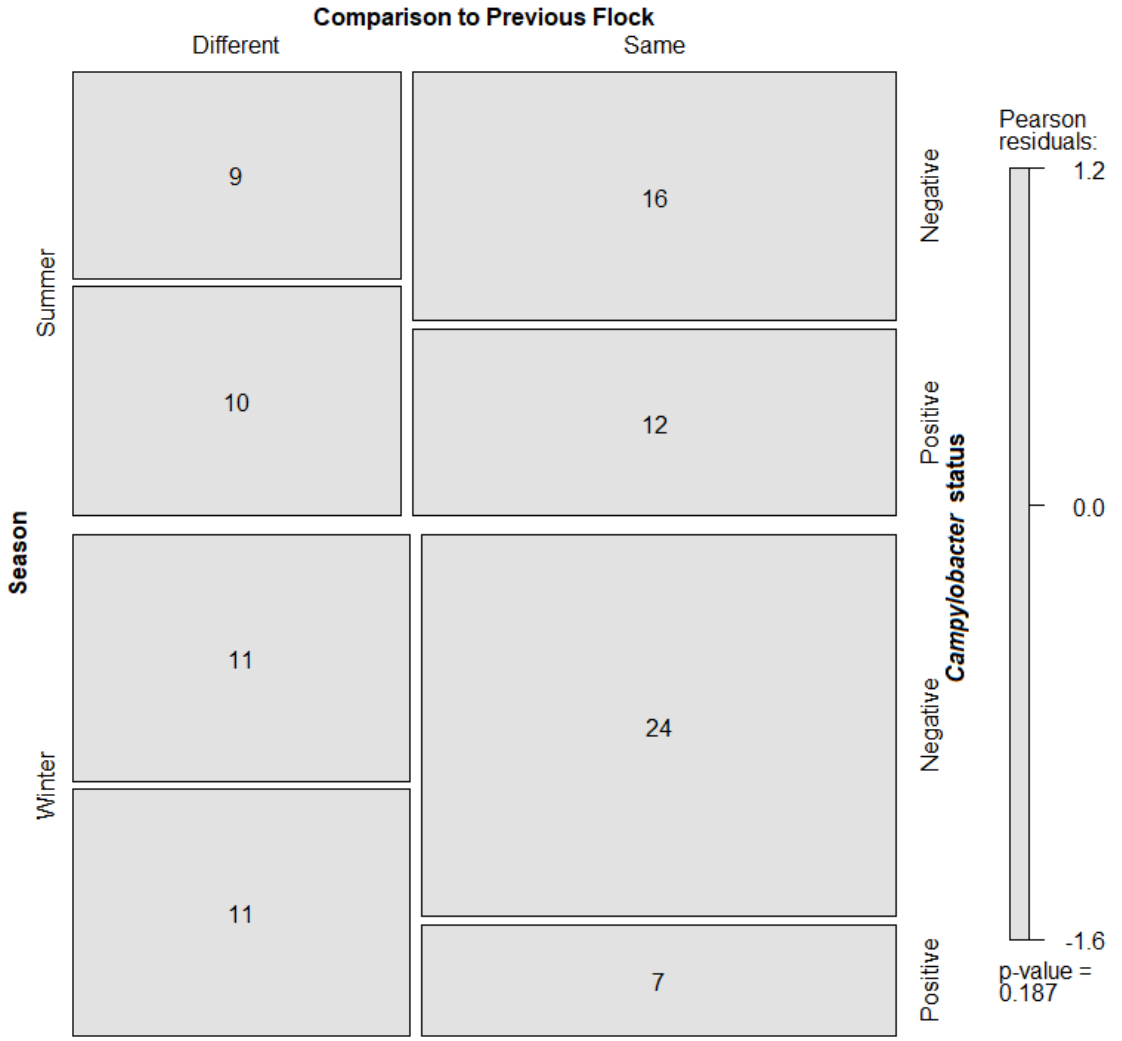

Figure 4.7: Mosaic plots displaying frequencies of positive and negative Campylobacter detections at final clearance and whether the same Campylobacter detection result was observed in the preceding flock, along with Pearson residuals and Chi-Squared Test p-value, by season (summer defined as April to September, winter as October to March).

4.4 Discussion

In this study, materials entering and surrounding chicken houses were assessed for *Campylobacter* spp. presence, in addition to routine monitoring of the *Campylobacter* infection status of chicken flocks kept within the same houses. Through statistical comparison, the author aimed to assess the likelihood of breaches in biosecurity, such as air that could be passed through ventilation, personnel entering the chicken house and consumable materials routinely brought inside, as origins of *Campylobacter* infection in the chicken flocks housed within. However, detections of *Campylobacter* across all sample typed did not reveal any widespread contamination, and so no hypothesis of external origins of *Campylobacter* infection could be supported by the work conducted.

There is limited evidence in the findings presented here that materials required at the beginning of a broiler chicken production cycle, such as bedding and feed, are contaminated with *Campylobacter* spp. and hence are unlikely to be a primary source of subsequent flock infections. There are comparable studies in published literature, such as Battersby, Whyte and Bolton (2016b), which tested chick paper for contamination with *Campylobacter* using PCR-based techniques and found no contamination present on any samples, across several farms and production cycles. This provides good evidence to back up the conventional wisdom in research of *Campylobacter* in broiler chickens that vertical transmission does not play a role in flock acquisition of the bacterium (Bull *et al.*, 2006; Callicott *et al.*, 2006).

Campylobacter was found on chick starter feed on two occasions in this study, and hence represents a rare event which cannot be connected to subsequent flock infection. Feed has been explored as a potential route of *Campylobacter* infection in prior published research and confirms our presented findings as being absent of *Campylobacter* on delivery from the supplying feedmill (Bull *et al.*, 2006; Hansson *et al.*, 2007), but it has been suggested as a potential intra-flock vector, becoming contaminated once in the poultry house and communicating *Campylobacter* between individual birds (Bull *et al.*, 2006; Alves *et al.*, 2017). One surprising finding from the results presented here is the presence of a strong response on all feed samples when analysed by PCR according to the 16S rRNA gene primers described by Lund et al. (2004)

for *Campylobacter* detection. The feed used throughout this project is made exclusively by Avara Foods Ltd. for use with their own flocks and primarily consists of wheat, soya bean meal and vegetable oil (Avara Foods Ltd., personal correspondence, 2018). No sequences in the wheat or soya genome were discovered upon an investigation using NCBI BLAST (NCBI, 1988) which matched to any significant degree with the PCR primers, suggesting that it is not a coincidental match in the products which make up the feed that is causing the spurious result. A match was, however, found with a yet-unclassified *Brachyspira* spp. isolated in swine, and the observed spurious amplification did not manifest when using primers specifically designed to exclude it. *Brachyspira* are known to infect avian species and can cause a disease known as spirochaetosis, although non-pathogenic strains which colonise chickens also exist (Medhanie *et al.*, 2013; Mappley, La Ragione and Woodward, 2014). The results presented here do not necessarily suggest the presence of *Brachyspira* as there is potential for the sequence to match a yet unidentified bacterial species, nor do they suggest the presence of a live pathogen in the feed as it is heat-treated prior to delivery and the PCR will readily pick up the DNA of dead cells. Overall, the presented work suggests that *Campylobacter* spp. were not present in feed, as presented to the poultry house from the feedmill, at any prevalence that would suggest it as a primary disease vector.

Of flocks that were *Campylobacter* positive at clearance, fewer acquired their infection over the period between thinning and clearance (45%) than already had acquired an infection prior to thinning. These findings align with another study of UK broiler flocks by Goddard et al. (2014), which found that, of flocks that were positive at clearance and had previously been thinned, 48% acquired their infection within two days of thinning. However, since no comparison can be made in the novel work presented in the current chapter to equivalent flocks that were not thinned, any conclusions from it concerning whether the practice of thinning has an impact on *Campylobacter* acquisition are limited. Most studies of UK broiler chicken flocks agree that the practice of thinning does represent a substantial risk to flock infection (Allen *et al.*, 2008; Lawes *et al.*, 2012; Goddard *et al.*, 2014; Higham *et al.*, 2018). However, no current studies, to the knowledge of the author, have found evidence of variability in the risk posed by thinning on flock *Campylobacter* infection between seasons. A

significant difference is presented here in the proportions of flocks acquiring *Campylobacter* post-thin between summer and winter (Figure 4.6). This is consistent with a decreased risk of *Campylobacter* acquisition brought about by a breach in biosecurity during winter months.

No *Campylobacter* was detected within the farm surroundings outside of an active and ongoing infection of a flock (Figure 4.2). *Campylobacter* spp. were found on two occasions on bootsocks collected in the farm environment. These detections happened on two separate farms during concurrent production cycles. On both occasions, detection occurred only after an infection had begun within the chicken house under study. This finding is in keeping with the hypothesis that movement of *Campylobacter* in the farm environment is predominantly from inside the chicken house to outside. During related research, Bull et al. (2006) found that *Campylobacter* originating from a positive chicken flock were readily detectable in the air outside of the infected house. Both farms in the study detailed in this section that had *Campylobacter*-positive environmental samples had ventilation that extracted from the poultry house directly onto the margins between houses, and thus could potentially be more prone to contaminating the environment than houses which extract from the roof ridge, at an altitude away from farm personnel. The fact that no *Campylobacter* was found in the environment outside of these isolated incidents provided some evidence that *Campylobacter* infections in chicken flocks are not likely to originate from environmental contamination being carried in by farm staff. However, Battersby, Whyte and Bolton (2016a) demonstrated that chickens that did not come into contact with farm staff were significantly more protected from *Campylobacter* than those with regular staff contact, and so staff picking up these external *Campylobacter* originating from within the chicken shed may be responsible for intra-farm transmission, from house to house on the same farm, once an infection has begun in at least one house. (Battersby, Whyte and Bolton (2016b), contrary to the work presented in this study, detected *Campylobacter* on the tarmac apron outside poultry houses before the flocks contained within became *Campylobacter* positive, noting that levels detected on the tarmac increased once a flock had become positive. These swabs were, however, of a different type to the ones utilised in this chapter, consisting of sponges moistened in Maximum Recovery Diluent

(MRD), as opposed to the bootsocks. While bootsocks are likely to provide the more accurate representation of what is likely to be carried into the broiler house by farm personnel, the sponges used by Battersby, Whyte and Bolton (2016b) are more likely to dislodge organisms present due to the friction applied with the sponge compared to the brief contact made with the surface to be sampled with the bootsock. In pairwise comparisons, dampened swabs have been shown to be more effective at sampling poultry environments for human pathogens (Byrd *et al.*, 1997) and so the MRD applied to the sponge may have improved the ability of by Battersby, Whyte and Bolton (2016b) to recover organisms.

The air samples collected during the research presented in this chapter represent the only samples on which *Campylobacter* was detected in the environment surrounding the chicken house when *Campylobacter* was not already present and established within. However, no statistical link was found to suggest that a positive air sample at a bird age of five days can influence the terminal *Campylobacter* state of the associated production cycle, in part due to the limited sample number and resulting lack of statistical power. The device used was the same model that was successfully used in *Campylobacter* detection studies (Søndergaard *et al.*, 2014; Hoorfar *et al.*, 2020; Johannessen *et al.*, 2020), all of which managed to successfully use the same apparatus as utilised in the present study to detect airborne *Campylobacter* in chicken farm environments, and so any lack of detection is not likely due to the ineffectiveness of the sampling procedure. All the aforementioned studies, however, used the device for sampling within the interior of a chicken house, rather than the external environment as detailed in the work presented in this chapter, and so a direct comparison in findings cannot be made. Zweifel et al. (2008) attempted to culture *Campylobacter* from the air entering a chicken house across different bird ages but was unable to culture any from any sample taken. Bull et al. (2006) was able to detect *Campylobacter* in the air near a chicken house, but this was achieved by sampling downwind of a house containing *Campylobacter*-positive birds, and hence the organisms are likely to have originated from the infected flock. Based on the lack of detection noted on air samples in the work presented in this chapter, along with the proven effectiveness of the sampling methodology both in the pilot study and in research published by other authors, we hypothesise that airborne

transmission of *Campylobacter* is unlikely to be a contributing factor to flock infection. Evidence could not be found to suggest that poultry house ventilation is a substantial enough breach of biosecurity to allow a flock to become infected with *Campylobacter* through contaminated particulates.

4.5 Conclusions

This study found little evidence of *Campylobacter* contamination on bedding, feed or other materials routinely brought into contact with broiler chickens. There is also limited evidence of environmental *Campylobacter* from outside the chicken house providing a source of infection. For these reasons, the origin of *Campylobacter* seasonality is likely to originate from other factors that encourage proliferation of extant *Campylobacter* within the chicken house, rather than any seasonal emergence of *Campylobacter* reservoirs capable of transfer to housed chickens.

5 Extraintestinal spread, loading and species variation of *Campylobacter* in broiler chickens across seasons.

5.1 Introduction

The gut microbial composition of broiler chickens is of high commercial importance, not only because it is vital in its role of efficient conversion of feed to body mass and hence more saleable chicken meat (Torok *et al.*, 2011; Bae *et al.*, 2017), but also in its ability to help regulate and prevent pathogens from infecting the gastrointestinal tract, when it is properly established with appropriate species (Choi, Lee and Sul, 2015; Clavijo and Flórez, 2018; Rychlik, 2020). For this reason, feed additives with the potential to modulate gut microflora have been in recent development for use in *Campylobacter* control (Thibodeau *et al.*, 2015; Guyard-Nicodème *et al.*, 2016; Meunier *et al.*, 2016; Clavijo and Flórez, 2018), although this is still an emerging field of research with little impact on actual industrial practice as of present. Due to the emerging nature of this field of research, no good characterisation of a 'healthy' chicken gut has been put forward yet, with particular confoundment brought on by the potential impact of differences in diet and genetics across different production systems (Borda-Molina, Seifert and Camarinha-Silva, 2018).

Campylobacter infection amongst broiler chicken flocks is not a uniform disease, but instead can cause a range of pathologies. The breed of chicken has an impact on the resulting disease manifestation post-infection, with greater extraintestinal spread of *Campylobacter*, intensified clinical symptoms, and heightened immune responses manifesting in faster-growing breeds such as those used in commercial broiler chicken production (Williams *et al.*, 2013; Humphrey *et al.*, 2014). Different *C. jejuni* strains have been shown to induce highly varied immune responses from chicken intestinal tissues (John *et al.*, 2017), with some strains taking on distinct infection dynamics from others, both inside the chicken gut and in their systemic spread to other tissues (Chaloner *et al.*, 2014). There is also evidence to suggest that environmental stresses placed upon broiler chickens, such as heat stress, transport, and pre-slaughter feed withdrawal, can increase their susceptibility to colonisation by organisms such as *Campylobacter*, but the

underlying mechanisms have not been well-explored in literature to date (Humphrey, 2006; Rostagno, 2009; Quinteiro-Filho *et al.*, 2012; Verbrugghe *et al.*, 2012; Lara and Rostagno, 2013).

Campylobacter spp. have been noted to cause symptoms of hepatitis in broiler chickens, known as 'spotty liver disease' within the poultry industry (Jennings *et al.*, 2011; Crawshaw *et al.*, 2015; Van, Elshagmani, *et al.*, 2017). While in recent years the precise *Campylobacter* species responsible for causing spotty liver disease has been proposed as a novel species closely related to *C. jejuni*, named as *C. hepaticus* (Van *et al.*, 2016), *C. jejuni* has been noted to cause identical symptoms under certain conditions (Jennings *et al.*, 2011) and *C. hepaticus* has been found within the gut as well as the liver (Van, Gor, *et al.*, 2017). Since this is an emerging body of research, it is possible that various species of *Campylobacter* may be responsible for incidences of both spotty liver disease and gastrointestinal infection reported in the poultry industry.

It is known from prior research that both *Campylobacter* strains present in UK broiler chicken flocks, and the composition of the chicken gut microbiomes they inhabit, alter seasonally, although the interaction and magnitude of these factors are not known (Jorgensen *et al.*, 2011; Oakley *et al.*, 2018). Considering how the complex host-pathogen-environment interaction demonstrated in current research can be influenced by parameters that alter according to season, investigating this as a potential source of the seasonal trends observed in *Campylobacter* infections in broiler chickens is not only possible, but paramount. However, to date, this has not been conducted.

5.1.1 Chapter Aims

The aim of experiments detailed in this chapter was to ascertain whether the dominant species of *Campylobacter* infecting commercial broiler chicken flocks, and the loading of *Campylobacter* observed, altered seasonally. Additionally, further experiments sought to examine whether changes to the composition of the chicken gut microflora, gut damage caused by *Campylobacter* infection, and extraintestinal spread of *Campylobacter*, change between summer and winter. In this way, seasonal variations in the infection dynamics of *Campylobacter*, and the bird gut which it infects, can be

examined for explanatory factors in driving the observed seasonality of *Campylobacter* infections.

Specifically, objectives were to:

- Evaluate whether the species or loading of *Campylobacter* observed infecting broiler chicken flocks changed across the year.
- Assess the damage acquired by the chicken gut upon *Campylobacter* infection, and whether it differed with season or species.
- Assess extraintestinal spread of *Campylobacter* to the liver, and whether it differed with season or species.
- Measure the community 16S rRNA gene composition of the chicken gut and evaluate whether any changes occurred upon infection with *Campylobacter*, or if there are apparent differences across seasons.

This was achieved by monitoring eleven farms over a period of 16 months and recording the *Campylobacter* status of the flocks daily. Following this, a subset of four farms were monitored for an additional 12 months afterwards, with environmental samples collected simultaneously alongside those collected for *Campylobacter* detection.

5.2 Methods

For the first ten cycles of the project, the flocks observed were the same as those described as observed in Section 3. Of these, farms 1A, 1B, 2A and 3A were observed for a further seven production cycles (cycles 11 to 17). These additional cycles were observed between April 2020 and March 2021. Cycles 11 to 17 shall henceforth be referred to as 'Phase Two' of the project, and all previous cycles as 'Phase One', as reported in Section 2.2.

5.2.1 Bootsock Collection and Testing

The bootsocks collected and tested for the research conducted in this section are the same as those detailed in Section 3.2.2, occurring through Phase One of the project. These were collected by the farmer as part of the first welfare check of the day.

5.2.2 Viscera Sample Collection

Viscera collection and testing only occurred over Phase Two of the project and was carried out as detailed in Section 2.5 on ten birds per clearance per farm. This gave one set of ileal contents, caecal contents, liver section and ileal section for every bird sampled, stored at -20°C after collection.

5.2.2.1 PCR Testing

For ileal and caecal contents, DNA extraction was performed using a QIAGEN QIAamp Fast Stool Mini Kit (QIAGEN, Hilden, Germany), as detailed in Section 2.6. Lysis was conducted at 95°C to ensure that total bacterial DNA was extracted. Each extract was tested for *Campylobacter* presence using a multiplex qPCR as detailed in Section 2.7, with subsequent confirmatory PCR and gel electrophoresis as per Section 2.8 if species identity could not be ascertained by the primary qPCR.

5.2.2.2 Ileal Section Assessment

Histological preparations were carried out by a third party within NHS Wales. Tissue specimens were fixed and embedded in paraffin, sections cut and stained with haematoxylin and eosin (H&E) using standard NHS histology protocols. These were then photographed using a microscopy system and examined in Aperio ImageScope 12.4.6 (Leica Biosystems Imaging, Inc., Wetzlar, Germany). On each viable micrograph, ten villus lengths and ten corresponding crypt depths were measured in the software using the ruler annotation tool, as demonstrated in Figure 5.1.

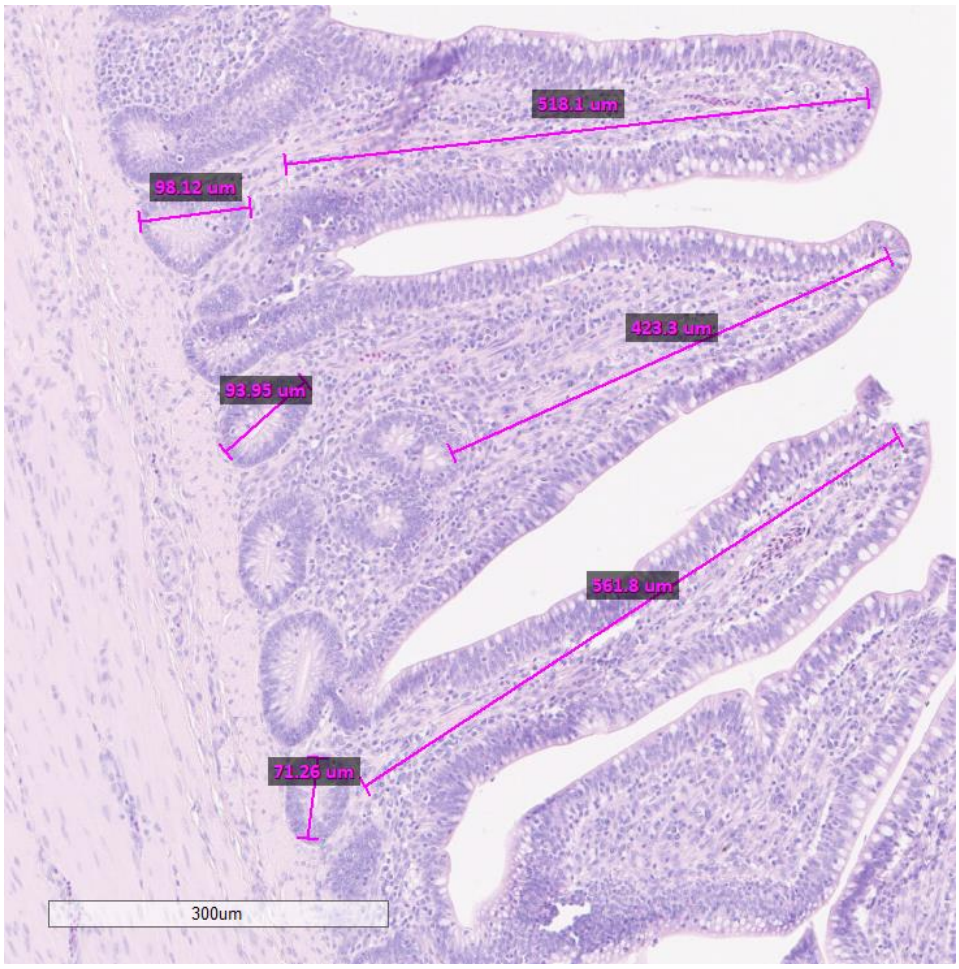

Figure 5.1: Example of chicken ileal tissue, stained with H&E, with crypt depth and villus length recorded. Three villi lengths are given on the right of the image, three crypt depths inside the circular structures on the left.

5.2.3 Community 16S rRNA Gene Analysis

The DNA extracted from caecal and ileal contents, as described in Section 2.5, were further assessed for their microbiome composition. This was conducted by a third party within Swansea University, using the Illumina MiSeq system (Illumina Inc., San Diego, CA, USA). The resulting reads were analysed using the mothur software suite v1.46.1 (Schloss *et al.*, 2009), following the SOP for preparation of a single dataset described by (Kozich *et al.*, 2013). Accepted reads were limited to any between 248 and 256 base pairs in length, with no ambiguous base pairs. All reads were aligned with the SILVA ribosomal RNA database v132 (Quast *et al.*, 2013), consisting of 695,171 ribosomal RNA reference sequences with at least a 1% dissimilarity to each other. All chimaeric sequences were excluded from the final dataset.

5.2.4 Statistical Methods

All analyses were constructed in R (R Core Team, 2020) as detailed in Section 2.10.

For testing the independence of categorical variables, the Chi-Squared Test was used (Section 2.10.2). Graphing and visualisation were conducted using ggplot2 (Valero-Mora, 2010; Wickham, 2016) and vcd (Meyer, Zeileis and Hornik, 2006). Graphs and visualisations were constructed using ggplot2 (Valero-Mora, 2010; Wickham, 2016) and vcd (Meyer, Zeileis and Hornik, 2006). For all plots describing the constituent model partials of GAMs, model partial values are centred with a Y-axis value of zero representing the mean value for the variable modelled in the graph. Therefore, positive values indicate when an explanatory variable, on the X-axis, has affected the model in a way that moves the modelled variable in a positive direction away from the mean, and vice-versa, with Y-axis magnitude showing any change in the variable predicted by the model away from the mean. For graphing each model partial, all other variables not represented by the Y-axis but incorporated in the model are held at their mean value. For GAM model partial graphs describing categorical variables, such as farm, the X and Y axes are reversed.

5.2.4.1 Shannon Diversity Index

The Shannon Diversity Index (SDI) is a means of measuring the diversity of a given sample set, given the proportion of each of its constituent sample groupings, such as species or genera, present. In this study, it was calculated as described by Shannon (1948).

The formula for SDI is given as:

$$SDI = -\sum p_i \times \ln(p_i)$$

In which p_i is the proportion of the entire set of constituting of sample group i.

5.3 Results

5.3.1 *Campylobacter* Species Across Seasons

Of the 119 production cycles tested, *Campylobacter* was present in 47 of them (39%), of which 37 were *C. jejuni* (79%), 3 were *C. coli* (6%) and 7 were a coinfection of *C. coli* and *C. jejuni* (15%). Farms 1B, 1C, 2B, 3B and 3D never acquired an infection with *C. coli*, either on its own or as part of a coinfection. Figure 5.2 demonstrates the proportions of each species detected on each farm throughout the present study.

Farm 3A had a higher proportion of *C. coli* among *Campylobacter* infections than other farms. Only farms 3A and 3C had infections with only *C. coli* present.

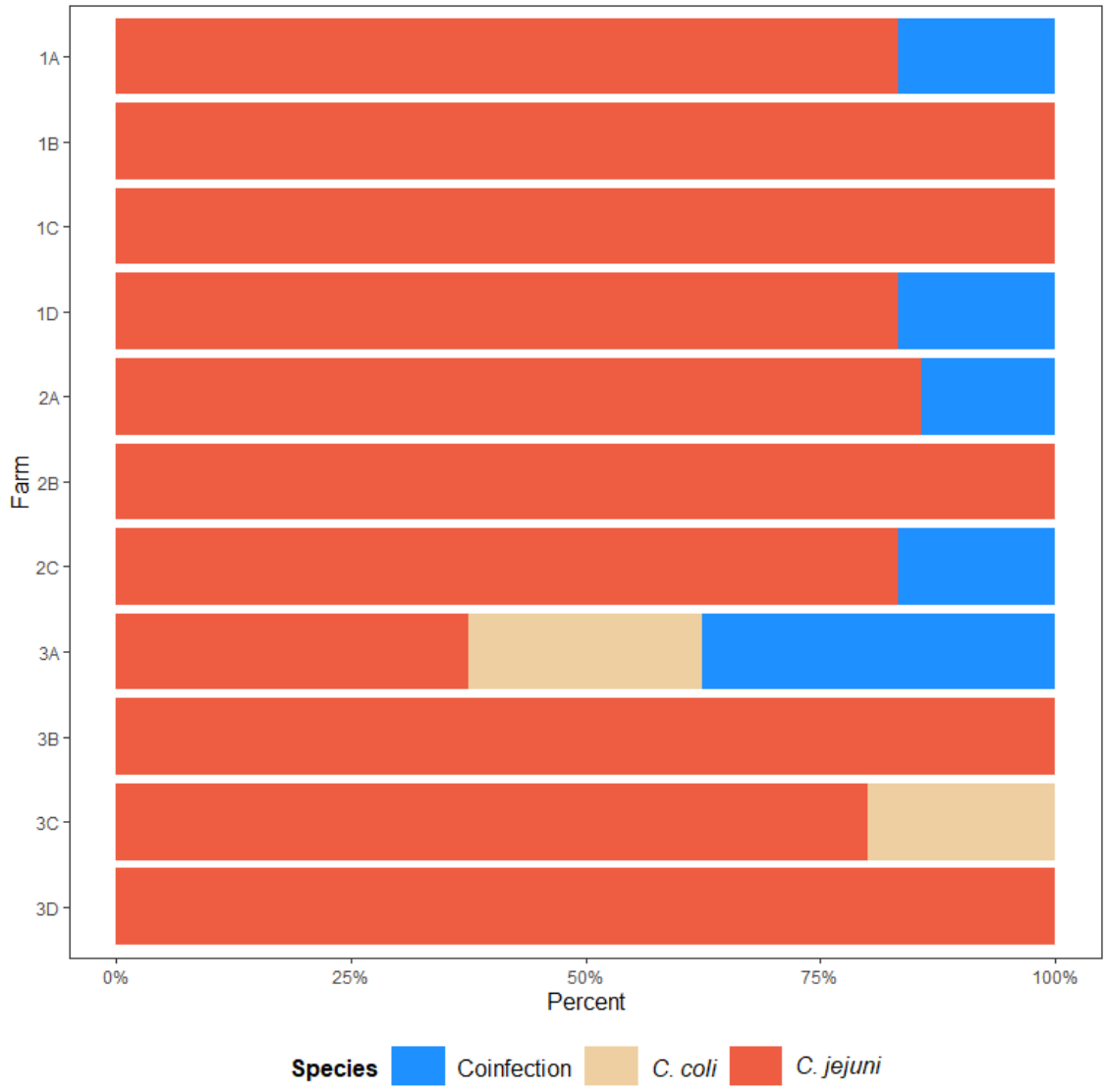

Figure 5.2: Proportions of each Campylobacter species tested for present on each farm in the study.

To assess whether different species are more likely to appear at certain times of year than others, a Chi-Square analysis with Yates' Correction was conducted (Figure 5.3). While it was not found that there is a statistically significant difference in the ratios observed in *C. jejuni* and *C. coli* between summer and winter (p = 0.150), infections resulting from *C. coli* alone were only present in summer months, and of the ten infections observed with *C. coli* present, seven of them occurred between April and September.

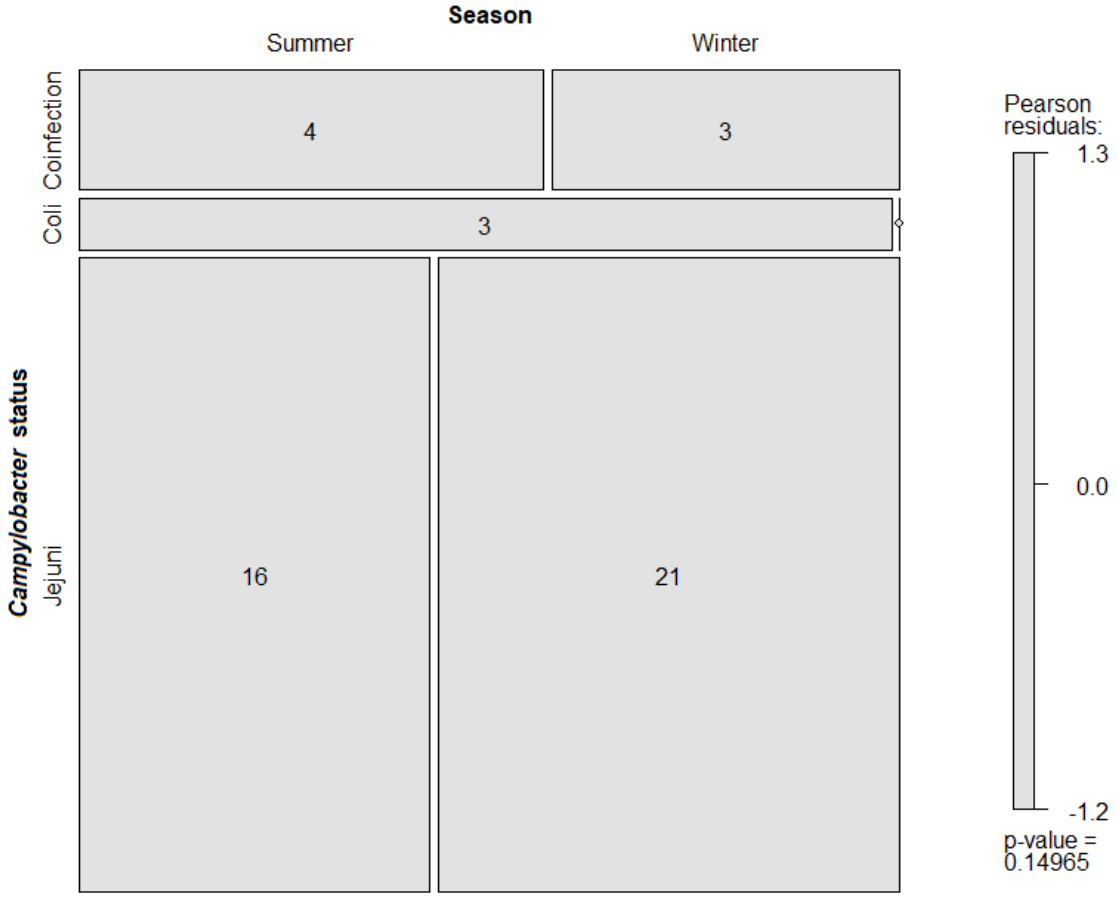

Figure 5.3: Mosaic plot displaying the frequencies of infections in broiler chicken flocks by Campylobacter jejuni, Campylobacter coli, and coinfections of the two organisms, against season (summer defined as April to September, winter as October to March).

5.3.2 Damage to the Chicken Gut

To assess damage sustained by the chicken gut, sections taken from the ilea of flocks slaughtered through Phase Two were analysed as per the method described in Section

5.2.2.2. Histological preparations were photographed using microscopy and the villus-to-crypt ratio was determined in Aperio ImageScope 12.4.6 (Leica Biosystems Imaging, Inc., Wetzlar, Germany). In total, 87 ileal sections were assessed out of the 100 collected, with the remainder excluded due to an inability to distinguish gut features correctly, through improper slide preparation or sample degradation.

A summary of analysed samples can be found in Table 5.1.

Table 5.1: Summary of villi lengths (µm), crypt depths (µm) and villus:crypt ratios of chickens sampled from four different farms across three production cycles. n = 10 villi and 10 crypts per sample, 87 samples total. Standard deviation = 286.0 for villus lengths, 62.3 for crypt depths. Grand mean = 676.7µm for villus lengths, 123.3µm for crypt depths.

Villus Length	**Cycle 14**	**Cycle 15**	**Cycle 17**
1A	481.1		1197.0
1B	534.7	658.5	
2A	855.4	619.7	639.6
3A	586.6		868.5
Crypt Depth	**Cycle 14**	**Cycle 15**	**Cycle 17**
1A	136.9		129.0
1B	113.5	84.5	
2A	151.3	109.1	110.5
3A	134.3		127.7
Villus:Crypt Ratio	**Cycle 14**	**Cycle 15**	**Cycle 17**
1A	3.51		9.28
1B	4.71	7.79	
2A	5.65	5.68	5.79
3A	4.37		6.80

Due to issues with receiving and delivering necessary reagents through the COVID-19 pandemic in 2020, samples could only be collected over a relatively small length of time. Figure 5.5 gives the number of positive and negative ileal sections received over the project, with ileal contents tested by qPCR as described in Section 5.2.2.1 and

corresponding with a matching ileal section collected at the same time from the same chicken. Figure 5.4 gives villus:crypt ratios separated by season.

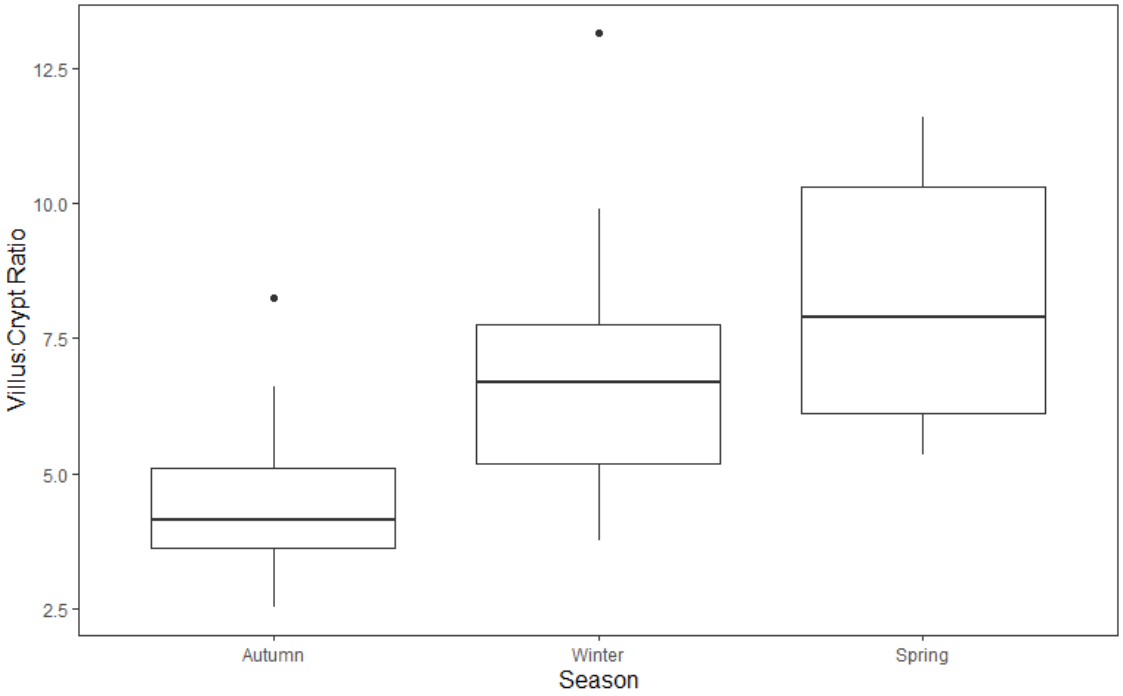

Figure 5.4: Villus:Crypt Ratios observed in chicken ileal sections, separated by season of year of collection. Autumn is defined as September and October, winter as November and December, and spring as February and March.

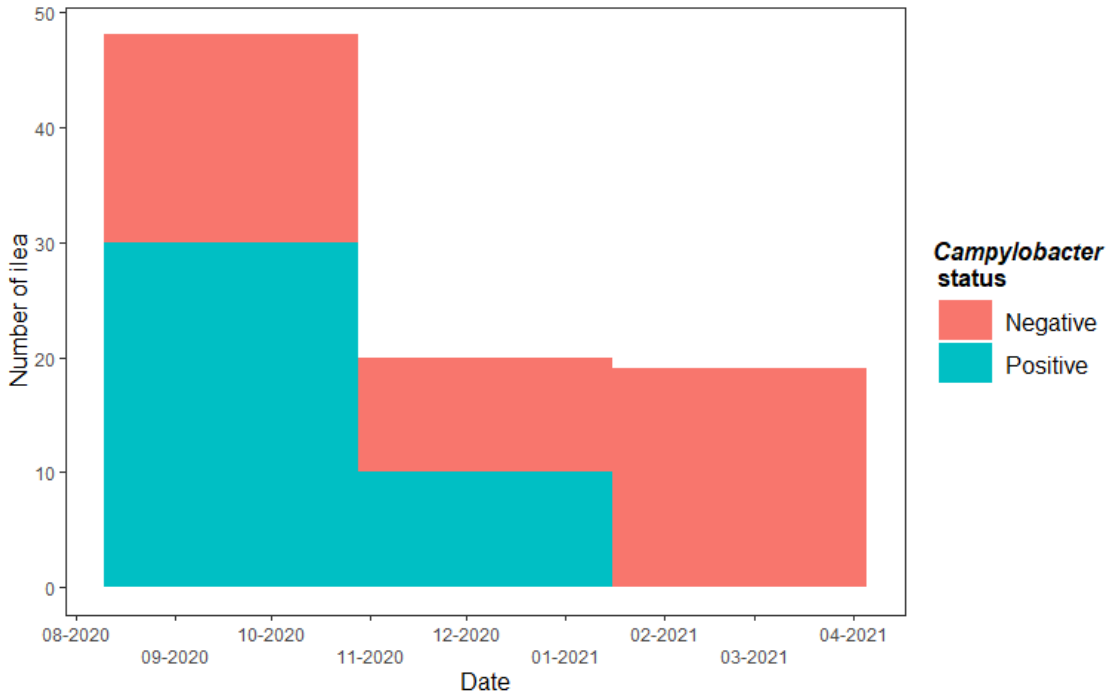

Figure 5.5: Histogram of the number of ileal sections from chickens both Campylobacter positive and negative, as collected and histologically analysed in Phase Two of the project.

Villus:crypt ratios and the number of negative ilea can be seen to increase together over the observation period (Figure 5.4, Figure 5.5). An ANOVA analysis testing the differences in villus:crypt ratios between seasons, shown in Figure 5.4, showed a statistically significant difference ($F = 38.580$, $p < 0.001$, $df = 2$). Subsequent comparison of means by Tukey's HSD revealed that the villus:crypt ratios observed were statistically distinct across all seasons ($p < 0.001$ between spring and autumn, $p < 0.001$ between winter and autumn, $p = 0.049$ between winter and spring).

Comparisons were made between the length of villi and depth of crypts in *Campylobacter* positive (n = 40) and negative (n = 47) birds through a two-way ANOVA, as described in Section 2.10.10. The villus:crypt ratio was found to be statistically significantly lower in ileal samples taken from chickens infected with *Campylobacter* ($F = 20.039$, $p < 0.001$, $df = 1$), with a mean villus:crypt ratio of 6.64 for negative birds and 4.70 for positive. Further ANOVA analyses revealed that villus length was significantly different between *Campylobacter* positive and negative samples ($F = 19.186$, $p < 0.001$, $df = 1$; mean negative length 770 µm, mean positive length 567 µm), but not crypt depth ($F = 1.064$, $p = 0.305$, $df = 1$). The farm the bird was raised on was found to have a statistically significant impact on villus length ($F = 8.733$, $p < 0.001$) and crypt depth ($F = 6.464$, $p < 0.001$), but not the villus:crypt ratio ($F = 1.651$, $p = 0.184$, $df = 3$). The Tukey HSD results for ANOVA analyses of villus length and crypt depth against farm are given in Table 5.2.

Table 5.2: p-values for Tukey's HSD Tests, comparing villus length and crypt depth on histological examination of chicken ileal sections against farm of chicken origin (1A, 1B, 2A, 3A). p-values less than 0.05 are highlighted in bold.

Villus Length

	1A	1B	2A
1B	**< 0.001**		
2A	0.349	**0.008**	
3A	0.145	0.175	0.891

Crypt Depth

	1A	1B	2A
1B	**0.001**		
2A	0.580	0.058	
3A	1.000	**0.008**	0.714

The differences between *Campylobacter* positive and negative flocks for observed parameters are displayed in the boxplot presented in Figure 5.6.

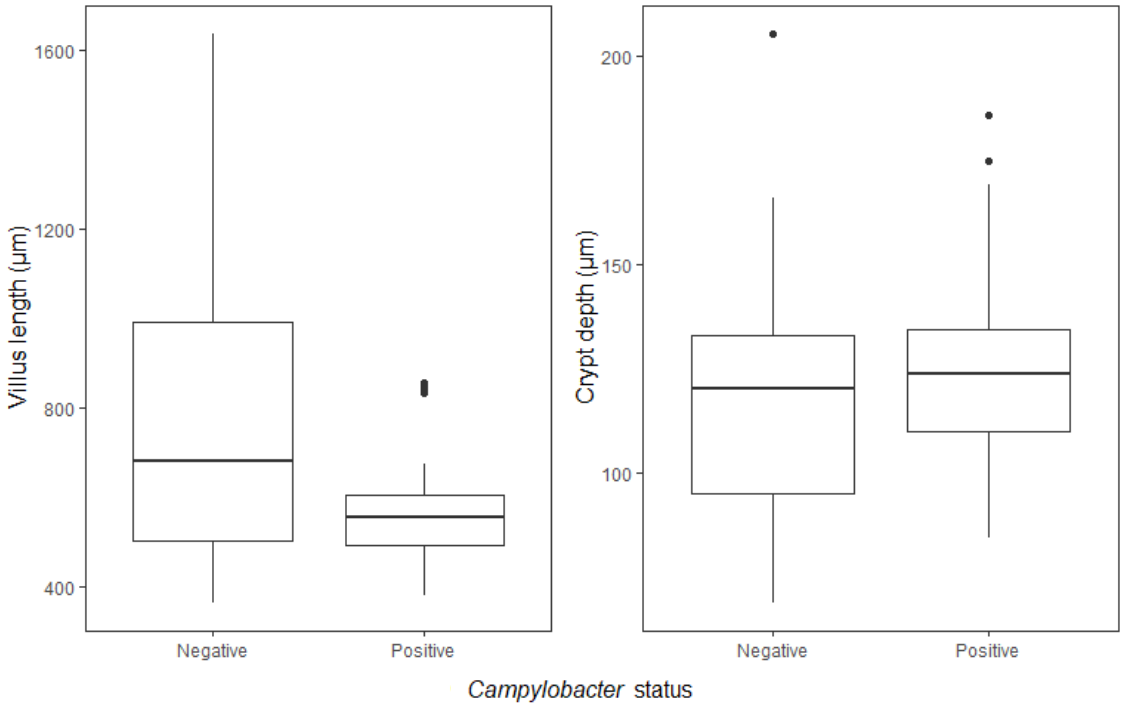

Figure 5.6: Villus lengths and crypt depths for Campylobacter positive and negative ileal samples taken from slaughtered chickens through Phase Two of the presented project.

To assess the interaction of season and *Campylobacter* status in determining the extent of damage to the chicken gut, a two-way ANOVA was conducted, with *Campylobacter* detection at qPCR, season and farm as factors used to describe the length of villi observed in ileal sections. It was found that there was no statistical impact from *Campylobacter* detection ($F = 1.413$, $p = 0.238$, $df = 1$) and the interaction of season and *Campylobacter* detection ($F = 0.027$, $p = 0.869$, $df = 1$), but there was from season ($F = 13.780$, $p < 0.001$, $df = 2$) and farm ($F = 9.371$, $p < 0.001$, $df = 3$). Statistically significant results of this analysis are shown in Figure 5.7.

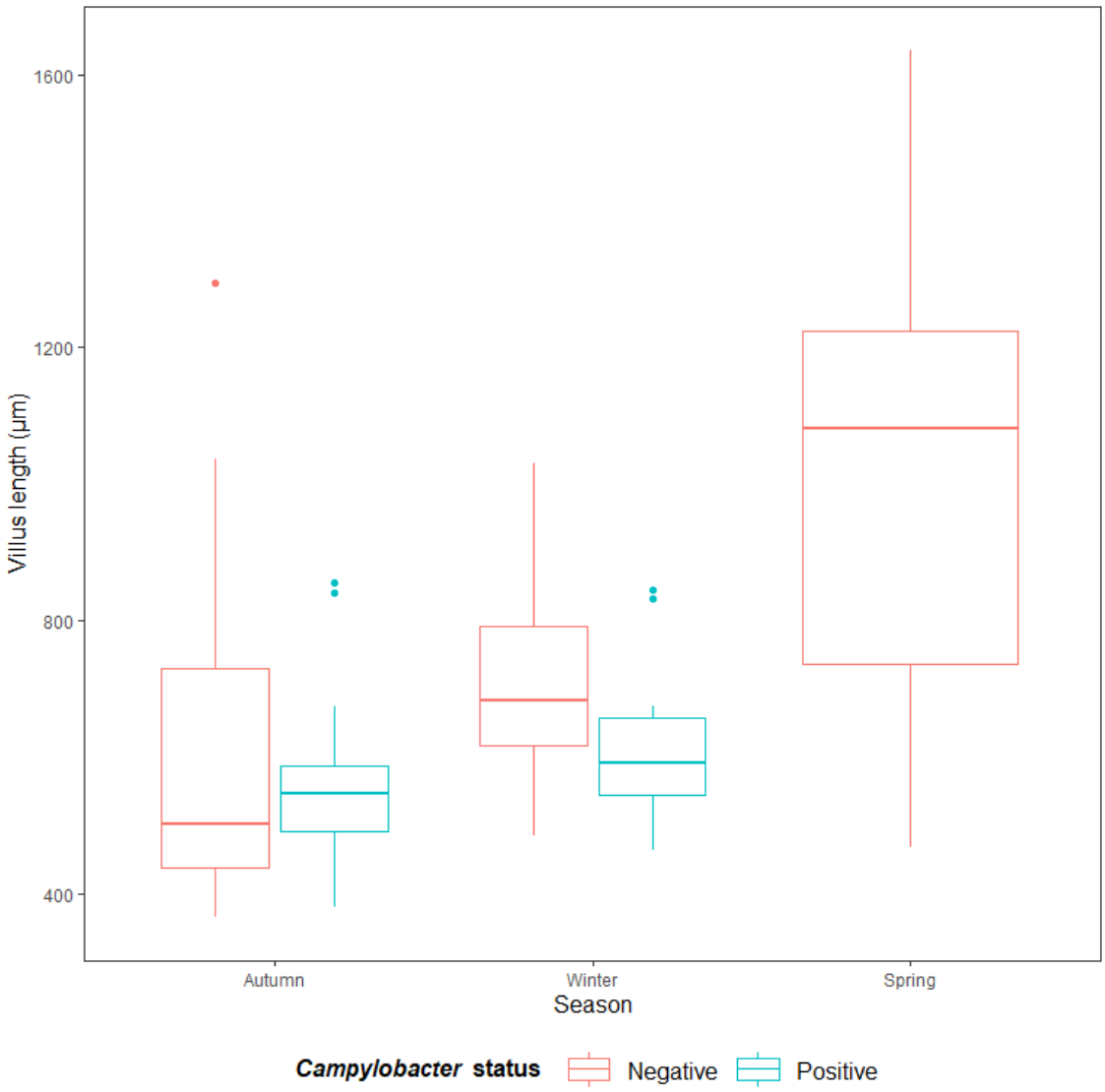

Figure 5.7: Villus length in chicken ileal sections separated by season of year of collection and Campylobacter detection at qPCR. Autumn is defined as September and October, winter as November and December, and spring as February and March.

Average body weights from each flock at slaughter were provided from the records of Avara Foods Ltd. In order to assess if the observed differences in gut villus length manifested in different final weights of each flock, a Spearman's Rho calculation was conducted between the recorded average body weights and villus length of each flock assessed (n = 9). However, no correlation could be found ($p = 0.810$, $\rho = -0.10$).

5.3.3 Bacterial Loading of *Campylobacter* Across Seasons

To assess the loading of *Campylobacter*, caecal samples from final bird clearances were analysed using quantitative PCR, described in Section 2.7. An initial DNA concentration for each sample was measured using a NanoDrop One (Thermo Scientific, Waltham, MA, USA) and, using the C_q values of each PCR, a copy number present within each sample for each *Campylobacter* gene tested for was also calculated using the formulae described in Section 2.7.2. From this, it is possible to calculate the number of copies of *Campylobacter* genes present in the initial known concentration of DNA extracted. Overall, this constituted 100 samples. Figure 5.8 presents the results separated by farm and season.

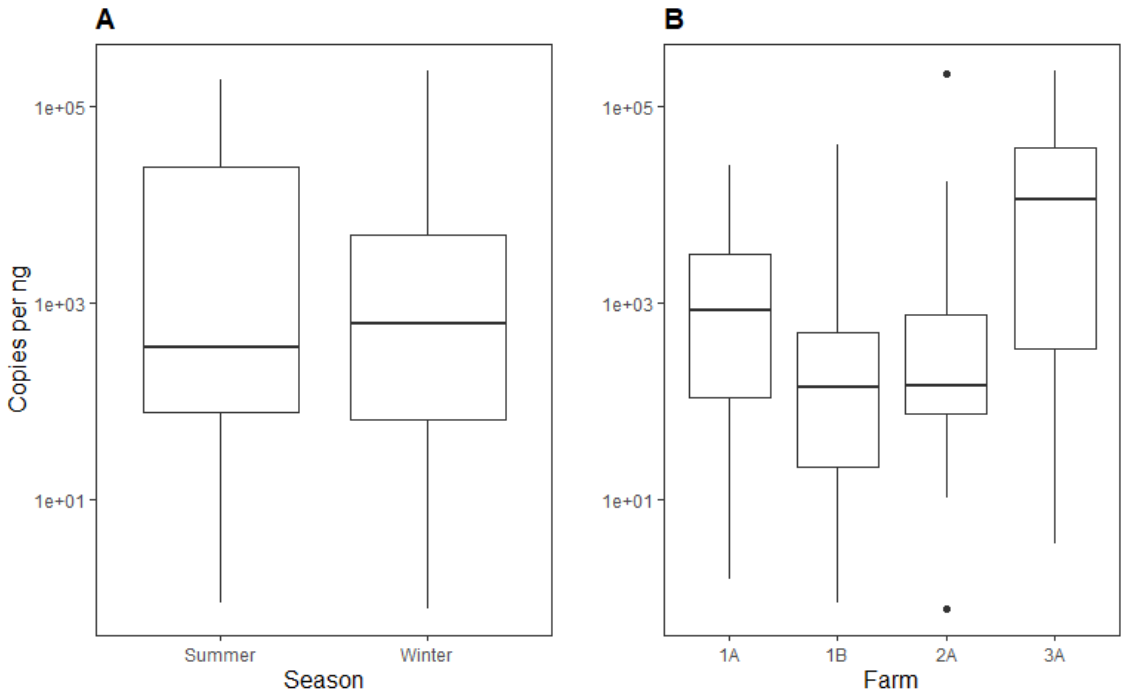

Figure 5.8: Boxplots demonstrating the variation in copy numbers of Campylobacter observed in caecal sample total DNA extracts, separated by season (Figure 5.8A, left) and by farm (Figure 5.8B, right). n=100 samples. Y-axes are logarithmic.

To test whether season has an impact on the levels of *Campylobacter* observed in caecal samples, a GAM was constructed, using DNA concentration, season, and farm as predictors in the copy number of *Campylobacter* observed. The output measures from this model are detailed in Figure 5.9.

According to the model, Farm 3A was found to have significantly higher levels of *Campylobacter* loading than other farms tested (p = 0.033). No significant difference was observed across season (p = 0.195). A positive correlation was detected with the concentration of DNA detected on the NanoDrop One (p = 0.002).

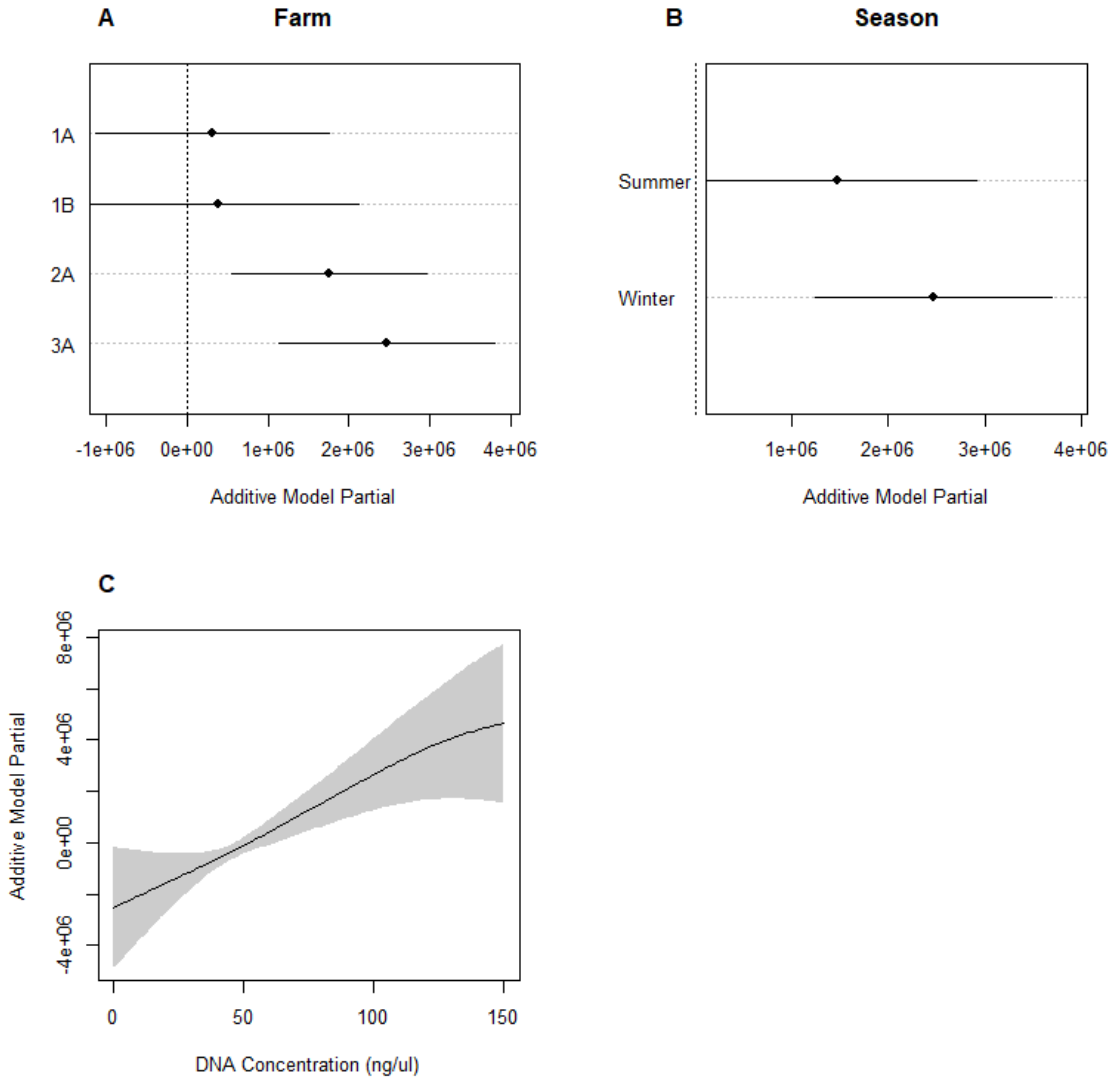

Figure 5.9: Details from a Generalised Additive Model output, predicting the impact of farm (Figure 5.9A, top left), season (Figure 5.9B, top right; p = 0.195) and DNA concentration recorded on a NanoDrop One device (Figure 5.9C, bottom left; p = 0.002) on the copy number of Campylobacter observed in caecal samples obtained from cleared broiler chicken flocks (n = 100). Summer defined as April to September, winter as October to March.

To assess the bacterial load of *Campylobacter* on positive bootsocks across seasons, the qPCR enumeration data analysed in Phase One of the project were assessed across seasons. To achieve this, a GAM was constructed, using the month of the year, the number of days the sample was taken after the infection first began in the flock and the concentration of the DNA in the sample to predict the number of copies of *Campylobacter* DNA, calculated from the qPCR C_q values as detailed in Section 2.7.2, observed in the quantitative PCR output. This is demonstrated in Figure 5.10.

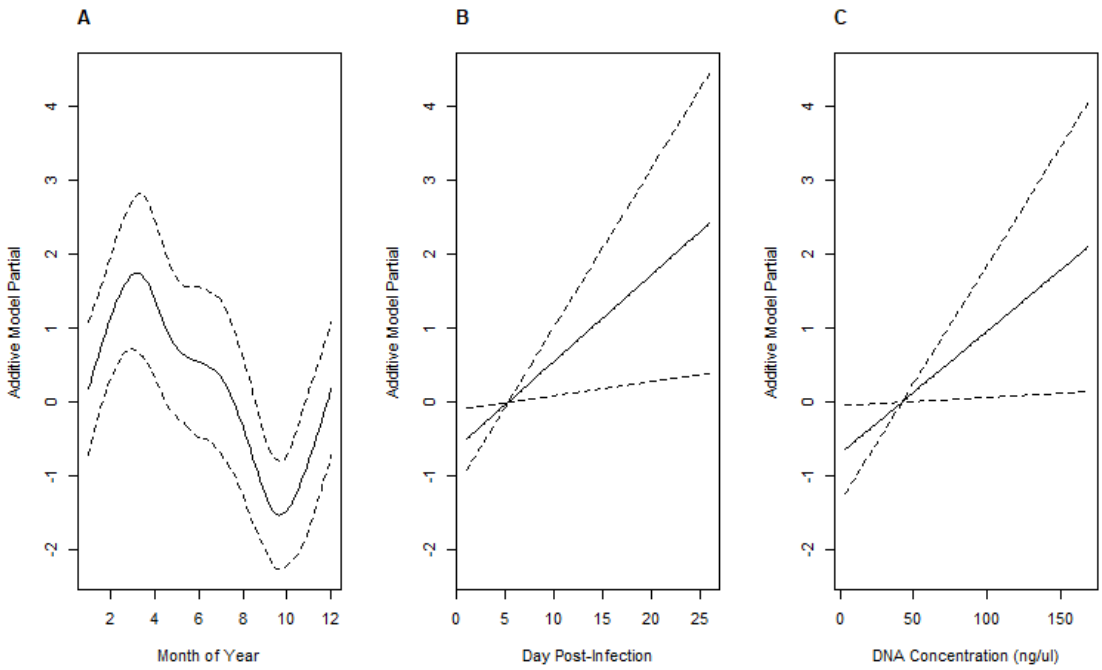

Figure 5.10: Details from a Generalised Additive Model output, predicting the impact of month (Figure 5.10A, left; p < 0.001), days since the onset of infection (Figure 5.10B, middle; p = 0.018) and extracted DNA concentration (Figure 5.10C, right; p = 0.033) on the logarithmic (base 2) transformation of the number of copies of Campylobacter DNA detected in bootsock samples collected inside chicken houses (n = 189).

All three parameters were found to be statistically significant. The month of year was found to be statistically linked to *Campylobacter* abundance in the sample ($p < 0.001$), with a maximum occurring around April and a minimum occurring around October. The copy number of *Campylobacter* was found to be linearly associated with the number of days after a *Campylobacter* infection began that the sample was taken ($p = 0.018$) and the total concentration of DNA that was obtained after extraction from the bootsock sample ($p = 0.033$).

5.3.4 Extraintestinal Spread of *Campylobacter*

To assess whether more virulent *Campylobacter* strains occurred at certain times of year, all collected liver samples (n = 80) were tested by qPCR as detailed in Section 5.2.2.1. The results of this testing is given in Figure 5.11.

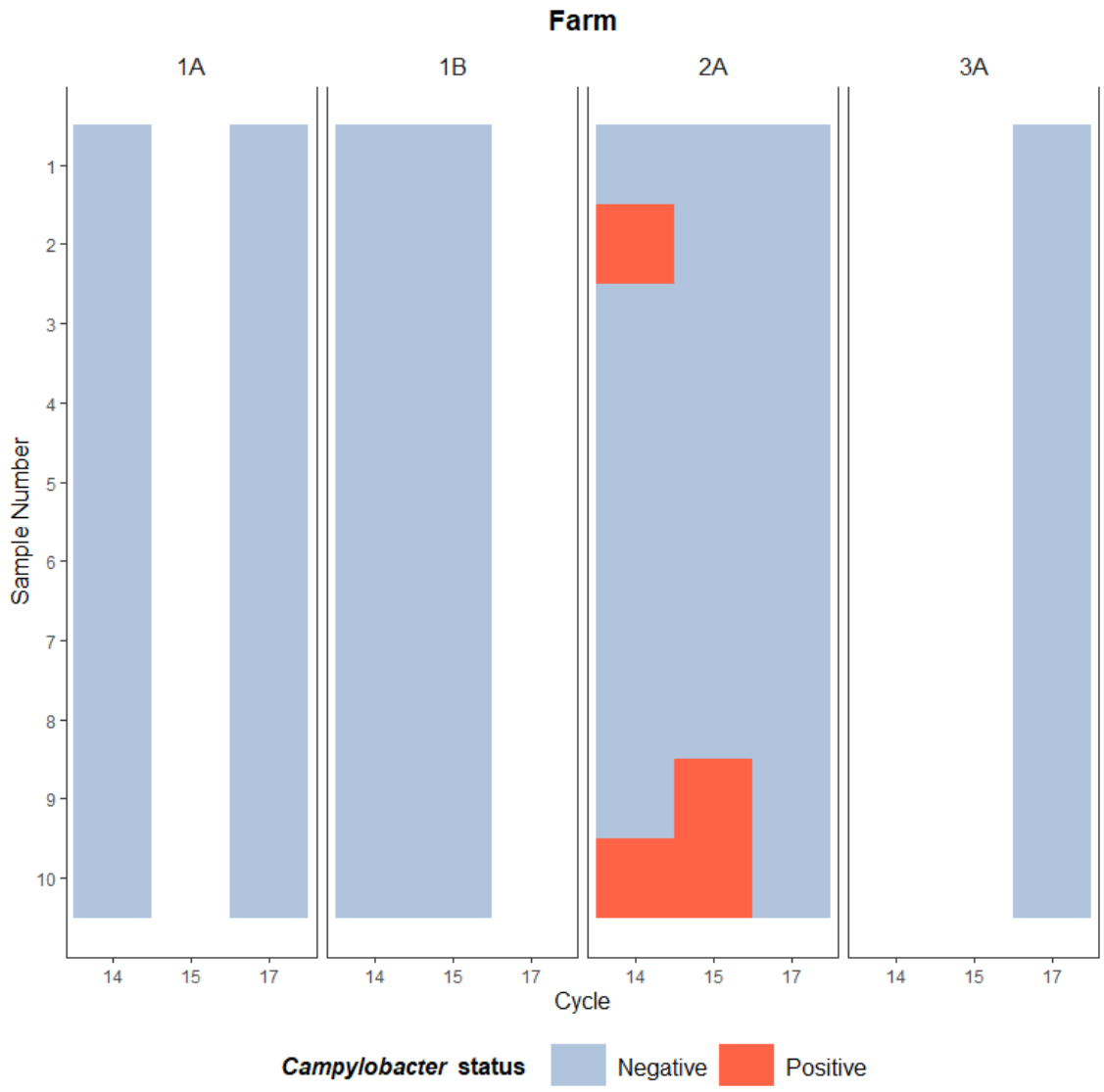

Figure 5.11: Campylobacter detections in broiler chicken livers, by qPCR across four farms and three production cycles.

Only four positive liver detections were made, two each in cycles 14 and 15 on farm 2A. These four livers all came from chickens that also had *Campylobacter* positive caecal contents. However, bootsocks collected from farm 2A across both cycles 14 and 15, both interior and exterior, did not test positive for *Campylobacter* at any point.

Welch's Two-Sample t-Tests were conducted comparing *Campylobacter* detection in the liver of a chicken with matching villus lengths (p = 0.650, df = 3.848) and crypt depths

(p = 0.539, df = 3.581); neither showed a statistically significant difference between positive and negative groups.

5.3.5 The Chicken Gut Microbiome and *Campylobacter*

Community 16S rRNA gene data was derived from DNA extracted from the caecal contents of 191 birds, collected across four farms and five production cycles. A summary of the proportions of each phylum seen for each farm and production cycle can be found in Figure 5.12.

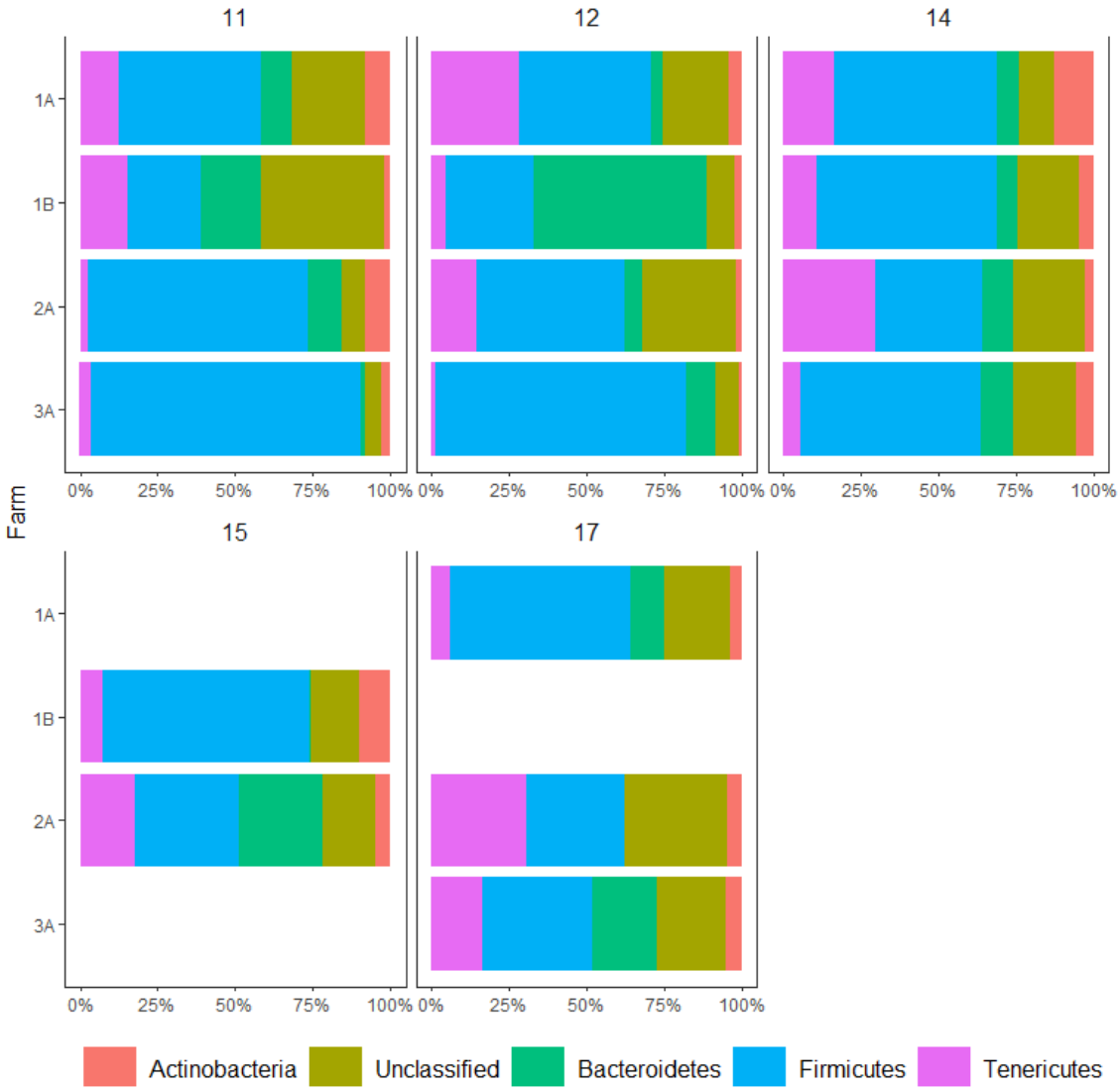

Figure 5.12: Proportions of abundance of different phyla of bacteria observed in 16S microbiome analysis of broiler chicken caecal contents (1,225,542 reads from 191 samples), separated by farm and production cycle. Any phyla with under 5000 reads (Cyanobacteria, Epsilonbacteraeota, Fusobacteria, Patescibacteria, Proteobacteria, Synergistetes and Verrucomicrobia) were excluded from this graph due to lack of visibility.

Most reads were from Firmicutes (50.1%), followed by unclassified bacteria (20.1%), Tenericutes (13.1%), Bacteroidetes (11.8%) and Actinobacteria (4.5%). Seven other phyla (Cyanobacteria, Epsilonbacteraeota, Fusobacteria, Patescibacteria, Proteobacteria, Synergistetes and Verrucomicrobia) constituted 0.3% of observed reads combined.

Figure 5.13 gives the proportions of *Campylobacter* positive and negative caecal contents across seasons, after testing by qPCR as described in Section 5.2.2.1. A peak in positive detections can be observed to last from July to October.

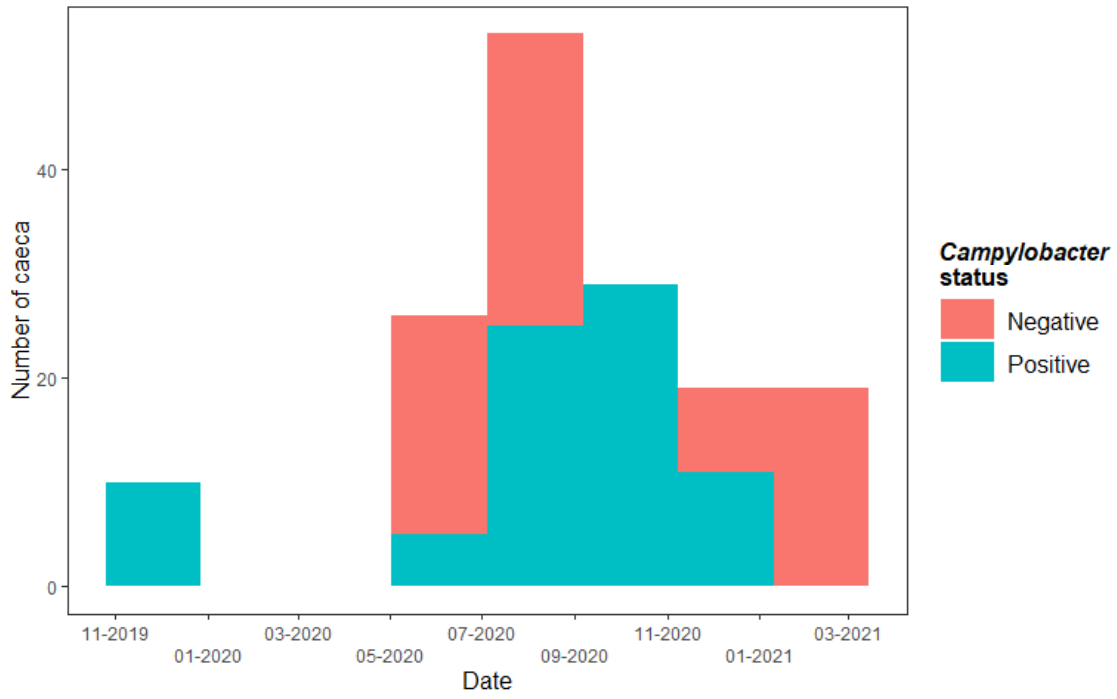

Figure 5.13 Histogram of the number of caecal contents with and without Campylobacter on qPCR testing.

The Shannon Diversity Index (SDI; Section 5.2.4.1) was calculated, to describe diversity across genera, for each sample with over 1,000 reads (n = 186, mean = 1.931). To assess whether differences in diversity of genera are apparent in the caecal contents of broiler chickens across seasons, and with the presence or absence of *Campylobacter* detection in the caeca, an ANOVA analysis was constructed. The Shannon Diversity Index of the caeca, as explored in this analysis can be seen in Figure 5.14.

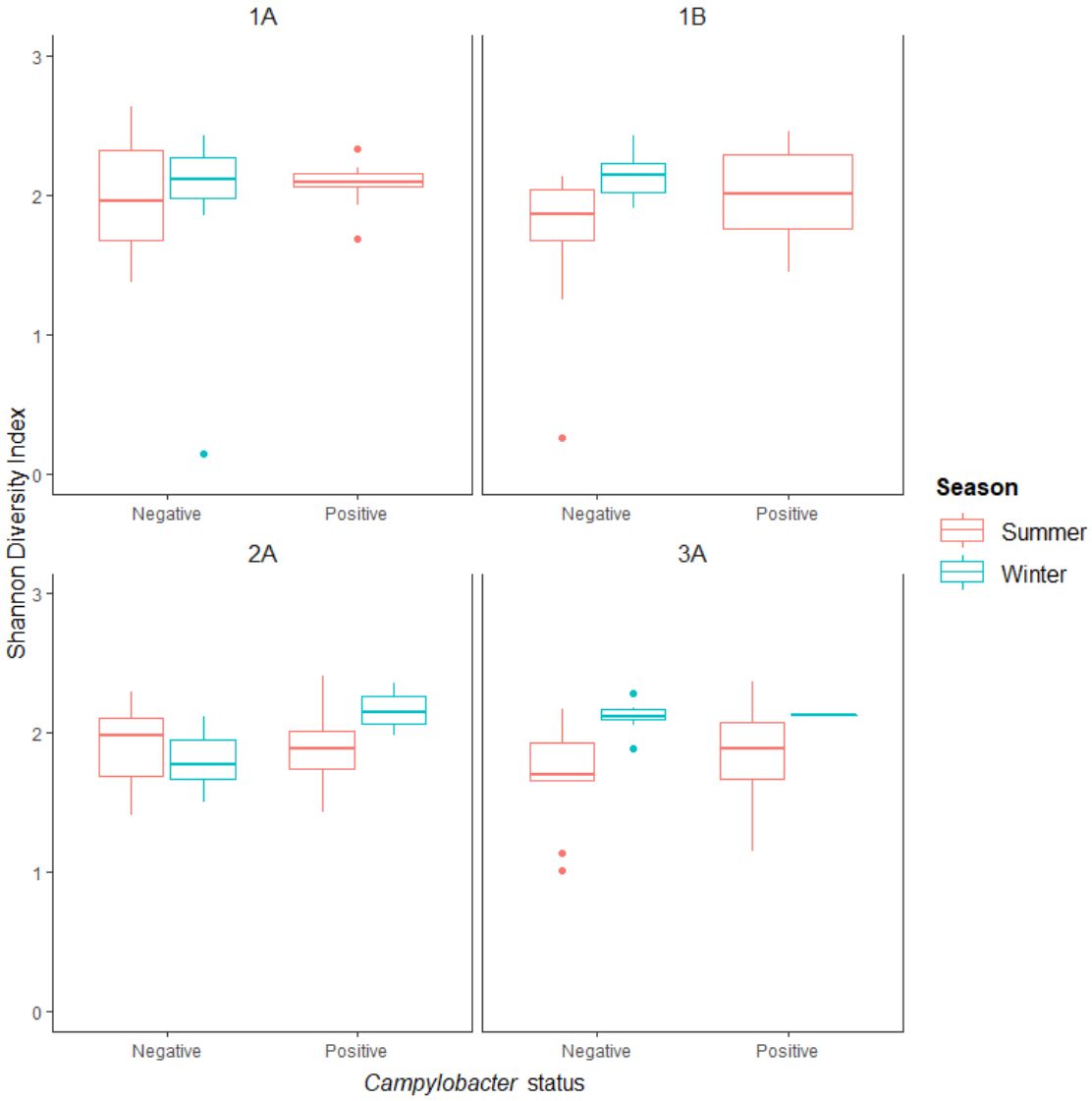

Figure 5.14: Boxplots demonstrating the variation in Shannon Diversity Index amongst the caecal contents of broiler chickens (n=186) across season of collection, farm and whether Campylobacter was detected in the same sample at qPCR.

In the information provided in Figure 5.14, the season of sample collection ($F = 5.772$, $p = 0.017$) and *Campylobacter* status ($F = 4.938$, $p = 0.028$) of each caecum proved to have a statistically significant impact on SDI, but the interaction of these parameters was not statistically significant ($F = 3.465$, $p = 0.06$). Average SDI was greater in winter than summer, and in *Campylobacter* positive samples.

To understand whether any differences in the proportions of each observed phylum in caecal contents influenced whether a flock was *Campylobacter* positive, a fitted GAM was constructed with binomial data derived from the *Campylobacter* status of each bird

with at least 1,000 bacterial reads (1 for positive, 0 for negative; n = 189), derived from PCR testing of caecal contents, using the natural logarithm of the count of each phylum observed in the same caeca, farm, season and the number of total bacteria observed per sample as predictor variables (Figure 5.15). The logarithmic transformation was applied to enable a more meaningful analysis of the data, given the distribution observed.

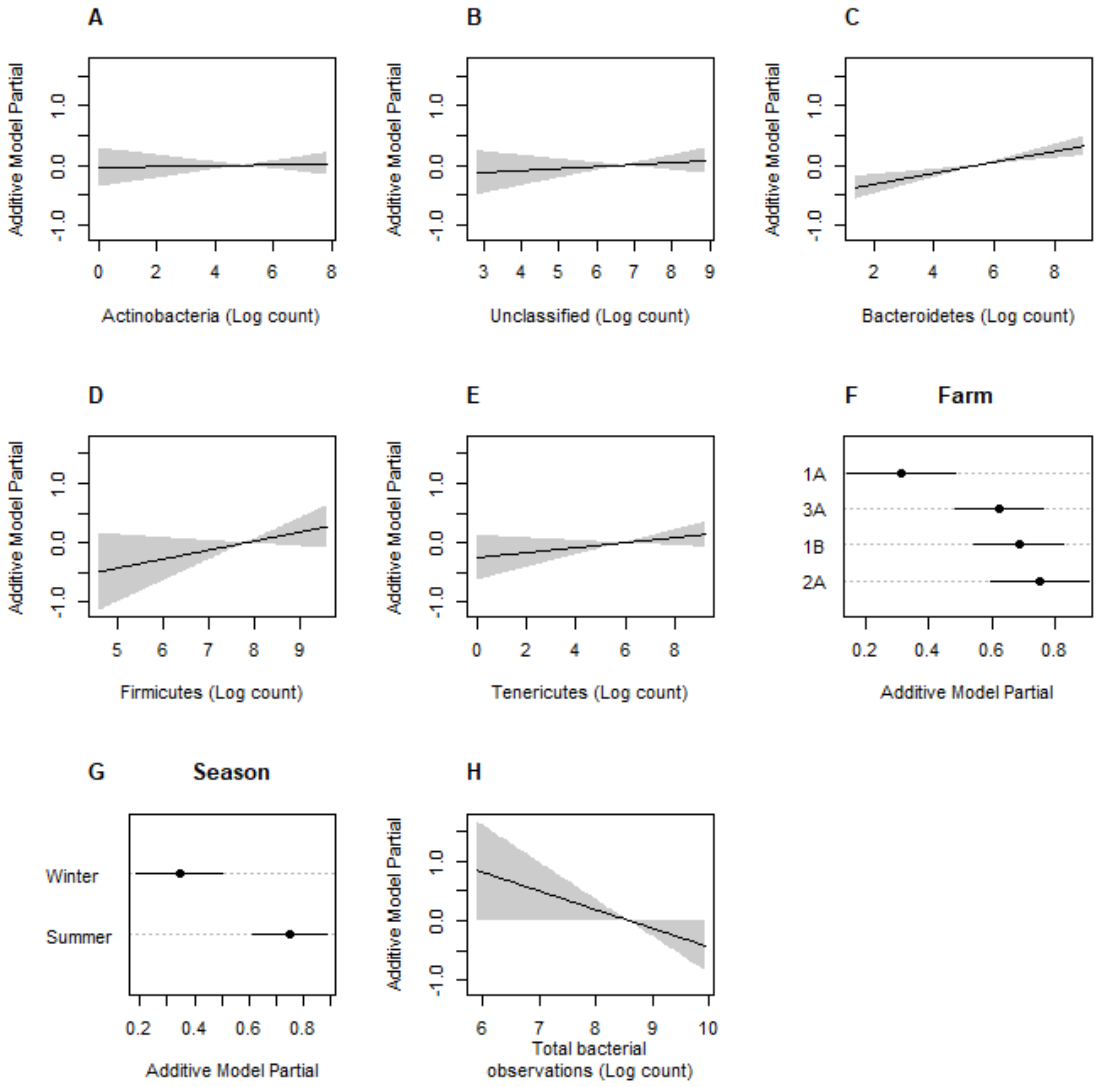

Figure 5.15: Details from a Generalised Additive Model output, predicting the impact of the natural logarithm counts of Actinobacteria (Figure 5.15A; p = 0.817), unclassified bacteria (Figure 5.15B; p = 0.493), Bacteroidetes (Figure 5.15C; p < 0.001), Firmicutes (Figure 5.15D; p = 0.135) and Tenericutes (Figure 5.15E; p = 0.191) in caecal contents, farm (Figure 5.15F), season (Figure 5.15G; p < 0.001), and the natural logarithm of the count of all bacteria observed per sample (Figure 5.15H; p = 0.045) on the probability of a broiler chicken flock being Campylobacter positive at final clearance.

Some farms were more prone to *Campylobacter* infection than others (p < 0.05 for all farms), and *Campylobacter* was more prevalent in summer than winter (p < 0.001). The presence of Bacteroidetes was associated with an increased probability of a

Campylobacter infection ($p < 0.001$) and the total count of bacteria was negatively correlated with the probability of a *Campylobacter* infection ($p = 0.045$).

To test if there were differences in the proportions of any of the genera of bacteria discovered in 16S sequencing in *Campylobacter* positive and negative caeca, a SIMPER analysis was conducted, comparing the proportions of each of the 71 genera of bacteria detected. A summary of the most statistically significant groups is presented in Table 5.3.

Table 5.3: Genera of bacteria present in community 16S analysis of broiler chicken caeca content with a p-value of less than 0.05 for dissimilarity between Campylobacter positive and negative samples at SIMPER analysis, along with the cumulative contribution made to difference between groups (%).

Genus	Abundance (*Campylobacter* positive)	Abundance (*Campylobacter* negative)	Cumulative contribution (%)	p-value
Campylobacter	7.8	4.3	98.8	0.001
Clostridiales (unclassified)	50.1	97.6	95.8	0.003
Bilophilia	0.7	1.3	99.8	0.008
Gastranaerophilales	1.3	0.6	99.7	0.010
Helicobacter	3.3	1.5	99.5	0.012
Bacteroidia (unclassified)	46.9	135.1	87.8	0.020
Ruminococcaceae (unclassified)	328.4	472.5	69.9	0.030
Desulfovibrio	0.6	0.0	100.0	0.031
Flavonifractor	2.4	3.2	99.6	0.035
Odoribacter	513.7	366.5	61.4	0.039
Megasphaera	169.2	53.8	84.9	0.046
Bacillales (unclassified)	0.5	1.0	99.8	0.048

Of species observed to be significantly different between positive and negative caeca, six increased in abundance when the caecum was *Campylobacter* positive, and six decreased. All had been previously observed in published research of chicken gut or caecal microbiota (Torok et al., 2011; Sergeant et al., 2014; Maki and Looft, 2018; Kubasova et al., 2019b; Rychlik, 2020; Xiao et al., 2021). *Odoribacter* and unclassified Ruminococcaceae were the largest groups present, each constituting around 7% of the average composition of caecal samples, with the total species presented in Table 5.3 accounting for over 18% combined. A general trend was present of Gram-positive organisms being lower in abundance and Gram-negative organisms being higher in abundance in *Campylobacter* positive samples. The only exceptions to this were Gram-positive *Megasphaera*, which was more abundant in *Campylobacter* positive samples, and Gram-negative *Bilophila* and unclassified Bacteroidia, which were both less abundant in *Campylobacter* positive samples.

To test if there were differences in the proportions of any of the genera of bacteria discovered in 16S sequencing across seasons, a SIMPER analysis was conducted, comparing the proportions of each of the 71 genera of bacteria detected. A summary of the most statistically significant groups is presented in Table 5.4. Five genera proved to be statistically significantly different both between *Campylobacter* positive and negative samples and between samples collected in summer and winter: *Campylobacter, Clostridiales* (unclassified), *Bacteroidia* (unclassified), *Odoribacter* and *Bacillales* (unclassified). *Ruminococcaceae* was almost statistically significantly different between seasons ($p = 0.055$). In samples collected during the summer, over which time a peak in infections is expected, proportional changes in keeping with those observed in *Campylobacter* positive caeca were seen in *Bacteroidia* (unclassified), *Bacillales* (unclassified) and *Clostridiales* (unclassified). *Campylobacter* and *Odoribacter* both decreased in summer, whereas they had increased with *Campylobacter* infection.

Table 5.4: Genera of bacteria present in community 16S analysis of broiler chicken caeca content with a p-value of less than 0.05 for dissimilarity between samples collected in summer and winter at SIMPER analysis, along with the cumulative contribution made to difference between groups (%). Summer is defined as April to September, winter as October to March.

Genus	Abundance (Summer)	Abundance (*Winter*)	Cumulative contribution (%)	p-value
Coriobacteriales (unclassified)	1.2	14.0	98.5	0.001
Bacillales (unclassified)	0.4	1.6	99.7	0.001
Mollicutes (unclassified)	39.0	197.6	79.5	0.003
Clostridioles	0.1	0.5	100.0	0.006
Erysipelotrichaceae (unclassified)	225.8	425.1	69.2	0.007
Clostridiales (unclassified)	58.4	119.5	95.7	0.007
Faecalitalea	92.9	156.7	89.6	0.010
Bacteroidia (unclassified)	65.4	166.9	81.9	0.028
Tyzzerella	1.1	4.2	99.5	0.028
Firmicutes (unclassified)	224.9	387.5	73.2	0.030
Campylobacter	5.1	8.8	98.6	0.033
Atopobiaceae (unclassified)	14.3	65.4	96.5	0.037
Tyzzerella (3)	0.0	0.1	100.0	0.047
Lactobacillus	8.5	14.6	98.1	0.048
Odoribacter	377.0	615.7	60.1	0.049

Since *Campylobacter* 16S detections did not correlate across summer and winter as it did between positive and negative flocks, a two-way ANOVA was conducted, with *Campylobacter* detection at qPCR, season and farm as factors used to describe the abundance of *Campylobacter* 16S detections. Figure 5.16 shows variation in *Campylobacter* abundance in caeca separated by *Campylobacter* detection at qPCR and season, and Table 5.5 and Table 5.6 gives resultant p-values from the ANOVA analysis.

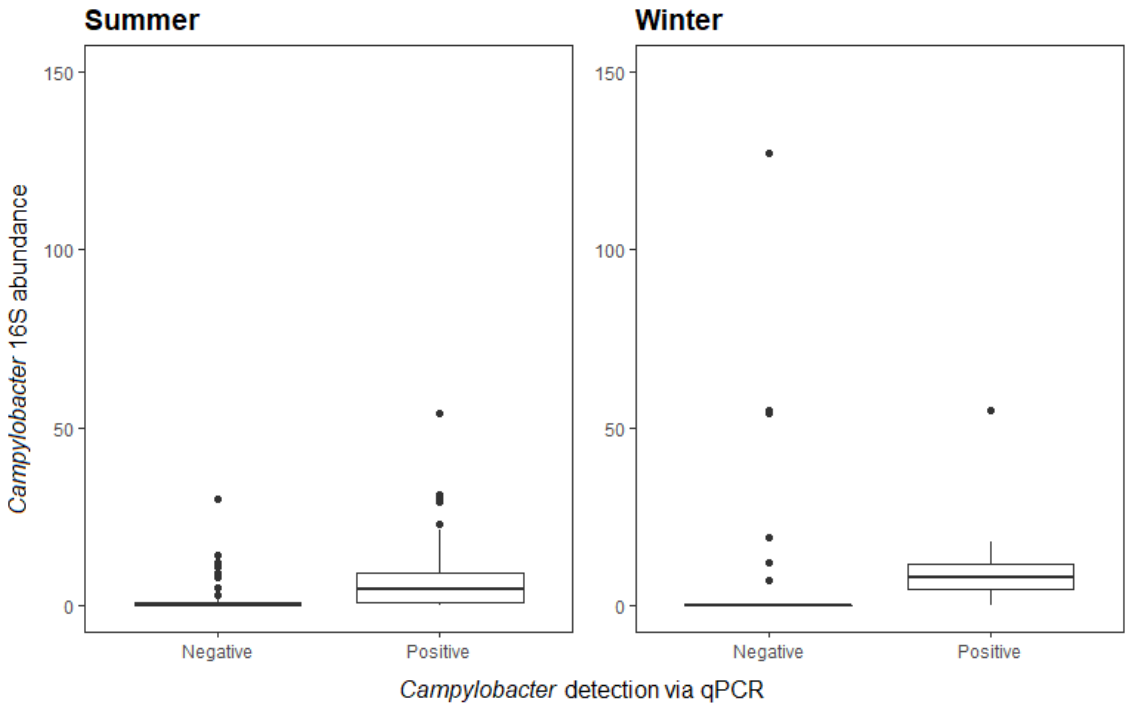

Figure 5.16: Campylobacter 16S copy numbers in chicken caecal contents, separated by Campylobacter detection via qPCR and season. Summer is defined as April to September, winter as October to March.

Table 5.5: p-values from an ANOVA analysis of Campylobacter 16S abundance in chicken caecal contents.

Parameter	**p-value**
Campylobacter detection via qPCR	0.061
Season	0.012
Farm	< 0.001
Interaction between *Campylobacter* detection via qPCR and season	0.946

Table 5.6: p-values for Tukey's HSD Tests, comparing Campylobacter 16S abundance in chicken caecal contents against farm of chicken origin (1A, 1B, 2A, 3A). p-values less than 0.05 are highlighted in bold.

	1A	1B	2A
1B	0.421		
2A	0.940	0.680	
3A	0.055	**< 0.001**	**0.004**

Table 5.5 shows that season and *Campylobacter* detection via qPCR showed no interaction in determining the number of *Campylobacter* 16S detections in caecal contents. Farm proved to be highly statistically significant in terms of *Campylobacter* 16S abundance. Farm 3C had the highest copy numbers, with an average of 13.5 detections per sample, and farm 1B had the lowest, with an average of 1.6 detections per sample. While season proved to be statistically significant in the ANOVA detailed in Table 5.5, a subsequent Welch's Two-Sample t-Test comparing the abundance of *Campylobacter* 16S in samples positive at qPCR testing collected in summer (n = 80, mean = 7.2) and winter (n = 11, mean = 11.9) showed no significant difference between seasons (p = 0.318, df = 84.411).

In order to assess whether gross proportional changes in the caecal phyla occur across both seasons and *Campylobacter* status, a two-way ANOVA was conducted, with *Campylobacter* detection at qPCR, season and farm as factors used to describe the ratio of Firmicutes to Bacteroidetes observed in caecal contents. The results of this can be seen in Table 5.7. No groups were statistically distinct from one another.

Table 5.7: p-values from an ANOVA analysis of the Firmicutes:Bacteroidetes ratio in chicken caecal contents.

Parameter	p-value
Campylobacter detection via qPCR	0.259
Season	0.124
Farm	0.432
Interaction between *Campylobacter* detection via qPCR and season	0.254

5.4 Discussion

This chapter aimed to investigate whether physiological or microbiological changes to the chicken gut altered between seasons, and if such changes could be associated with *Campylobacter* incidence. This was achieved by examining the extent of gut damage and 16S rRNA gene composition of caecal contents of broiler chickens, of known *Campylobacter* status at qPCR examination of caecal contents, across seasons. Significant changes were found in the microbial composition of chicken caeca, both between summer and winter and in *Campylobacter* positive and negative birds. There was a notable similarity in the changes that occurred between seasons and those that occurred between *Campylobacter* positive and negative, suggesting that there is interaction between *Campylobacter* infection, season, and chicken gut microbiota. Additionally, gut damage was observed to be significantly increased in summer months, which may have an impact on *Campylobacter* infection dynamics at that time of year.

The findings of this chapter corroborate most published research, in that *C. jejuni* is the dominant species of *Campylobacter* observed in broiler chickens, followed by *C. coli* (Bull *et al.*, 2006; Näther *et al.*, 2009; Jorgensen *et al.*, 2011). However, no direct statistical association could be found between season and the prevalence of either *C. jejuni* or *C. coli*. Differences in the proportions of *C. coli* and *C. jejuni* in Italian broiler

chicken flocks was found across seasons in work by Manfreda et al. (2006), with higher proportions of *C. jejuni* found in winter. This is anecdotally supported in the study presented in the current chapter by the fact that *C. coli* infections were exclusively observed in summer months, but due to a very low statistical power (n = 3) this cannot be resolved through statistical analysis. Näther et al. (2009) performed a similar study, across 75 farms, and similarly determined no difference in the proportion of different species across seasons, with an approximate split of 70% *C. jejuni* and 30% *C. coli* observed year-round. However, Näther et al. (2009) detected far less *C. jejuni* - *C. coli* coinfections than were found in the work presented in this chapter. This could be due to the culture- and enrichment-based methodology of Näther et al. (2009), which is known to have the potential to introduce bias in the species of *Campylobacter* that are recovered (Williams *et al.*, 2012), particularly in dealing with samples containing mixed *Campylobacter* species when compared to detection by PCR (Arnold *et al.*, 2015). For these reasons, it can be stated that the findings of species proportionality can be interpreted as having a more accurate representation of the actual composition of infections on commercial farms, especially in terms of mixed populations of *Campylobacter* spp.

The bacterial load of *Campylobacter* on bootsocks varied with season. However, the peak in loading was observed in March and the lowest values were observed in October. The GAM displayed in Figure 5.10 implies that this seasonal pattern alone is associated with an approximately four-fold change in *Campylobacter* enumeration. This pattern precedes the seasonality of incidence conventionally observed on UK broiler flocks by around three months (Ellis-Iversen *et al.*, 2009; Lawes *et al.*, 2012). A comparable finding to this pattern of timing was reported by Stern et al. (2001), who found that *Campylobacter* contamination of excreted caecal contents among US broiler flocks followed a seasonal pattern with a spring peak and autumn dip, while contamination of faecal samples followed the more conventional summer peak and winter dip pattern. This is suggestive of broader changes to chicken intestinal microflora, with regular seasonal changes which may prove conducive to *Campylobacter* infection. Since *Campylobacter* infection rates do not increase at the same time as loading levels, it also suggests that it may be the faecal shedding of *Campylobacter*, the process which allows

the organism to spread from one chicken to another, that follows a seasonal pattern, with higher shedding in warmer months. This may point towards a more severe form of the resultant illness being more likely to occur in summer, possibly due to modulations in the chicken gut microbiota or heat stress.

In the present study, ileal villus length was found to be 26% shorter, on average, in broiler chickens which had been infected with *Campylobacter*. The atrophy of intestinal villi has previously been reported as a prominent characteristic of *Campylobacter* infection in chickens (Lamb-Rosteski *et al.*, 2008; Humphrey *et al.*, 2014; Awad *et al.*, 2015), possibly due to the production of Cytolethal Distending Toxin (Awad *et al.*, 2015), and is exacerbated in the faster-growing breeds of chicken used in commercial production (Humphrey *et al.*, 2014). Additionally, upon further statistical elucidation, it was revealed that the season of sample collection played a larger, statistically significant role in villus length than *Campylobacter* status, with a clear trend towards improved gut integrity as the seasons associated with increased *Campylobacter* infections waned. Inflammation and microbial composition of the chicken gut are known to have a bidirectional relationship, each being capable of dramatically altering the other (Kogut *et al.*, 2018), and so the damage observed in this chapter may be indicative of physiological damage in response to pressures other than *Campylobacter* infection. The observed gut damage may be indicative of heat stress (Quinteiro-Filho *et al.*, 2012; Varasteh *et al.*, 2015; Nanto-Hara *et al.*, 2020; Ruff *et al.*, 2020) and therefore serves as an explanation of how *Campylobacter* infections spread more readily within a chicken house in summer, due to diarrhoea brought on by an inflamed lower gastrointestinal tract.

Campylobacter was only detected in the livers of chickens on four occasions (Figure 5.11). No statistical difference could be discerned between *Campylobacter* positive and negative livers in the level of gut damage observed. However, all four of those detections occurred on the same farm over two consecutive production cycles, and all occurred in birds whose caecal contents tested positive for *C. jejuni*. Colonisation of the liver by *C. jejuni* is posited to be strain-dependent, or to be reliant on other predisposing factors being present for successful invasion of the liver (Jennings *et al.*, 2011). It is also possible that *C. hepaticus*, a close relative of *C. jejuni* which shows a high level of genetic likeness

to it, could be the cause of *Campylobacter* presence in the liver (Van *et al.*, 2016; Van, Gor, *et al.*, 2017), and the genomic similarity of these organisms could have resulted in a false attribution by the qPCR method used for speciation. In either instance, the repeat detection of *Campylobacter* in the livers of chickens from the same chicken house suggests that it may have been the same strain or species of *Campylobacter* in each of these instances. The fact that these occurred across successive production cycles indicates that these infections may have originated from a common source that is capable of repeat infections of that chicken house.

The Shannon Diversity Index of broiler chicken caecal contents changed both with season and *Campylobacter* infection (Figure 5.14). Bacterial diversity in the chicken gut is known to be significant in determining growth performance outcomes (Bae *et al.*, 2017), and hence these findings may be commercially relevant. However, while the presented work here is highly indicative of an increase in diversity in winter months, this contradicts the prior findings of Oakley et al. (2018), who noted a decrease in the number of species present at the same time of year. It was also noted that *Campylobacter* positive caeca from the work described in this chapter were consistently more diverse than negative caeca. Sofka et al. (2015) assessed diversity across *Campylobacter* status amongst faecal samples and, while a statistical significance was not put forward, a tendency towards increased diversity when samples are *Campylobacter* negative was noted. However, this project also identified a statistically significant link between *Campylobacter* positive flocks and the total abundance of bacteria, with both the gross amount of bacterial 16S rRNA gene DNA decreasing under infection conditions, along with the abundance of most phyla identified as contributing to the changes in microflora composition observed when *Campylobacter* positive. For this reason, the increase in diversity may be due to a reduction in the population of formerly numerous genera of bacteria, as a reduction in the most populous genera will result in a higher proportion of the gut composition belonging to otherwise rarer species, thereby increasing the SDI.

Firmicutes were observed to constitute the most numerous phylum out of all those detected in chicken caeca (50.1%), an observation consistent with the vast majority of prior studies of chicken intestinal microbiota (Clavijo and Flórez, 2018; Kers *et al.*, 2018),

and hence provides evidence that reported results are comparable with existing research. Distinct changes to the microbial composition of caeca were found to be present both when *Campylobacter* was detectable via qPCR, or across different seasons. Thibodeau et al. (2015) noted a decrease in *Mollicutes* spp. when a *Campylobacter* infection was present in the chicken gut, and similarly the work conducted in this chapter suggests that a decrease in *Mollicutes* spp. occurs in summer months compared to winter. *Mollicutes* species are generally pathogenic, such as *Mycoplasma gallisepticum* which causes chronic respiratory disease in chickens (Saif, 2020). *Ruminococcaceae* and *Clostridiales* were also noted to have decreased under *Campylobacter* positive conditions in the work presented in this chapter. Two previous studies have suggested that these increase with *Campylobacter* infection (Kaakoush *et al.*, 2014; Connerton *et al.*, 2018), but Thibodeau et al. (2015) suggested that some species of *Clostridia* show up to a five-fold reduction in the presence of *Campylobacter*. *Megasphaera*, a butyrate-producing bacterial phylum usually associated with healthy chicken gut flora (Maki and Looft, 2018; Rychlik, 2020), was found to have increased with *Campylobacter* infection, despite purported associations between butyrate and resistance against pathogens such as *Campylobacter* in literature (Sunkara *et al.*, 2011; Zhou *et al.*, 2014; Fan *et al.*, 2021). These changes are all suggestive of immunological modulation of the gut brought about by the challenge represented by *Campylobacter* (Clavijo and Flórez, 2018) and active responses to infection. The inconsistencies between studies highlight the extent that additional factors that vary between research projects, such as the breed of chicken used, the gut bacteria of the breeder birds from which the study birds originated, or dietary regime employed, might have on the initial conditions of the gut (Borda-Molina, Seifert and Camarinha-Silva, 2018; Clavijo and Flórez, 2018) and hence the changes they undergo in response to an infection challenge.

Changes to the microbial composition of chicken caecal contents were noted as occurring seasonally in this study. For the most part, these changes were remarkably similar as those observed between *Campylobacter* positive and negative samples, with summer caecal contents aligning well with *Campylobacter* positive caecal traits. In both summer and *Campylobacter* positive caeca, the greatest magnitude of change from winter or negative caeca was in a loss of Gram-positive organisms, with an unclassified

Clostridiales spp. and other Firmicutes being particularly prominent in defining both. Of all the phyla of bacteria observed in chicken caeca throughout the study, only Bacteroidetes showed a correlation with *Campylobacter* infection of the same caecum (Figure 5.15), a finding put forward anecdotally but not statistically by Sofka et al. (2015). Firmicutes and Bacteroidetes are considered to be the two most important phyla in the human gut, with the Firmicutes:Bacteroidetes Ratio widely used as a broad monitoring statistic of gut dysbiosis (Stojanov, Berlec and Štrukelj, 2020). This statistic has also been posited as being biologically relevant to chicken gut regulation (Xu *et al.*, 2016). Alterations of this ratio in both positive and negative directions are indicative of poor broiler performance or external stressors (Bae *et al.*, 2017; Shi *et al.*, 2019; Yin *et al.*, 2021). Between the finding in Figure 5.15 that Bacteroidetes are significantly increased under *Campylobacter* infection conditions and the parallel trend of Firmicutes populations significantly decreasing in both summer and in *Campylobacter* positive caeca, there is evidence of gut dysregulation of the same type happening both in warmer months and during Campylobacter infection. However, due to the relatively low number of samples and narrow window of time over which they were collected, it is not possible to statistically resolve how much of the observed changes were due to *Campylobacter* infection or season independently. Higher rates of Campylobacter infection occurred over the summer and this will put an inevitable skew on some of the analyses due to interaction. The analysis in Table 5.7 which attempted to resolve whether Firmicutes:Bacteroidetes ratios were distinctly different across both seasons and *Campylobacter* status did not show any statistically significant result. However, since the two SIMPER analyses conducted (Table 5.3 and Table 5.4) showed that different genera of bacteria belonging to the same phylum were significant in the differences between summer and winter caeca and the differences between *Campylobacter* positive and negative caeca, there is evidence that these observed changes are, to a degree, independent of one another.

5.5 Conclusions

This work demonstrates that chicken gut microflora undergoes seasonal alterations that are in keeping with the changes seen when compromised by *Campylobacter* infection. Several key correlations have been identified as important in linking season,

Campylobacter infection and the integrity and composition of the chicken gastrointestinal tract. For this reason, seasonal patterns of *Campylobacter* infection may be explained through seasonal changes to the chicken gut microbiome.

6 General Discussion

6.1 Seasonality of *Campylobacter* in commercial broiler chicken flocks

Human cases of campylobacteriosis are often directly attributable to prior exposure to chicken meat contaminated with *Campylobacter* spp. (Cody *et al.*, 2010; Silva *et al.*, 2011; Skarp, Hänninen and Rautelin, 2016), and therefore infected broiler chickens destined for retail as fresh meat pose a significant public health risk. With an estimated 250,000 annual cases of human *Campylobacter* infection in the UK (McCarthy *et al.*, 2021) and mandated targets in UK law to reduce chicken carcass contamination (European Union, 2017; National Archives, 2017), there is significant demand within the poultry industry for farm-based interventions to prevent the inception of *Campylobacter* infection in commercial broiler chickens raised for meat.

In the UK, *Campylobacter* infection of commercial chicken flocks occurs in a predictable seasonal pattern, with a peak in infections during summers (McDowell *et al.*, 2008; Jorgensen *et al.*, 2011). However, the origins of this seasonality remain relatively unexplained by research up to the present (Hansson *et al.*, 2016). Understanding what drives seasonal trends in *Campylobacter* infection amongst chicken flocks could reveal insights into how flocks initially become infected, and hence lead to improvements in biosecurity and help keep *Campylobacter* out of the food supply chain.

6.2 Outcomes of research objectives

The work carried out in this thesis was based upon five initial research objectives, detailed in Section 1.7.2 and based on gaps in current knowledge on *Campylobacter* seasonality amongst commercial broiler chicken flocks. The resultant findings shall be detailed in the present chapter.

By assessing whether broiler chicken flocks became *Campylobacter* positive sooner in their lives during summer in Section 3.3.8, it was found that season did not have an impact on the bird age at which infections began. Analyses of chicken house construction parameters and *Campylobacter* incidence, as described in Section 3.3.7, suggested that both wooden posted house construction and side inlet ventilation systems are linked to increased risk of *Campylobacter* infection of the flocks contained within. Through monitoring *Campylobacter* contamination of the farm yard

environment surrounding the chicken house and comparing to the *Campylobacter* status of the flock housed within, as detailed in Sections 4.3.2 and 0, it was determined that *Campylobacter* originating outside of the chicken house is not a likely origin of flock infections.

Comparison of the microbial composition of chickens across seasons and both with and without a detectable *Campylobacter* infection, as per Section 5.3.5, revealed that seasonal changes occur to the proportions of different species within the chicken gut microbiota, with summer gut composition having distinct parallels with those seen under a *Campylobacter* infection state. Assessment of damage sustained to the chicken gut throughout the project, as detailed in Section 5.3.2, showed decreased villus lengths in summer months, indicating a higher level of damage or inflammation at this time.

6.3 Chicken house construction impacts flock *Campylobacter* incidence.

The construction of the house containing the observed chicken flocks proved to be a key factor in determining the likelihood that the flock contained within went on to acquire a *Campylobacter* infection. Specifically, wooden posted houses were found to be more prone to infection than their steel and clearspan counterparts. Other factors, such as heating system, also were implicated in the likelihood of *Campylobacter* acquisition. These house construction parameters proved to have a higher impact upon the probability of a given chicken flock becoming infected by *Campylobacter* than any other recorded parameter throughout Phase One of the presented body of work. While they are not able to be directly associated with the original aim of elucidating the origins of seasonal trends in *Campylobacter* infections, the findings based on house construction are worthy of note for their potential utility in reducing *Campylobacter* burden in the poultry industry.

It is possible that the observed relationships of certain house ventilation systems and construction materials to *Campylobacter* infection of the flocks kept within could have connections to *Campylobacter* infection seasonality. A combination of insect exposure risk and temperature and humidity management issues during warmer months may result in some housing systems being more able to cope with seasonal disease challenges than others. Recent research also suggests that changes in chicken house

disinfection routine can modulate the gut microflora and *Campylobacter* burden of the chickens housed within immediately post-cleaning (Fan *et al.*, 2021). Since wooden surfaces are not as readily decontaminated as other materials (Welker *et al.*, 1997) and the work presented in Section 5.3.5 indicates that pronounced seasonal changes occur to the populations of bacteria within the chicken gastrointestinal tract, it is possible that there is a link between the broiler house construction material, how easily it can be cleaned between production cycles and how susceptible the chicken gut environment is to pathogenic colonisation by *Campylobacter*. Post-disinfection drying of surfaces contaminated by chickens infected with *Campylobacter* is critical for effective inactivation of any residual *Campylobacter* (Morgan *et al.*, 2022), and different materials will dry at different rates (Mujumdar, 2000). Environmental humidity, which impacts drying time, is heightened in colder months as seen in Figure 3.8, but the temperature differential between inside and outside the poultry house is greater in winter. As disinfection occurs in the days preceding the arrival of new chicks, temperatures inside the house are elevated so they will be at the required 34°C at time of placement. Since air can hold more moisture at higher temperatures, the relative humidity of incoming cold air is drastically reduced as it is heated by the house heating system (University of Kentucky, 2022), thereby increasing the drying potential of a poultry house in cold environmental conditions. However, it should be noted that UK broiler flocks tend to experience issues when drying litter and bedding materials during the chicken rearing process in winter months (Hermans *et al.*, 2006), contrary to what may be expected. The issue of 'wet litter' is linked to inadequate ventilation (Dunlop *et al.*, 2016), a control parameter that is indeed usually minimised in cold environments to reduce excessive heat loss from the chicken house (Aviagen, 2010), but challenges in drying the chicken house environment when birds are present does not necessarily hold true when there are no birds present. For this reason, there is cause to believe that the differences in house construction materials and ventilation systems may interact with disinfection procedures through their inherent impact on drying, and consequently this may impact the vulnerability of chickens contained within the house to *Campylobacter* infection. This effect may become more pronounced under certain weather conditions, due to the impacts of humidity and temperature on the ability to dry out the contents of the poultry

house, thereby allowing *Campylobacter* a greater chance of surviving the disinfection process.

6.4 Modulations in the chicken gut microflora occur across seasons and with *Campylobacter*.

Numerous, congruent changes occurred in the chicken gut microbiota, both across seasons and between *Campylobacter* positive and negative samples. Seasonal differences in the composition of broiler chicken intestinal microbiota have been detailed in prior research in the field (Oakley *et al.*, 2018), along with observed changes that occur with heat stress on the birds (Quinteiro-Filho *et al.*, 2012; Shi *et al.*, 2019; Rostagno, 2020; Cao *et al.*, 2021; He, Maltecca and Tiezzi, 2021; Yin *et al.*, 2021). The known interaction between chicken gastrointestinal microbiota and *Campylobacter* is not unidirectional, with evidence in existing literature both for the pressure that gut microflora can impart upon *Campylobacter* (Chintoan-Uta *et al.*, 2020; Almansour *et al.*, 2021; Almansour, 2022) and for the impact that *Campylobacter* can have on the constituent species in the chicken gut (Johansen *et al.*, 2006; Sofka *et al.*, 2015; Connerton *et al.*, 2018). Indeed, some authors have noted this two-way interaction (Kaakoush *et al.*, 2014; Sakaridis *et al.*, 2018) and the difficulties it can place on knowing whether statistically significant changes arise from *Campylobacter* infection or initial differences in gut microbiota between chicken populations. The composition of bacteria within the chicken gut is determined to a great extent by environmental factors, as chickens evolved to acquire their microbiota from adult hens, which is generally not possible in commercial chicken production due to the hatchery systems in place (Kubasova et al., 2019a; Rychlik, 2020). For this reason, the level of biosecurity, diet and housing conditions that young chicks are exposed to greatly determine their future intestinal microflora (Kers *et al.*, 2018). In this way, the chicken house environment, thermal stressors and *Campylobacter* presence all feed into determining the species present in the chicken gut, and hence how capable the chicken gut is at dealing with a pathogen challenge such as *Campylobacter*. This explains why each farm was statistically distinct in analyses throughout this project, due to differences in house microbial composition and style of management employed by the farmer, and why it has been a

challenge up to the present to identify the core factors which are responsible for *Campylobacter* seasonality.

Changes in the chicken gut associated with *Campylobacter* infection tended towards alterations in the gross numbers of bacteria, reflected in the lower total bacterial count numbers and increase in diversity observed in Section 5.3.5. This contrasts with the seasonal changes observed, which instead tended to reflect in gut damage and differences in the abundance of bacterial species present in the gut. This difference may suggest that each parameter is indeed having an independent impact on the chicken, to a degree, and that the analyses conducted here can reflect this.

Physical damage to the chicken gut was observed when a *Campylobacter* infection was detectable by qPCR. This is in keeping with the current scientific consensus that *Campylobacter* should not be considered a typical component of the gut microbiota of broiler chickens (Smith *et al.*, 2005; Humphrey *et al.*, 2014; Wigley, 2015; Pielsticker *et al.*, 2016) and indicates the extent of damage that infection can bring about. However, damage reduced over the course of the observation period during Phase Two of the project, transitioning from a period of high risk of *Campylobacter* infection to one of lower probability, and it was this temporal trend which was by far more strongly associated with the observed shortening of villi. The integrity of the chicken gut is known to be directly negatively affected by both acute and chronic thermal stress (Nanto-Hara *et al.*, 2020; Mazzoni *et al.*, 2022), including the observed reduction in villus length (Nanto-Hara *et al.*, 2020; Rostagno, 2020), immunological inflammation responses (Varasteh *et al.*, 2015; Ahmad *et al.*, 2022) and increased permeability of the intestinal lumen to the point of gastrointestinal leakage (Ruff *et al.*, 2020). *Campylobacter* infects the chicken gut through adhesion and invasion into the epithelial cells of the lumen (Hermans *et al.*, 2011), and thus the damage that the gut appears to have sustained during warmer months, independent of detectable levels of *Campylobacter* in the flock, is likely to provide a boost in virulence of the bacterium.

6.5 Environmental sources are not responsible for *Campylobacter* infections in broiler chickens.

Potential environmental sources of *Campylobacter* were surveyed for causal connection with acquisition of *Campylobacter* by concurrent broiler chicken flocks, detailed in Chapter 4. However, very little evidence was found of environmental reservoirs of *Campylobacter* with potential to breach the biosecurity of a chicken house. Bootsocks collected along the paths farmers walk to enter the chicken houses were consistently absent of *Campylobacter* unless an infection was already ongoing in the chicken house. Few detections were made in samples of the air around the chicken house, and a scant minority of samples collected from materials introduced to the chicken house at initial chick placement tested positive for *Campylobacter*.

When compared to the findings of other published research which found considerably more environmental *Campylobacter* outside of periods of flock infection on similar sample types and locations, a temptation is present to question aspects of the methodologies employed in this thesis. Purely molecular methodologies were used here, to minimise species biasing from culturing in selective media (Williams *et al.*, 2012) and remove the necessity for any *Campylobacter* spp. present on swabs or within samples to be still living at the point of testing. Bacterial DNA can survive at room temperature in a state where it can be extracted and amplified by PCR for at least one year (Young *et al.*, 2007). Throughout the project, samples were refrigerated or frozen upon return to the laboratory after experiencing at most one production cycle (up to 40 days) at ambient temperatures, and testing of stored samples proved to give positive qPCR results at timepoints up to 12 months after collection. Consecutive positives are also seen throughout the presented body of work in this thesis, with the majority of flocks remaining *Campylobacter* positive in daily bootsock testing following the first detection of *Campylobacter*, demonstrating the repeatable efficacy of the testing regime employed here.

Previous research in isolating *Campylobacter* from rural environments has shown that its seasonal prevalence does not correspond with infection patterns observed in humans or chickens (Jones *et al.*, 2017). Additionally, presence of environmental *Campylobacter* on farms which routinely produce *Campylobacter* positive chickens has

been shown to be no higher than on farms which do not (Hansson *et al.*, 2007). Prior studies have also found low detection rates similar to those presented here (Thakur et al., 2013; 0.8% of samples) when testing swabs of the farm yard adjacent to the chicken house, or samples of bedding and feed. However, despite its fastidious requirements for active proliferation, environmental persistence of *Campylobacter* in soils, waters and other materials can pose a risk of infection to chickens (Bronowski, James and Winstanley, 2014), and persistent environmental *Campylobacter* in areas such as chicken house anterooms has been identified as a known risk factor for chicken flock *Campylobacter* infection (Ellis-Iversen *et al.*, 2012). Overall, based on the analyses conducted as part of the research presented here and knowledge from prior studies, environmental loading of *Campylobacter* seems an unlikely origin for the seasonal trends observed in chicken flock infections. However, this leaves the authors unable to comment on the likely ultimate origin of *Campylobacter* among broiler chickens, due to limited evidence within the presented work for any likely sources. *Campylobacter* transmission into the chicken house from outside may therefore comprise a 'rare event' that can seed multiple repeat infections in subsequent production cycles once it has established itself in the chicken house environment.

6.6 Seasonality of *Campylobacter* infections is highly multi-factorial, and its origins remain obscure.

The presented body of research identified no one single underlying factor that contributes most heavily to *Campylobacter* seasonal variations in commercial chicken flocks. Instead, the author hypothesises that many smaller factors each contribute to the overall picture of seasonality. Many factors that were anticipated to have a causal link to *Campylobacter* infection rates in broiler chickens in the working hypotheses of this thesis either showed no associations with season or were not significant sources of *Campylobacter.*

Given the lack of detection of environmental *Campylobacter* throughout the study, the author does not posit direct transmission of *Campylobacter* from surfaces, aerosols or dust as being likely origins of campylobacteriosis in commercial chicken production. Similarly, transmission via materials such as bedding, water and feed, introduced throughout the poultry process across biosecure barriers, is unlikely to be responsible

for the onset of observed infections in this project. Considering the tendency of certain farms towards infection by rarer species of *Campylobacter*, as seen in Section 5.3.1, there is some evidence for re-infection of a given chicken house, cycle after cycle, from a source such as the interior of the poultry house when insufficiently disinfected between flocks. This is given further credence by the fact that houses constructed of timber were observed to be more prone to *Campylobacter* acquisition by flocks of chickens contained within, as this material choice is not as readily sanitised as the sheet metal that more modern houses are constructed from (Welker *et al.*, 1997).

There is evidence throughout this thesis that signs of heat stress, such as intestinal damage, are apparent in warmer months, and that these may be conducive to *Campylobacter*'s ability to infect chickens. As observed in Figure 3.13, a loss of control of chicken house temperatures was noted in summer months, resulting in temperatures exceeding those recommended for the chickens. However, a statistical link between acute heat stress events and the onset of *Campylobacter* could not be established. In fact, the onset of *Campylobacter* infection was not impacted by season, as observed in Section 3.3.8, with the mean age of acquisition of *Campylobacter* remaining constant year-round. This suggests that the heat stress symptoms observed are more subtle in origin than simply elevated temperatures. Over winter months, the temperature differential between the heated interior of the chicken house and the exterior are much higher than in summer, resulting in lower relative humidity within the chicken house in winter as the heated air is capable of holding more moisture (University of Kentucky, 2022). Chickens primarily thermoregulate through direct body-to-air heat transfer, and can supplement this when temperatures are raised beyond a level they can tolerate by evaporative means through panting (Aviagen, 2010). For this reason, thermal stresses can be more effectively handled by chickens when humidity is lower. In this way, the effect of elevated relative humidity in chicken houses in summer combined with ineffective means of controlling house temperatures may induce symptoms of thermal stress in chickens, which in turn may lead to an environment within the chicken gut conducive to *Campylobacter* infection.

6.7 Suggestions on future research

Technologies have been specifically developed for housed chicken production in hot geographical climates, such as evaporative cooling pads, water misters and tunnel ventilation. These have been shown to adequately reduce internal house temperatures to within tolerable welfare conditions for chicken production (Datekin, Karaca and Yildiz, 2009; Dunlop and McAuley, 2021) and are not currently in widespread use in UK broiler chicken production. Research into the impact of climate change trends on commercial chicken production suggests that regions which previously did not usually employ any cooling method beyond active ventilation are now considering implementing in-house evaporative cooling measures, or even air conditioning (Izar-Tenorio *et al.*, 2020). Given the trends observed in UK climate in recent years, more cooling has been suggested as a future welfare and productivity prerogative in management of all kinds of livestock, not just chicken production (Wreford and Topp, 2020). For this reason, future experiments assessing the feasibility in the UK of broiler production systems and house ventilation or cooling designs adapted to warmer climates could be conducted for their impact on *Campylobacter* control.

Control of chicken gut bacteria already plays an important role in the poultry industry's pathogen reduction strategy, with numerous commercial bacterial probiotic products in regular use in UK hatcheries specifically with the intent of seeding the guts of newly hatched chicks with bacteria which can outcompete pathogens such as *Campylobacter* spp. (Heimesaat *et al.*, 2021; Fortomaris *et al.*, 2022). These products are a relatively recent innovation and are still under constant improvement and efficacy testing by their manufacturers. While it is known, both from the work conducted in this thesis and prior findings, that chicken gut microbiota is altered by both elevated temperatures and pathogenic infections (He, Maltecca and Tiezzi, 2021), the inverse also appears to be true, with evidence of resistance to both heat stress and *Campylobacter* infection being conferred from certain bacterial species which can be introduced to the chicken gut (Chintoan-Uta *et al.*, 2020; Almansour *et al.*, 2021; Jiang *et al.*, 2021; Almansour, 2022). Development of new probiotics which take into account recent findings in the interaction between gut microbiota, heat stress and immunological modulation could provide effective control of *Campylobacter* across seasons.

Additionally, feed additive solutions could be explored for long-term, continued microbial modulation of the gut as a form of *Campylobacter* management (Molnár *et al.*, 2015; Guyard-Nicodème *et al.*, 2016; Clavijo and Flórez, 2018).

It is not only the observed changes in gut microbiota which could be a target of future research, but also the damage sustained to the gut during periods of heightened *Campylobacter* infections. Probiotics and feed additives have been put forward as a means of alleviating these symptoms (Varasteh *et al.*, 2015; Ahmad *et al.*, 2022), but utilising broader approaches such as employing more thermotolerant breeds of chicken (Kennedy, Lichoti and Ommeh, 2022; Liang *et al.*, 2022) or employing thermal conditioning methods on young chicks (Ouchi *et al.*, 2021) may prove to improve the resilience of UK poultry producers to both thermal stress-induced production losses and *Campylobacter* infections.

6.8 Project hindrances, their impact on stated objectives and critique of the presented study

As stated in Section 1.7.2, the original purpose of the work contained in this thesis is to explain what drives seasonal patterns in *Campylobacter* infections among commercial broiler chicken flocks. To this purpose, all of the experiments carried out were originally intended to take place over an unbroken span of many months, in an attempt to examine parameters across a range of seasons. However, due to issues caused by the COVID-19 pandemic, farmers withdrawing from the project or missed sample collections, there are unavoidable gaps in the dataset when compared to the originally planned number to be collected and assessed. For these reasons, and others, in some cases it may not be possible to resolve statistical differences or infer seasonal trends, due to incomplete sampling regimes and reduced sample numbers. Originally, the methodology of Phase Two was to be informed by the findings of Phase One, resulting in experiments tailored to answering the open questions left behind after analysing the results of the first phase, but the restrictions of the pandemic resulted in a scaling back of efforts to what was practical to implement at that time. The presented results represent the extent of what could be ascertained under the limitations that the circumstances imposed upon the work conducted.

Whilst this thesis is able to give light to some of the many factors which lead to the observed seasonal trends in *Campylobacter* infection among broiler chickens, it ultimately cannot give a conclusive, comprehensive answer to the original question posed. This is due to limitations in the presented work that manifested across the study period. The eleven farms engaged with the study were self-selected from a recruitment drive among the 168 farms supplying Avara Foods Ltd., and therefore only represent those engaged enough with research to participate. This may have biased the farm selection towards farmers more likely to take current research into account or otherwise have practices in their management style that may not truly be reflective of widespread industry practice. It also confines the farms involved in the study to only those in a single geographic region, following the production guidelines of a single company, and hence the results will only pertain as true to these exact circumstances. Future studies in this field may wish to broaden the scope and number of farms to those under different parent companies, or in other regions or nations, to assess if findings are more universal than what can be ascertained here.

Another necessary practical limitation to the study was the transit time and storage temperature of the routine bootsock swabs tested throughout both phases of the project, as described in Section 3.2.2, which may have resulted in some degradation of the DNA detected. However, bacterial DNA is surprisingly robust post cell death and will persist for many months (Young *et al.*, 2007), and the repeated detection on subsequent days of *Campylobacter* in this study pays testament to this. Due to the limitations of time and resources, it was also not possible to test every individual bootsock collected, and instead a method of selecting samples for test which identifies the first day of infection was devised, as detailed in Section 3.2.2. This may have resulted in some short-duration infections being missed if they fell in the four-day window between negative samples. The qPCR-based approach to *Campylobacter* detection employed throughout this thesis is also not without flaws; Since no culture-based approach was used, there can be no measurement of the viability of *Campylobacter* detected, and so some detections in this project may have been of dead bacteria that could not have had any impact on chicken infection.

One of the major limiting factors of this research has been the length of time given for research to be conducted over. When analysing seasonal trends, multiple years are generally favoured for gaining statistical significance and ensuring that unique events that affect a single year can be accounted for. This was not the case with this project, as, due to the phased design and constraints on timespan, the longest continuous period of testing occurred for a span of 18 months. This limitation is reflected in the fact that the usual reduction of cases of *Campylobacter* infection among broiler chickens was not observed over the winter of 2018-2019 in Phase One, but was observed in the winter of 2019-2020 and throughout Phase Two of the project. This curtailed the statistical power of any analyses into *Campylobacter* seasonality attempted in Section 3.3. A significant improvement to this study could have been gained through extending the period of time over which samples were collected into multiple continuous years.

6.9 Implications of findings presented in this thesis on the poultry industry

Based on the understanding gained in Section 3.3.7 on the impact of house construction on the likelihood of a chicken house to become *Campylobacter* positive, efforts should be made to modernise older farms with wooden houses and LPG heating. Particular attention should be made to aspects of house design concerning surfaces on which *Campylobacter* could evade disinfection and the resilience of the heating and ventilation systems against fluctuations in temperature in the outdoor environment, to minimise the possibility of *Campylobacter* carrying from the previously placed flock to the subsequent one and reduce possible stressors that could cause the infection to take hold and spread.

The presented study has highlighted changes that could be implemented in the poultry industry. Exposed wooden surfaces inside of poultry houses should be minimsed, and heating systems that are capable of effective drying, both post-disinfection and of soiled litter, should be employed. Ventilation intakes should be high enough to be clear of risk of particulate or insect transmission from the farm environment. Long-term goals for improving chicken gut health and thermal resilience should be implemented to ensure that the industry evolves to cope with the increasing pressures of modern poultry production.

Bibliography

ADAS (2019) *Impacts of the better chicken commitment on the UK broiler sector*. National Farmers' Union.

Adkin, A., Hartnett, E., Jordan, L., Newell, D. and Davison, H. (2006) 'Use of a systematic review to assist the development of Campylobacter control strategies in broilers', *Journal of Applied Microbiology*, 100(2), pp. 306–315. Available at: https://doi.org/10.1111/j.1365-2672.2005.02781.x.

Agunos, A., Waddell, L., Léger, D. and Taboada, E. (2014) 'A systematic review characterizing on-farm sources of Campylobacter spp. for broiler chickens', *PLoS ONE*, 9(8), p. e104905. Available at: https://doi.org/10.1371/journal.pone.0104905.

Ahmad, R., Yu, Y.H., Hsiao, F.S.H., Su, C.H., Liu, H.C., Tobin, I., Zhang, G. and Cheng, Y.H. (2022) 'Influence of Heat Stress on Poultry Growth Performance, Intestinal Inflammation, and Immune Function and Potential Mitigation by Probiotics', *Animals*, 12(17). Available at: https://doi.org/10.3390/ani12172297.

Allen, V.M., Ridley, A.M., Harris, J.A., Newell, D.G. and Powell, L. (2011) 'Influence of production system on the rate of onset of Campylobacter colonization in chicken flocks reared extensively in the United Kingdom', *British Poultry Science*, 52(1), pp. 30–39. Available at: https://doi.org/10.1080/00071668.2010.537306.

Allen, V.M., Weaver, H., Ridley, A.M., Harris, J.A., Sharma, M., Emery, J., Sparks, N., Lewis, M. and Edge, S. (2008) 'Sources and Spread of Thermophilic Campylobacter spp. during Partial Depopulation of Broiler Chicken Flocks', *Journal of Food Protection*, 71(2), pp. 264–270. Available at: https://doi.org/10.4315/0362-028X-71.2.264.

Allos, B.M. (2001) 'Campylobacter jejuni infections: Update on emerging issues and trends', *Clinical Infectious Diseases*, 32(8), pp. 1201–1206. Available at: https://doi.org/10.1086/319760.

Almansour, A. (2022) *The role of microbiota on Campylobacter jejuni colonization and growth performance in broiler chickens*. University of Arkansas.

Almansour, A., Fu, Y., Alenezi, T., Bansal, M., Alrubaye, B., Wang, H. and Sun, X. (2021) 'Microbiota from specific pathogen-free mice reduces campylobacter jejuni chicken colonization', *Pathogens*, 10(11). Available at: https://doi.org/10.3390/pathogens10111387.

Alter, T. (2016) 'Prevention and mitigation strategies for Campylobacter with focus on poultry production', in *Campylobacter: Features, Detection, and Prevention of Foodborne Disease*, pp. 111–129. Available at: https://doi.org/10.1016/B978-0-12-803623-5.00006-X.

Alves, M.B.R., Fonseca, B.B., Melo, R.T., Mendonça, E.P., Nalevaiko, P.C., Girão, L.C., Monteiro, G.P., Silva, P.L. and Rossi, D.A. (2017) 'Feed can be a source of Campylobacter jejuni infection in broilers', *British Poultry Science*, 58(1), pp. 46–49. Available at: https://doi.org/10.1080/00071668.2016.1258691.

Arnold, M.E., Jones, E.M., Lawes, J.R., Vidal, A.B., Clifton-Hadley, F.A., Rodgers, J.D. and Powell, L.F. (2015) 'Bayesian analysis of culture and PCR methods for detection of Campylobacter spp. in broiler caecal samples', *Epidemiology and Infection*, 143(2), pp. 298–307. Available at: https://doi.org/10.1017/S0950268814000454.

Asuming-Bediako, N., Parry-Hanson Kunadu, A., Abraham, S. and Habib, I. (2019) 'Campylobacter at the Human-Food Interface: The African Perspective.', *Pathogens*, 8(2), pp. 1–30. Available at: https://doi.org/10.3390/pathogens8020087.

Avara Foods (2022) *Animal Welfare Report*. Available at: https://www.avarafoods.co.uk/getmedia/c80b51cb-6b5a-49cb-b311-459d1cdff820/Avara-Welfare-Report-2022.pdf.

AVEC (2021) *Annual Report*. Brussels: AVEC. Available at: https://avec-poultry.eu/wp-content/uploads/2021/09/6226-AVEC-annual-report-2021_64.pdf.

Aviagen (2010) *Environmental Management in the Broiler House*. Newbridge, Scotland: Aviagen Ltd. Available at: https://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross_Environmental_Management_in_the_Broiler_House.pdf.

Aviagen (2018) *Ross Broiler Management Handbook, technical document no: 1118-AVNR-032*. Newbridge, Scotland: Aviagen Ltd. Available at: http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-BroilerHandbook2018-EN.pdf.

Awad, W.A., Hess, C. and Hess, M. (2018) 'Re-thinking the chicken –Campylobacter jejuni interaction: a review', *Avian Pathology*, 47(4), pp. 352–363. Available at: https://doi.org/10.1080/03079457.2018.1475724.

Awad, W.A., Molnár, A., Aschenbach, J.R., Ghareeb, K., Khayal, B., Hess, C., Liebhart, D., Dublecz, K. and Hess, M. (2015) 'Campylobacter infection in chickens modulates the intestinal epithelial barrier function', *Innate Immunity*, 21(2), pp. 151–160. Available at: https://doi.org/10.1177/1753425914521648.

Bae, Y., Koo, B., Lee, S., Mo, J., Oh, K. and Mo, I.P. (2017) 'Bacterial diversity and its relationship to growth performance of broilers', *Korean Journal of Veterinary Research*, 57(3), pp. 159–167.

Baffone, W., Casaroli, A., Citterio, B., Pierfelici, L., Campana, R., Vittoria, E., Guaglianone, E. and Donelli, G. (2006) 'Campylobacter jejuni loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model', *International Journal of Food Microbiology*, 107(1), pp. 83–91. Available at: https://doi.org/10.1016/j.ijfoodmicro.2005.08.015.

Bang, D.D., Borck, B., Nielsen, E.M., Scheutz, F., Pedersen, K. and Madsen, M. (2004) 'Detection of seven virulence and toxin genes of Campylobacter jejuni isolates from Danish turkeys by PCR and cytolethal distending toxin production of the isolates', *Journal of Food Protection*, 67(10), pp. 2171–2177. Available at: https://doi.org/10.4315/0362-028x-67.10.2171.

Battersby, T., Walsh, D., Whyte, P. and Bolton, D. (2017) 'Evaluating and improving terminal hygiene practices on broiler farms to prevent Campylobacter cross-contamination between flocks', *Food Microbiology*, 64, pp. 1–6. Available at: https://doi.org/10.1016/j.fm.2016.11.018.

Battersby, T., Whyte, P. and Bolton, D. (2016a) 'Protecting broilers against Campylobacter infection by preventing direct contact between farm staff and broilers', *Food Control*, 69, pp. 346–351. Available at: https://doi.org/10.1016/j.foodcont.2016.04.053.

Battersby, T., Whyte, P. and Bolton, D. (2016b) 'The pattern of Campylobacter contamination on broiler farms; external and internal sources', *Journal of Applied Microbiology*, 120(4), pp. 1108–1118. Available at: https://doi.org/10.1111/jam.13066.

Bennett, C.E., Thomas, R., Williams, M., Zalasiewicz, J., Edgeworth, M., Miller, H., Coles, B., Foster, A., Burton, E.J. and Marume, U. (2018) 'The broiler chicken as a signal of a human reconfigured biosphere', *Royal Society Open Science*, 5(12). Available at: https://doi.org/10.1098/rsos.180325.

Berrang, M.E., Meinersmann, R.J., Cox, N.A. and Thompson, T.M. (2018) 'Multilocus Sequence Subtypes of Campylobacter Detected on the Surface and from Internal Tissues of Retail Chicken Livers', *Journal of Food Protection*, 81(9), pp. 1535–1539. Available at: https://doi.org/10.4315/0362-028X.JFP-18-131.

BIOHAZ (2010) 'Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU', *EFSA Journal*, 8(1), pp. 1–89. Available at: https://doi.org/10.2903/j.efsa.2010.1437.Available.

BIOHAZ (2011) 'Scientific Opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain', *EFSA Journal*, 9(4), pp. 1–141. Available at: https://doi.org/10.2903/j.efsa.2011.2105.

Blaser, M.J. (1997) 'Epidemiologic and clinical features of Campylobacter jejuni infections', *Journal of Infectious Diseases*, 176(6 Suppl.), pp. 103–105. Available at: https://doi.org/10.1086/513780.

Blaser, M.J., Berkowitz, I.D., LaForce, F.M., Cravens, J., Reller, L.B. and Wang, W.L. (1979) 'Campylobacter enteritis: Clinical and epidemiologic features', *Annals of*

Internal Medicine, 91(2), pp. 179–185. Available at: https://doi.org/10.7326/0003-4819-91-2-179.

Bolton, D.J. (2015) 'Campylobacter virulence and survival factors', *Food Microbiology*, 48, pp. 99–108. Available at: https://doi.org/10.1016/j.fm.2014.11.017.

Borda-Molina, D., Seifert, J. and Camarinha-Silva, A. (2018) 'Current Perspectives of the Chicken Gastrointestinal Tract and Its Microbiome', *Computational and Structural Biotechnology Journal*, 16, pp. 131–139. Available at: https://doi.org/10.1016/j.csbj.2018.03.002.

Breslow, N.E. (1975) 'Analysis of Survival Data under the Proportional Hazards Model', *International Statistical Review / Revue Internationale de Statistique*, 43(1), p. 45. Available at: https://doi.org/10.2307/1402659.

Bronowski, C., James, C.E. and Winstanley, C. (2014) 'Role of environmental survival in transmission of Campylobacter jejuni', *FEMS Microbiology Letters*, 356(1), pp. 8–19. Available at: https://doi.org/10.1111/1574-6968.12488.

Bull, S.A., Allen, V.M., Domingue, G., Jørgensen, F., Frost, J.A., Ure, R., Whyte, R., Tinker, D., Corry, J.E.L., Gillard-King, J. and Humphrey, T.J. (2006) 'Sources of Campylobacter spp. colonizing housed broiler flocks during rearing', *Applied and Environmental Microbiology*, 72(1), pp. 645–652. Available at: https://doi.org/10.1128/AEM.72.1.645-652.2006.

Butzler, J.P. (2004) 'Campylobacter, from obscurity to celebrity', *Clinical Microbiology and Infection*, 10(10), pp. 868–876. Available at: https://doi.org/10.1111/j.1469-0691.2004.00983.x.

Byers, J.A. (2011) 'Analysis of vertical distributions and effective flight layers of insects: Three-dimensional simulation of flying insects and catch at trap heights', *Environmental Entomology*, 40(5), pp. 1210–1222. Available at: https://doi.org/10.1603/EN11043.

Byrd, J.A., Corrier, D.E., DeLoach, J.R. and Nisbet, D.J. (1997) 'Comparison of Drag-Swab Environmental Protocols for the Isolation of Salmonella in Poultry Houses', *Avian Diseases*, 41(3), pp. 709–713. Available at: https://doi.org/10.2307/1592165.

Callicott, K.A., Frioriksdóttir, V., Reiersen, J., Lowman, R., Bisaillon, J.R., Gunnarsson, E., Berndtson, E., Hiett, K.L., Needleman, D.S. and Stern, N.J. (2006) 'Lack of evidence for vertical transmission of Campylobacter spp. in chickens', *Applied and Environmental Microbiology*, 72(9), pp. 5794–5798. Available at: https://doi.org/10.1128/AEM.02991-05.

Cao, C., Chowdhury, V.S., Cline, M.A. and Gilbert, E.R. (2021) 'The Microbiota-Gut-Brain Axis During Heat Stress in Chickens: A Review', *Frontiers in Physiology*, 12(October). Available at: https://doi.org/10.3389/fphys.2021.752265.

de Castro Burbarelli, M.F., do Valle Polycarpo, G., Lelis, K.D., Granghelli, C.A., de Pinho, A.C.C., Queiroz, S.R.A., Fernandes, A.M., de Souza, R.L.M., Moro, M.E.G., de Andrade Bordin, R. and de Albuquerque, R. (2018) 'Cleaning and disinfection programs against Campylobacter jejuni for broiler chickens: Productive performance, microbiological assessment and characterization', *Poultry Science*, 96(9), pp. 3188–3198. Available at: https://doi.org/10.3382/ps/pex153.

Chaloner, G., Wigley, P., Humphrey, S., Kemmett, K., Lacharme-Lora, L., Humphrey, T. and Williams, N. (2014) 'Dynamics of dual infection with Campylobacter jejuni strains in chickens reveals distinct strain-to-strain variation in infection ecology', *Applied and Environmental Microbiology*, 80(20), pp. 6366–6372. Available at: https://doi.org/10.1128/AEM.01901-14.

Chaveerach, P., Huurne, H.M., Lipman, L.J. and Knapen, F. Van (2003) 'Survival and Resuscitation of Ten Strains of Campylobacter jejuni and Campylobacter coli under Acid Conditions', *American Society for Microbiology*, 69(1), pp. 711–714. Available at: https://doi.org/10.1128/AEM.69.1.711.

Chen, T., Small, D.A., McDermott, M.K., Bentley, W.E. and Payne, G.F. (2003) 'Enzymatic methods for in situ cell entrapment and cell release', *Biomacromolecules*, 4(6), pp. 1558–1563. Available at: https://doi.org/10.1021/bm034145k.

Chinivasagam, H.N., Tran, T., Maddock, L., Gale, A. and Blackall, P.J. (2009) 'Mechanically ventilated broiler sheds: A possible source of aerosolized Salmonella, Campylobacter, and Escherichia coli', *Applied and Environmental Microbiology*, 75(23), pp. 7417–7425. Available at: https://doi.org/10.1128/AEM.01380-09.

Chintoan-Uta, C., Wisedchanwet, T., Glendinning, L., Bremner, A., Psifidi, A., Vervelde, L., Watson, K., Watson, M. and Stevens, M.P. (2020) 'Role of cecal microbiota in the differential resistance of inbred chicken lines to colonization by campylobacter jejuni', *Applied and Environmental Microbiology*, 86(7). Available at: https://doi.org/10.1128/AEM.02607-19.

Choi, K.Y., Lee, T.K. and Sul, W.J. (2015) 'Metagenomic analysis of chicken gut microbiota for improving metabolism and health of chickens - A review', *Asian-Australasian Journal of Animal Sciences*, 28(9), pp. 1217–1225. Available at: https://doi.org/10.5713/ajas.15.0026.

Chowdhury, S., Sandberg, M., Themudo, G.E. and Ersbøll, A.K. (2012) 'The effect of presence of infected neighbouring farms for the Campylobacter infection status in Danish broiler farms', *Spatial and Spatio-temporal Epidemiology*, 3(4), pp. 311–322. Available at: https://doi.org/10.1016/j.sste.2012.06.001.

Chowdhury, S., Themudo, G.E., Sandberg, M. and Ersboll, A.K. (2013) 'Spatio-temporal patterns of Campylobacter colonization in Danish broilers', *Epidemiology and Infection*, 141(5), pp. 997–1008. Available at: https://doi.org/10.1017/S0950268812001446.

Christensen, J.E., Pacheco, S.A. and Konkel, M.E. (2009) 'Identification of a Campylobacter jejuni-secreted protein required for maximal invasion of host cells', *Molecular Microbiology*, 73(4), pp. 650–662. Available at: https://doi.org/10.1111/j.1365-2958.2009.06797.x.

Clarke, K.R. (1993) 'Non-parametric multivariate analyses of changes in community structure', *Australian Journal of Ecology*, 18(1), pp. 117–143. Available at: https://doi.org/10.1111/j.1442-9993.1993.tb00438.x.

Clavijo, V. and Flórez, M.J.V. (2018) 'The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review', *Poultry Science*, 97(3), pp. 1006–1021. Available at: https://doi.org/10.3382/ps/pex359.

Cody, A.J., Colles, F.M., Sheppard, S.K. and Maiden, M.C.J. (2010) 'Where does campylobacter come from? A molecular odyssey', *Advances in Experimental Medicine and Biology*, 659, pp. 47–56. Available at: https://doi.org/10.1007/978-1-4419-0981-7_4.

Connerton, P.L., Richards, P.J., Lafontaine, G.M., O'Kane, P.M., Ghaffar, N., Cummings, N.J., Smith, D.L., Fish, N.M. and Connerton, I.F. (2018) 'The effect of the timing of exposure to Campylobacter jejuni on the gut microbiome and inflammatory responses of broiler chickens', *Microbiome*, 6, p. 88. Available at: https://doi.org/10.1186/s40168-018-0477-5.

Cook, K.L. and Bolster, C.H. (2007) 'Survival of Campylobacter jejuni and Escherichia coli in groundwater during prolonged starvation at low temperatures', *Journal of Applied Microbiology*, 103(3), pp. 573–583. Available at: https://doi.org/10.1111/j.1365-2672.2006.03285.x.

Cox, D.R. (1972) 'Regression Models and Life-Tables', *Journal of the Royal Statistical Society: Series B (Methodological)*, 34(2), pp. 187–202. Available at: https://doi.org/10.1111/j.2517-6161.1972.tb00899.x.

Crawshaw, T.R., Chanter, J.I., Young, S.C.L., Cawthraw, S., Whatmore, A.M., Koylass, M.S., Vidal, A.B., Salguero, F.J. and Irvine, R.M. (2015) 'Isolation of a novel thermophilic Campylobacter from cases of spotty liver disease in laying hens and experimental reproduction of infection and microscopic pathology', *Veterinary Microbiology*, 179(3–4), pp. 315–321. Available at: https://doi.org/10.1016/j.vetmic.2015.06.008.

Datekin, M., Karaca, C. and Yildiz, Y. (2009) 'Long axis heat distribution in a tunnel-ventilated broiler house equipped with an evaporative pad cooling system', *Animal Production Science*, 49(12), pp. 1125–1131. Available at: https://doi.org/10.1071/EA08077.

David, J.M., Pollari, F., Pintar, K.D.M., Nesbitt, A., Butler, A.J. and Ravel, A. (2017) 'Do contamination of and exposure to chicken meat and water drive the temporal dynamics of Campylobacter cases?', *Epidemiology and Infection*, 145(15), pp. 1391–3203. Available at: https://doi.org/10.1017/S0950268817002199.

Dekeyser, P., Gossuin-Detrain, M., Butzler, J.P. and Sternon, J. (1972) 'Acute enteritis due to related vibrio: First positive stool cultures', *Journal of Infectious Diseases*, 125(4), pp. 390–392. Available at: https://doi.org/10.1093/infdis/125.4.390.

Department for Environment Food and Rural Affairs (2018) *Code of practice for the welfare of meat chickens and meat breeding chickens*, *National Archives*. DEFRA. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/694013/meat-chicken-code-march2018.pdf.

Department for Environment Food and Rural Affairs (2022) *Latest poultry and poultry meat statistics*, *National statistics*. Available at: https://www.gov.uk/government/statistics/poultry-and-poultry-meat-statistics (Accessed: 12 May 2022).

Van Deun, K., Pasmans, F., Ducatelle, R., Flahou, B., Vissenberg, K., Martel, A., Van den Broeck, W., Van Immerseel, F. and Haesebrouck, F. (2008) 'Colonization strategy of Campylobacter jejuni results in persistent infection of the chicken gut', *Veterinary Microbiology*, 130(3–4), pp. 285–297. Available at: https://doi.org/10.1016/j.vetmic.2007.11.027.

Dingle, K.E., Colles, F.M., Wareing, D.R.A.A., Ure, R., Fox, A.J., Bolton, F.E., Bootsma, H.J., Willems, R.J.L., Urwin, R. and Maiden, M.C.J. (2001) 'Multilocus Sequence Typing System for Campylobacter jejuni', *Journal of Clinical Microbiology*, 39(1), pp. 14–23. Available at: https://doi.org/10.1128/JCM.39.1.14.

Dixon, P. (2003) 'VEGAN, a package of R functions for community ecology', *Journal of Vegetation Science*, 14(6), pp. 927–930. Available at: https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

Duarte, A., Botteldoorn, N., Miller, W.G., Coucke, W., Martiny, D., Hallin, M., Seliwiorstow, T., De Zutter, L., Uyttendaele, M., Vandenberg, O. and Dierick, K. (2019) 'Relation between broiler and human Campylobacter jejuni strains isolated in Belgium from 2011 to 2013', *Journal of Applied Microbiology*, 126(1), pp. 277–287. Available at: https://doi.org/10.1111/jam.14132.

Dunlop, M.W. and McAuley, J. (2021) 'Direct surface wetting sprinkler system to reduce the use of evaporative cooling pads in meat chicken production: indoor thermal environment, water usage, litter moisture content, live market weights, and mortalities', *Poultry Science*, 100(5), p. 101078. Available at: https://doi.org/10.1016/j.psj.2021.101078.

Dunlop, M.W., Moss, A.F., Groves, P.J., Wilkinson, S.J., Stuetz, R.M. and Selle, P.H. (2016) 'The multidimensional causal factors of "wet litter" in chicken-meat production', *Science of the Total Environment*, 562, pp. 766–776. Available at: https://doi.org/10.1016/j.scitotenv.2016.03.147.

Duong, T. and Konkel, M.E. (2009) 'Comparative studies of Campylobacter jejuni genomic diversity reveal the importance of core and dispensable genes in the biology of this enigmatic food-borne pathogen', *Current Opinion in Biotechnology*, 20(2), pp. 158–165. Available at: https://doi.org/10.1016/j.copbio.2009.03.004.

EFSA (2010) 'Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses in the EU, 2008 - Part A: Campylobacter and Salmonella prevalence estimates', *EFSA Journal*, 8(3). Available at: https://doi.org/10.2903/j.efsa.2010.1503.

EFSA (2014) *Campylobacter Fact Sheet*. Parma, Italy: EFSA. Available at: https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/factsheetcampylobacter.pdf.

EFSA and ECDC (2018) 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017', *EFSA Journal*, 16(12). Available at: https://doi.org/10.2903/j.efsa.2018.5500.

EFSA and ECDC (2019) ‘The European Union One Health 2018 Zoonoses Report’, *EFSA Journal*, 17(12). Available at: https://doi.org/10.2903/j.efsa.2019.5926.

EFSA and ECDC (2021) ‘The European Union One Health 2019 Zoonoses Report’, *EFSA Journal*, 19(2). Available at: https://doi.org/10.2903/j.efsa.2021.6406.

Ellis-Iversen, J., Jorgensen, F., Bull, S., Powell, L., Cook, A.J. and Humphrey, T.J. (2009) ‘Risk factors for Campylobacter colonisation during rearing of broiler flocks in Great Britain’, *Preventive Veterinary Medicine*, 89(3–4), pp. 178–184. Available at: https://doi.org/10.1016/j.prevetmed.2009.02.004.

Ellis-Iversen, J., Ridley, A., Morris, V., Sowa, A., Harris, J., Atterbury, R., Sparks, N. and Allen, V. (2012) ‘Persistent environmental reservoirs on farms as risk factors for Campylobacter in commercial poultry’, *Epidemiology and Infection*, 140(5), pp. 916–924. Available at: https://doi.org/10.1017/S095026881100118X.

Encyclopedia Of Life (2020) *Campylobacter Names*. Available at: https://eol.org/pages/83126/names (Accessed: 3 April 2020).

Escherich, T. (1886) ‘Über das Vorkommen von Vibrionen im Darmkanal und den Stuhlgängen der Säuglinge’, *Münchener Medizinische Wochenschrift*, 33, pp. 815–817.

European Commission (2017) *Overview report on the mitigation measures in place for Campylobacter spp. in poultry*. Available at: https://doi.org/10.2875/1113.

European Union (2017) ‘Commission Regulation (EU) 2017/1495’, *Official Journal*, L218(60), pp. 1–6.

Evans, S.J. and Sayers, A.R. (2000) ‘A longitudinal study of campylobacter infection of broiler flocks in Great Britain’, *Preventive Veterinary Medicine*, 46(3), pp. 209–223. Available at: https://doi.org/10.1016/S0167-5877(00)00143-4.

Fan, Y., Forgie, A.J., Ju, T., Marcolla, C., Inglis, T., McMullen, L.M., Willing, B.P. and Korver, D.R. (2021) ‘The use of disinfectant in barn cleaning alters microbial composition and increases carriage of Campylobacter jejuni in broiler chickens’,

bioRxiv, p. 2021.10.29.466552. Available at: https://doi.org/10.1101/2021.10.29.466552.

Fernandes, A.M., Balasegaram, S., Willis, C., Wimalarathna, H.M.L., Maiden, M.C. and McCarthy, N.D. (2015) 'Partial Failure of Milk Pasteurization as a Risk for the Transmission of Campylobacter From Cattle to Humans', *Clinical Infectious Diseases*, 61(6), pp. 903–909. Available at: https://doi.org/10.1093/cid/civ431.

Ferrero, R.L. and Lee, A. (1988) 'Motility of Campylobacter jejuni in a viscous environment: comparison with conventional rod-shaped bacteria.', *Journal of general microbiology*, 134(1), pp. 53–59. Available at: https://doi.org/10.1099/00221287-134-1-53.

Food And Drink Business Europe (2017) *SonoSteam Technology Tackling Campylobacter in Poultry*. Available at: https://www.fdbusiness.com/sonosteam-technology-tackling-campylobacter-in-poultry/ (Accessed: 24 September 2023).

Food Standards Agency (2015) *Campylobacter contamination in fresh whole UK-produced chilled chickens at retail: October – December 2015*. Available at: https://www.food.gov.uk/sites/default/files/campylobacter-survey-report-oct-dec-2015.pdf.

Fortomaris, P., Argyriadou, A., Papaioannou, N. and Franzo, G. (2022) 'Effects of a Multi-genus Synbiotic (PoultryStar sol) on Gut Health and Performance of Broiler Breeders', *Journal of World's Poultry Research*, 12(4), pp. 212–229.

Frosth, S., Karlsson-Lindsjö, O., Niazi, A., Fernström, L.L. and Hansson, I. (2020) 'Identification of transmission routes of campylobacter and on-farm measures to reduce campylobacter in chicken', *Pathogens*, 9(5), pp. 1–17. Available at: https://doi.org/10.3390/pathogens9050363.

Fujikoshi, Y. (1993) 'Two-way ANOVA models with unbalanced data', *Discrete Mathematics*, 116(1–3), pp. 315–334. Available at: https://doi.org/10.1016/0012-365X(93)90410-U.

Garrity, G.M., Bell, J.A. and Lilburn, T. (2015) ' Helicobacteraceae fam. nov. ', *Bergey's Manual of Systematics of Archaea and Bacteria*, pp. 1–1. Available at: https://doi.org/10.1002/9781118960608.fbm00211.

Ghareeb, K., Awad, W.A., Mohnl, M., Schatzmayr, G. and Böhm, J. (2013) 'Control strategies for Campylobacter infection in poultry production', *World's Poultry Science Journal*, 69(1), pp. 57–76. Available at: https://doi.org/10.1017/S0043933913000068.

Gibbens, J.C., Pascoe, S.J.S., Evans, S.J., Davies, R.H. and Sayers, A.R. (2001) 'A trial of biosecurity as a means to control Campylobacter infection of broiler chickens', *Preventive Veterinary Medicine*, 48(2), pp. 85–99. Available at: https://doi.org/10.1016/S0167-5877(00)00189-6.

Goddard, A.D., Arnold, M.E., Allen, V.M. and Snary, E.L. (2014) 'Estimating the time at which commercial broiler flocks in Great Britain become infected with Campylobacter: A Bayesian approach', *Epidemiology and Infection*, 142(9), pp. 1884–1892. Available at: https://doi.org/10.1017/S0950268813002926.

Godschalk, P.C.R., Kuijf, M.L., Li, J., St. Michael, F., Ang, C.W., Jacobs, B.C., Karwaski, M.F., Brochu, D., Moterassed, A., Endtz, H.P., Van Belkum, A. and Gilbert, M. (2007) 'Structural characterization of Campylobacter jejuni lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndromes', *Infection and Immunity*, 75(3), pp. 1245–1254. Available at: https://doi.org/10.1128/IAI.00872-06.

Gölz, G., Kittler, S., Malakauskas, M. and Alter, T. (2018) 'Survival of Campylobacter in the Food Chain and the Environment', *Current Clinical Microbiology Reports*, 5(2), pp. 126–134. Available at: https://doi.org/10.1007/s40588-018-0092-z.

Google (2020) *Google Maps*. Available at: https://www.google.com/maps (Accessed: 22 June 2020).

Griekspoor, P., Colles, F.M., McCarthy, N.D., Hansbro, P.M., Ashhurst-Smith, C., Olsen, B., Hasselquist, D., Maiden, M.C.J. and Waldenstr??m, J. (2013) 'Marked host specificity and lack of phylogeographic population structure of Campylobacter jejuni in

wild birds', *Molecular Ecology*, 22(5), pp. 1463–1472. Available at: https://doi.org/10.1111/mec.12144.

Gundogdu, O., Bentley, S.D., Holden, M.T., Parkhill, J., Dorrell, N. and Wren, B.W. (2007) 'Re-annotation and re-analysis of the Campylobacter jejuni NCTC11168 genome sequence', *BMC Genomics*, 8, pp. 1–8. Available at: https://doi.org/10.1186/1471-2164-8-162.

Guyard-Nicodème, M., Keita, A., Quesne, S., Amelot, M., Poezevara, T., Le Berre, B., Sánchez, J., Vesseur, P., Martín, A., Medel, P. and Chemaly, M. (2016) 'Efficacy of feed additives against Campylobacter in live broilers during the entire rearing period', *Poultry Science*, 95(2), pp. 298–305. Available at: https://doi.org/10.3382/ps/pev303.

Hald, B., Rattenborg, E. and Madsen, M. (2001) 'Role of batch depletion of broiler houses on the occurrence of Campylobacter spp. in chicken flocks', *Letters in Applied Microbiology*, 32(4), pp. 253–256. Available at: https://doi.org/10.1046/j.1472-765X.2001.00896.x.

Hald, B., Skov, M.N., Nielsen, E.M., Rahbek, C., Madsen, J.J., Wainø, M., Chriél, M., Nordentoft, S., Baggesen, D.L. and Madsen, M. (2016) 'Campylobacter jejuni and Campylobacter coli in wild birds on Danish livestock farms', *Acta Veterinaria Scandinavica*, 58, p. 11. Available at: https://doi.org/10.1186/s13028-016-0192-9.

Hald, B., Skovgård, H., Bang, D.D., Pedersen, K., Dybdahl, J., Jespersen, J.B. and Madsen, M. (2004) 'Flies and Campylobacter infection of broiler flocks', *Emerging Infectious Diseases*, 10(8), pp. 1490–1492. Available at: https://doi.org/10.3201/eid1008.040129.

Hannu, T., Mattila, L., Rautelin, H., Pelkonen, P., Lahdenne, P., Siitonen, A. and Leirisalo-Repo, M. (2002) 'Campylobacter-triggered reactive arthritis: a population-based study', *Rheumatology*, 41(3), pp. 312–318. Available at: https://doi.org/10.1093/rheumatology/41.3.312.

Hansson, I., Engvall, E.O., Vågsholm, I. and Nyman, A. (2010) 'Risk factors associated with the presence of Campylobacter-positive broiler flocks in Sweden.',

Preventive Veterinary Medicine, 96(1–2), pp. 114–121. Available at: https://doi.org/10.1016/j.prevetmed.2010.05.007.

Hansson, I., Sandberg, M., Habib, I., Lowman, R. and Engvall, E. (2016) 'Knowledge gaps in control of Campylobacter for prevention of campylobacteriosis', *Transboundary and Emerging Diseases*, 65(Suppl. 1), pp. 30–48. Available at: https://doi.org/10.1111/tbed.12870.

Hansson, I., Vågsholm, I., Svensson, L. and Olsson Engvall, E. (2007) 'Correlations between Campylobacter spp. prevalence in the environment and broiler flocks', *Journal of Applied Microbiology*, 103(3), pp. 640–649. Available at: https://doi.org/10.1111/j.1365-2672.2007.03291.x.

Hartnack, S., Doherr, M.G., Alter, T., Toutounian-Mashad, K. and Greiner, M. (2009) 'Campylobacter monitoring in German broiler flocks: An explorative time series analysis', *Zoonoses and Public Health*, 56(3), pp. 117–128. Available at: https://doi.org/10.1111/j.1863-2378.2008.01184.x.

Hastie, T. and Tibshirani, R. (1986) 'Generalized additive models', *Statistical Science*, 1(3), pp. 297–310. Available at: https://doi.org/10.1214/ss/1177013604.

Havelaar, A.H., Mangen, M.J.J., De Koeijer, A.A., Bogaardt, M.J., Evers, E.G., Jacobs-Reitsma, W.F., Van Pelt, W., Wagenaar, J.A., De Wit, G.A., Van Der Zee, H. and Nauta, M.J. (2007) 'Effectiveness and efficiency of controlling Campylobacter on broiler chicken meat', *Risk Analysis*, 27(4), pp. 831–844. Available at: https://doi.org/10.1111/j.1539-6924.2007.00926.x.

Hazeleger, W.C., Jacobs-Reitsma, W.F., Lina, P.H.C., De Boer, A.G., Bosch, T., Van Hoek, A.H.A.M. and Beumer, R.R. (2018) 'Wild, insectivorous bats might be carriers of Campylobacter spp.', *PLoS ONE*, 13(1), p. e190647. Available at: https://doi.org/10.1371/journal.pone.0190647.

He, Y., Maltecca, C. and Tiezzi, F. (2021) 'Potential use of gut microbiota composition as a biomarker of heat stress in monogastric species: A review', *Animals*, 11(6). Available at: https://doi.org/10.3390/ani11061833.

Heimesaat, M.M., Weschka, D., Mousavi, S. and Bereswill, S. (2021) 'Treatment with the probiotic product Aviguard alleviates inflammatory responses during Campylobacter jejuni-induced acute enterocolitis in mice', *International Journal of Molecular Sciences*, 22(13). Available at: https://doi.org/10.3390/ijms22136683.

Hendrixson, D.R. and DiRita, V.J. (2004) 'Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract', *Molecular Microbiology*, 52(2), pp. 471–484. Available at: https://doi.org/10.1111/j.1365-2958.2004.03988.x.

Hermans, D., Van Deun, K., Martel, A., Van Immerseel, F., Messens, W., Heyndrickx, M., Haesebrouck, F. and Pasmans, F. (2011) 'Colonization factors of Campylobacter jejuni in the chicken gut', *Veterinary Research*, 42, p. 82. Available at: https://doi.org/10.1186/1297-9716-42-82.

Hermans, P.G., Fradkin, D., Muchnik, I.B. and Morgan, K.L. (2006) 'Prevalence of wet litter and the associated risk factors in broiler flocks in the United Kingdom', *Veterinary Record*, 158(18), pp. 615–622. Available at: https://doi.org/10.1136/vr.158.18.615.

Higham, L.E., Scott, C., Akehurst, K., Dring, D., Parnham, A., Waterman, M. and Bright, A. (2018) 'Effects of financial incentives and cessation of thinning on prevalence of Campylobacter: a longitudinal monitoring study on commercial broiler farms in the UK', *Veterinary Record*, p. 104823. Available at: https://doi.org/10.1136/vr.104823.

Hoorfar, J., Koláčková, I., Johannessen, G.S., Garofolo, G., Marotta, F., Wieczorek, K., Osek, J., Torp, M., Spilsberg, B., Sekse, C., Thornval, N.R. and Karpíšková, R. (2020) 'A Multicenter Proposal for a Fast Tool To Screen Biosecure Chicken Flocks for the Foodborne Pathogen Campylobacter', *Applied and environmental microbiology*, 86(20). Available at: https://doi.org/10.1128/AEM.01051-20.

Hornik, K. (2012) 'The Comprehensive R Archive Network', *WIREs Computational Statistics*, 4(4), pp. 394–398. Available at: https://doi.org/10.1002/wics.1212.

Horrocks, S.M., Anderson, R.C., Nisbet, D.J. and Ricke, S.C. (2009) 'Incidence and ecology of Campylobacter jejuni and coli in animals', *Anaerobe*, 15(1–2), pp. 18–25. Available at: https://doi.org/10.1016/j.anaerobe.2008.09.001.

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., Humphrey, T. and Wigley, P. (2014) 'Campylobacter jejuni is not merely a commensal in commercial broiler chickens and affects bird welfare', *mBio*, 5(4), pp. e01364-14. Available at: https://doi.org/10.1128/mBio.01364-14.

Humphrey, T. (2006) 'Are happy chickens safer chickens? Poultry welfare and disease susceptibility', *British Poultry Science*, 47(4), pp. 379–391. Available at: https://doi.org/10.1080/00071660600829084.

Ingresa-Capaccioni, S., González-Bodí, S., Jiménez-Trigos, E., Marco-Jiménez, F., Catalá, P., Vega, S. and Marin, C. (2014) 'Comparison of different sampling types across the rearing period in broiler flocks for isolation of Campylobacter spp.', *Poultry Science*, 94(4), pp. 766–771. Available at: https://doi.org/10.3382/ps/pev023.

Ishihara, K., Chuma, T., Andoh, M., Yamashita, M., Asakura, H. and Yamamoto, S. (2017) 'Effect of climatic elements on Campylobacter colonization in broiler flocks reared in southern Japan from 2008 to 2012', *Poultry science*, 96(4), pp. 931–937. Available at: https://doi.org/10.3382/ps/pew354.

Izar-Tenorio, J., Jaramillo, P., Griffin, W.M. and Small, M. (2020) 'Impacts of projected climate change scenarios on heating and cooling demand for industrial broiler chicken farming in the Eastern U.S', *Journal of Cleaner Production*, 255. Available at: https://doi.org/10.1016/j.jclepro.2020.120306.

Jennings, J.L., Sait, L.C., Perrett, C.A., Foster, C., Williams, L.K., Humphrey, T.J. and Cogan, T.A. (2011) 'Campylobacter jejuni is associated with, but not sufficient to cause vibrionic hepatitis in chickens', *Veterinary Microbiology*, 149(1–2), pp. 193–199. Available at: https://doi.org/10.1016/j.vetmic.2010.11.005.

Jiang, S., Yan, F.F., Hu, J.Y., Mohammed, A. and Cheng, H.W. (2021) 'Bacillus subtilis-based probiotic improves skeletal health and immunity in broiler chickens

exposed to heat stress', *Animals*, 11(6), pp. 1–21. Available at: https://doi.org/10.3390/ani11061494.

Johannessen, G.S., Garofolo, G., Di Serafino, G., Koláčková, I., Karpíšková, R., Wieczorek, K., Osek, J., Christensen, J., Torp, M. and Hoorfar, J. (2020) 'Campylobacter in chicken – Critical parameters for international, multicentre evaluation of air sampling and detection methods', *Food Microbiology*, 90(November 2019), p. 103455. Available at: https://doi.org/10.1016/j.fm.2020.103455.

Johansen, C.H., Bjerrum, L., Finster, K. and Pedersen, K. (2006) 'Effects of a Campylobacter jejuni infection on the development of the intestinal microflora of broiler chickens', *Poult Sci*, 85(4), pp. 579–587. Available at: https://doi.org/10.1093/ps/85.4.579.

John, D.A., Williams, L.K., Kanamarlapudi, V., Humphrey, T.J. and Wilkinson, T.S. (2017) 'The bacterial species Campylobacter jejuni Induce diverse innate immune responses in human and avian intestinal epithelial cells', *Frontiers in Microbiology*, 8, p. 1840. Available at: https://doi.org/10.3389/fmicb.2017.01840.

Jones, M.A., Marston, K.L., Woodall, C.A., Maskell, D.J., Linton, D., Karlyshev, A. V., Dorrell, N., Wren, B.W. and Barrow, P.A. (2004) 'Adaptation of Campylobacter jejuni NCTC11168 to high-level colonization of the avian gastrointestinal tract', *Infection and Immunity*, 72(7), pp. 3769–3776. Available at: https://doi.org/10.1128/IAI.72.7.3769-3776.2004.

Jones, N.R., Millman, C., van der Es, M., Hukelova, M., Forbes, K.J., Glover, C., Haldenby, S., Hunter, P.R., Jackson, K., O'Brien, S.J., Rigby, D., Strachan, N.J.C., Williams, N., Lake, I.R., O'Brien, S., Christley, R., Hertz-Fowler, C., Wigley, P., Winstanley, C., Diggle, P., Lake, I., Hiscock, K., Hunter, P., Forbes, K., Strachan, N., Griffith, R., Cross, P., Rushton, S., Humphrey, T., Bennett, M., Howard, D. and Wren, B. (2017) 'Novel sampling method for assessing human-pathogen interactions in the natural environment using boot socks and citizen scientists, with application to Campylobacter seasonality', *Applied and Environmental Microbiology*, 83(14), pp. e00162-17. Available at: https://doi.org/10.1128/AEM.00162-17.

Jonsson, M.E., Chriél, M., Norström, M. and Hofshagen, M. (2012) 'Effect of climate and farm environment on Campylobacter spp. colonisation in Norwegian broiler flocks', *Preventive Veterinary Medicine*, 107(1–2), pp. 95–104. Available at: https://doi.org/10.1016/j.prevetmed.2012.05.002.

Jonsson, M.E., Tafjord Heier, B., Norstrom, M. and Hofshagen, M. (2010) 'Analysis of simultaneous space-time clusters of Campylobacter spp. in humans and in broiler flocks using a multiple dataset approach', *International Journal of Health Geographics*, 9(1), p. 48. Available at: https://doi.org/10.1186/1476-072X-9-48.

Jore, S., Viljugrein, H., Brun, E., Heier, B.T., Borck, B., Ethelberg, S., Hakkinen, M., Kuusi, M., Reiersen, J., Hansson, I., Olsson Engvall, E., Løfdahl, M., Wagenaar, J.A., van Pelt, W. and Hofshagen, M. (2010) 'Trends in Campylobacter incidence in broilers and humans in six European countries, 1997-2007', *Preventive Veterinary Medicine*, 93, pp. 33–41. Available at: https://doi.org/10.1016/j.prevetmed.2009.09.015.

Jorgensen, F., Charlett, A., Swift, C., Corcionivoschi, N. and Nicola, C. (2019) *A microbiological survey of Campylobacter contamination in fresh whole UK-produced chilled chickens at retail sale*.

Jorgensen, F., Charlett, A., Swift, C., Painset, A. and Corcionivoschi, N. (2021) *A survey of the levels of Campylobacter spp. contamination and prevalence of selected antimicrobial resistance determinants in fresh whole UK-produced chilled chickens at retail sale (non-major retailers)*, *UK- wide survery of campylobacter contamination on fresh chickens at retail.* Available at: https://doi.org/10.46756/sci.fsa.xls618.

Jorgensen, F., Ellis-Iversen, J., Rushton, S., Bull, S.A., Harris, S.A., Bryan, S.J., Gonzalez, A. and Humphrey, T.J. (2011) 'Influence of season and geography on Campylobacter jejuni and C. coli subtypes in housed broiler flocks reared in Great Britain', *Applied and Environmental Microbiology*, 77(11), pp. 3741–3748. Available at: https://doi.org/10.1128/AEM.02444-10.

Kaakoush, N.O., Castaño-Rodríguez, N., Mitchell, H.M. and Man, S.M. (2015) 'Global epidemiology of Campylobacter infection', *Clinical Microbiology Reviews*, 28(3), pp. 687–720. Available at: https://doi.org/10.1128/CMR.00006-15.

Kaakoush, N.O., Sodhi, N., Chenu, J.W., Cox, J.M., Riordan, S.M. and Mitchell, H.M. (2014) 'The interplay between Campylobacter and Helicobacter species and other gastrointestinal microbiota of commercial broiler chickens', *Gut Pathogens*, 6, p. 18. Available at: https://doi.org/10.1186/1757-4749-6-18.

Kalupahana, R.S., Mughini-Gras, L., Kottawatta, S.A., Somarathne, S., Gamage, C. and Wagenaar, J.A. (2018) 'Weather correlates of Campylobacter prevalence in broilers at slaughter under tropical conditions in Sri Lanka', *Epidemiology and Infection*, 146(8), pp. 972–979. Available at: https://doi.org/10.1017/S0950268818000894.

Kennedy, M., Lichoti, J.K. and Ommeh, S.C. (2022) 'Breeding for heat stress tolerance in poultry', 21(1), pp. 49–61.

Kers, J.G., Velkers, F.C., Fischer, E.A.J., Hermes, G.D.A., Stegeman, J.A. and Smidt, H. (2018) 'Host and environmental factors affecting the intestinal microbiota in chickens', *Frontiers in Microbiology*, 9, p. 235. Available at: https://doi.org/10.3389/fmicb.2018.00235.

Ketley, J.M. (1997) 'Pathogeneis of enteric infection by Campylobacter', *Microbiology*, 143(1), pp. 5–21. Available at: https://doi.org/10.1099/00221287-143-1-5.

Klena, J.D., Parker, C.T., Knibb, K., Claire Ibbitt, J., Devane, P.M.L., Horn, S.T., Miller, W.G. and Konkel, M.E. (2004) 'Differentiation of Campylobacter coli, Campylobacter jejuni, Campylobacter lari, and Campylobacter upsaliensis by a multiplex PCR developed from the nucleotide sequence of the lipid A gene lpxA', *Journal of Clinical Microbiology*, 42(12), pp. 5549–5557. Available at: https://doi.org/10.1128/JCM.42.12.5549-5557.2004.

Kogut, M.H., Genovese, K.J., Swaggerty, C.L., He, H. and Broom, L. (2018) 'Inflammatory phenotypes in the intestine of poultry: not all inflammation is created equal', *Poultry Science*, 0(April), pp. 1–18. Available at: https://doi.org/10.3382/ps/pey087/4954697.

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson, D.E. and Cieplak, W. (1997) 'Identification and molecular cloning of a gene encoding a fibronectin- binding protein (CadF) from Campylobacter jejuni', *Molecular Microbiology*, 24(5), pp. 953–963. Available at: https://doi.org/10.1046/j.1365-2958.1997.4031771.x.

Konkel, M.E., Kim, B.J., Rivera-Amill, V. and Garvis, S.G. (2000) 'Identification of proteins required for the internalization of Campylobacter jejuni into cultured mammalian cells', *Advances in Experimental Medicine and Biology*, 473, pp. 215–224. Available at: https://doi.org/10.1007/978-1-4615-4143-1_22.

Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B. and Mickelson, J. (2004) 'Secretion of virulence proteins from Campylobacter jejuni is dependent on a functional flagellar export apparatus', *Journal of Bacteriology*, 186(11), pp. 3296–3303. Available at: https://doi.org/10.1128/JB.186.11.3296-3303.2004.

Koolman, L., Whyte, P. and Bolton, D.J. (2014) 'An investigation of broiler caecal Campylobacter counts at first and second thinning', *Journal of Applied Microbioloogy*, 117(3), pp. 876–881. Available at: https://doi.org/10.1111/jam.12580.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. and Rubel, F. (2006) 'World map of the Köppen-Geiger climate classification updated', *Meteorologische Zeitschrift*, 15(3), pp. 259–263. Available at: https://doi.org/10.1127/0941-2948/2006/0130.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. (2013) 'Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform', *Applied and Environmental Microbiology*, 79(17), pp. 5112–5120. Available at: https://doi.org/10.1128/AEM.01043-13.

Kruskal, W.H. and Wallis, W.A. (1952) 'Use of Ranks in One-Criterion Variance Analysis', *Journal of the American Statistical Association*, 47(260), pp. 583–621. Available at: https://doi.org/10.1080/01621459.1952.10483441.

Kryeziu, A.J., Mestani, N., Berisha, S. and Kamberi, M.A. (2018) 'The European performance indicators of broiler chickens as influenced by stocking density and sex',

Agronomy Research, 16(2), pp. 483–491. Available at: https://doi.org/10.15159/AR.18.040.

Kubasova, T., Kollarcikova, M., Crhanova, M., Karasova, D., Cejkova, D., Sebkova, A., Matiasovicova, J., Faldynova, M., Pokorna, A., Cizek, A. and Rychlik, I. (2019) 'Contact with adult hen affects development of caecal microbiota in newly hatched chicks', *PLoS ONE*, 14(3), pp. 1–13. Available at: https://doi.org/10.1371/journal.pone.0212446.

Kubasova, T., Kollarcikova, M., Crhanova, M., Karasova, D., Cejkova, D., Sebkova, A., Matiasovicova, J., Faldynova, M., Sisak, F., Babak, V., Pokorna, A., Cizek, A. and Rychlik, I. (2019) 'Gut anaerobes capable of chicken caecum colonisation', *Microorganisms*, 7(12), pp. 1–11. Available at: https://doi.org/10.3390/microorganisms7120597.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) 'MEGA X: Molecular evolutionary genetics analysis across computing platforms', *Molecular Biology and Evolution*, 35(6), pp. 1547–1549. Available at: https://doi.org/10.1093/molbev/msy096.

Kumar, S., Stecher, G. and Tamura, K. (2016) 'MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets', *Molecular Biology and Evolution*, 33(7), pp. 1870–1874. Available at: https://doi.org/10.1093/molbev/msw054.

Lake, I.R. (2017) 'Food-borne disease and climate change in the United Kingdom', *Environmental Health*, 16(Suppl.1), p. 117. Available at: https://doi.org/10.1186/s12940-017-0327-0.

Lake, I.R., Colón-González, F.J., Takkinen, J., Rossi, M., Sudre, B., Gomes Dias, J., Tavoschi, L., Joshi, A., Semenza, J.C. and Nichols, G. (2019) 'Exploring campylobacter seasonality across europe using the european surveillance system (TESSy), 2008 to 2016', *Eurosurveillance*, 24(13), pp. 1–12. Available at: https://doi.org/10.2807/1560-7917.ES.2019.24.13.180028.

Lamb-Rosteski, J.M., Kalischuk, L.D., Inglis, G.D. and Buret, A.G. (2008) 'Epidermal growth factor inhibits Campylobacter jejuni-induced claudin-4 disruption, loss of

epithelial barrier function, and Escherichia coli translocation', *Infection and Immunity*, 76(8), pp. 3390–3398. Available at: https://doi.org/10.1128/IAI.01698-07.

Lara, L. and Rostagno, M. (2013) 'Impact of Heat Stress on Poultry Production', *Animals*, 3(2), pp. 356–369. Available at: https://doi.org/10.3390/ani3020356.

Lawes, J.R., Vidal, A., Clifton-Hadley, F.A., Sayers, R., Rodgers, J., Snow, L., Evans, S.J. and Powell, L.F. (2012) 'Investigation of prevalence and risk factors for Campylobacter in broiler flocks at slaughter: Results from a UK survey', *Epidemiology and Infection*, 140(10), pp. 1725–1727. Available at: https://doi.org/10.1017/S0950268812000982.

Lee, M.D. and Newell, D.G. (2006) 'Campylobacter in Poultry: Filling an Ecological Niche', *Avian Diseases*, 50, pp. 1–9. Available at: https://doi.org/10.1637/7474-111605R.1.

Levy, A.J. (1946) 'A gastro-enteritis cutbreak probably due to a bovine strain of vibrio', *The Yale Journal of Biology and Medicine*, 18(1), pp. 243–258.

Liang, H.M., Chang, T.C., Lin, D.Y., Yang, K.T. and Cheng, F.Y. (2022) 'A comparative study of acute heat tolerance and meat quality in three chicken breeds', *Animal Science Journal*, 93(1), p. e13722. Available at: https://doi.org/10.1111/asj.13722.

Line, J.E. (2006) 'Influence of relative humidity on transmission of Campylobacter jejuni in broiler chickens', *Poultry Science*, 85(7), pp. 1145–1150. Available at: https://doi.org/10.1093/ps/85.7.1145.

Livak, K.J. and Schmittgen, T.D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method', *Methods*, 25(4), pp. 402–408. Available at: https://doi.org/10.1006/meth.2001.1262.

Llarena, A.K., Skjerve, E., Bjørkøy, S., Forseth, M., Winge, J., Hauge, S.J., Johannessen, G.S., Spilsberg, B. and Nagel-Alne, G.E. (2022) 'Rapid detection of Campylobacter spp. in chickens before slaughter', *Food Microbiology*, 103, p. 103949. Available at: https://doi.org/10.1016/j.fm.2021.103949.

Louis, V.R., Gillespie, I.A., O'Brien, S.J., Russek-Cohen, E., Pearson, A.D. and Colwell, R.R. (2005) 'Temperature-driven campylobacter seasonality in England and Wales', *Applied and Environmental Microbiology*, 71(1), pp. 85–92. Available at: https://doi.org/10.1128/AEM.71.1.85-92.2005.

Louwen, R., van Baarlen, P., van Vliet, A.H.M., van Belkum, A., Hays, J.P. and Endtz, H.P. (2012) 'Campylobacter bacteremia: A rare and under-reported event?', *European Journal of Microbiology and Immunology*, 2(1), pp. 76–87. Available at: https://doi.org/10.1556/EuJMI.2.2012.1.11.

Lund, M., Nordentoft, S., Pedersen, K. and Madsen, M. (2004) 'Detection of Campylobacter spp. in chicken fecal samples by real-time PCR', *Journal of Clinical Microbiology*, 42(11), pp. 5125–5132. Available at: https://doi.org/10.1128/JCM.42.11.5125-5132.2004.

MacRitchie, L.A., Hunter, C.J. and Strachan, N.J.C. (2014) 'Consumer acceptability of interventions to reduce Campylobacter in the poultry food chain', *Food Control*, 35, pp. 260–266. Available at: https://doi.org/10.1016/j.foodcont.2013.06.005.

Madden, R.H., Ball, H., Hutchison, M., Young, F. and Taylor, M. (2014) 'A quantitative comparison of sample matrices for the detection of Campylobacter spp. In broiler houses', *Romanian Biotechnological Letters*, 19(5), pp. 9785–9791.

Madden, R.H., Hutchison, M., Young, F. and Taylor, M. (2016) *Development of a rapid on-farm test for the detection of Campylobacter*. Available at: https://www.food.gov.uk/sites/default/files/fs241049afinalreport.pdf.

Maki, J.J. and Looft, T. (2018) 'Megasphaera stantonii sp. nov., a butyrate-producing bacterium isolated from the cecum of a healthy chicken', *International Journal of Systematic and Evolutionary Microbiology*, 68(11), pp. 3409–3415. Available at: https://doi.org/10.1099/ijsem.0.002991.

Man, S.M. (2011) 'The clinical importance of emerging Campylobacter species', *Nature Reviews Gastroenterology and Hepatology*, 8(12), pp. 669–685. Available at: https://doi.org/10.1038/nrgastro.2011.191.

Mandal, R.K., Jiang, T. and Kwon, Y.M. (2017) 'Essential genome of Campylobacter jejuni', *BMC genomics*, 18(1), p. 616. Available at: https://doi.org/10.1186/s12864-017-4032-8.

Manfreda, G., De Cesare, A., Bondioli, V., Stern, N.J. and Franchini, A. (2006) 'Enumeration and identity of Campylobacter spp. in Italian broilers', *Poultry Science*, 85(3), pp. 556–562. Available at: https://doi.org/10.1093/ps/85.3.556.

Mann, H.B. and Whitney, D.R. (1947) 'On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other', *The Annals of Mathematical Statistics*, 18(1), pp. 50–60. Available at: https://doi.org/10.1214/aoms/1177730491.

Mappley, L.J., La Ragione, R.M. and Woodward, M.J. (2014) 'Brachyspira and its role in avian intestinal spirochaetosis', *Veterinary Microbiology*, 168(2–4), pp. 245–260. Available at: https://doi.org/10.1016/j.vetmic.2013.11.019.

Massey, F.J. (1951) 'The Kolmogorov-Smirnov Test for Goodness of Fit', *Journal of the American Statistical Association*, 46(253), pp. 68–78. Available at: https://doi.org/10.1080/01621459.1951.10500769.

Matt, M., Nordentoft, S., Ian, K., Thomas, P., Heimo, L., Sandra, J. and Stüger, H.P. (2016) 'Estimating sensitivity and specificity of a PCR for boot socks to detect Campylobacter in broiler primary production using Bayesian latent class analysis', *Preventive Veterinary Medicine*, 128, pp. 51–57. Available at: https://doi.org/10.1016/j.prevetmed.2016.03.015.

Mazzoni, M., Zampiga, M., Clavenzani, P., Lattanzio, G., Tagliavia, C. and Sirri, F. (2022) 'Effect of chronic heat stress on gastrointestinal histology and expression of feed intake-regulatory hormones in broiler chickens', *Animal*, 16(8), p. 100600. Available at: https://doi.org/10.1016/j.animal.2022.100600.

McCarthy, N. (2001) 'Incidence of Guillain-Barre Syndrome following Infection with Campylobacter jejuni', *American Journal of Epidemiology*, 153(6), pp. 610–614. Available at: https://doi.org/10.1093/aje/153.6.610.

McCarthy, N., Taylor, E., Maiden, M., Cody, A., Jansen van Rensburg, M., Varga, M., Hedges, S., Swift, C., Jorgensen, F., Dallman, T., Painset, A. and Douglas, A. (2021) *Enhanced molecular-based (MLST/whole genome) surveillance and source attribution of Campylobacter infections in the UK*. Available at: https://doi.org/10.46756/sci.fsa.ksj135.

McDowell, S.W.J., Menzies, F.D., McBride, S.H., Oza, A.N., McKenna, J.P., Gordon, A.W. and Neill, S.D. (2008) 'Campylobacter spp. in conventional broiler flocks in Northern Ireland: Epidemiology and risk factors', *Preventive Veterinary Medicine*, 84(3–4), pp. 261–276. Available at: https://doi.org/10.1016/j.prevetmed.2007.12.010.

McFadyean, J. and Stockman, S. (1913) *Report of the Departmental Committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion. Appendix to part III, Abortion in sheep*. London.

Meade, K.G., Narciandi, F., Cahalane, S., Reiman, C., Allan, B. and O'Farrelly, C. (2009) 'Comparative in vivo infection models yield insights on early host immune response to Campylobacter in chickens.', *Immunogenetics*, 61(2), pp. 101–110. Available at: https://doi.org/10.1007/s00251-008-0346-7.

Medhanie, G.A., McEwen, S.A., Slavic, D. and Guerin, M.T. (2013) 'Brachyspira spp. and avian intestinal spirochaetosis: An epidemiological review', *World's Poultry Science Journal*, 69(3), pp. 541–552. Available at: https://doi.org/10.1017/S0043933913000561.

Meldrum, R.J., Griffiths, J.K., Smith, R.M.M. and Evans, M.R. (2005) 'The seasonality of human campylobacter infection and Campylobacter isolates from fresh, retail chicken in Wales', *Epidemiology and Infection*, 133, pp. 49–52. Available at: https://doi.org/10.1017/S0950268804003188.

MET Office (2022) *UK Climate Averages: Credenhill Observing Site*. Available at: https://www.metoffice.gov.uk/research/climate/maps-and-data/uk-climate-averages/gcq0233j7.

Meteoblue (2022) *Meteoblue*. Available at: https://www.meteoblue.com/ (Accessed: 30 December 2023).

Meunier, M., Guyard-Nicodème, M., Dory, D. and Chemaly, M. (2016) 'Control strategies against Campylobacter at the poultry production level: Biosecurity measures, feed additives and vaccination', *Journal of Applied Microbiology*, 120(5), pp. 1139–1173. Available at: https://doi.org/10.1111/jam.12986.

Meyer, D., Zeileis, A. and Hornik, K. (2006) 'The Strucplot Framework: Visualizing Multi-way Contingency Tables with vcd', *Journal of Statistical Software*, 17(3). Available at: https://doi.org/10.18637/jss.v017.i03.

Miller, C.E., Rock, J.D., Ridley, K.A., Williams, P.H. and Ketley, J.M. (2008) 'Utilization of lactoferrin-bound and transferrin-bound iron by Campylobacter jejuni', *Journal of Bacteriology*, 190(6), pp. 1900–1911. Available at: https://doi.org/10.1128/JB.01761-07.

Millman, C., Christley, R., Rigby, D., Dennis, D., O'Brien, S.J. and Williams, N. (2017) '"Catch 22": Biosecurity awareness, interpretation and practice amongst poultry catchers', *Preventive Veterinary Medicine*, 141, pp. 22–32. Available at: https://doi.org/10.1016/j.prevetmed.2017.04.002.

Molnár, A., Hess, C., Pál, L., Wágner, L., Awad, W.A., Husvéth, F., Hess, M. and Dublecz, K. (2015) 'Composition of diet modifies colonization dynamics of Campylobacter jejuni in broiler chickens', *Journal of Applied Microbiology*, 118(1), pp. 245–254. Available at: https://doi.org/10.1111/jam.12679.

Morgan, R.B., Sierra–Arguello, Y.M., Perdoncini, G., Borges, K.A., Furian, T.Q., Gomes, M.J.P., Lima, D., Salle, C.T.P., Moraes, H.L.S. and Nascimento, V.P. (2022) 'Comparison of transport crates contamination with Campylobacter spp. before and after the cleaning and disinfection procedure in broiler slaughterhouses', *Poultry Science*, 101(7), p. 101909. Available at: https://doi.org/10.1016/j.psj.2022.101909.

Morspan Ltd. (2021) *Morspan Construction Ltd. - Broiler Houses*. Available at: https://www.morspan.com/broiler-houses/ (Accessed: 4 August 2022).

Mujumdar, A. (2000) *Practical Guide to Industrial Drying*. Brossard, Quebec: Exergex Corporation.

Munir, M.T., Belloncle, C., Irle, M. and Federighi, M. (2019) 'Wood-based litter in poultry production: A review', *World's Poultry Science Journal*, 75(1), pp. 5–16. Available at: https://doi.org/10.1017/S0043933918000909.

Nachamkin, I., Yang, X.H. and Stern, N.J. (1993) 'Role of Campylobacter jejuni flagella as colonization factors for three- day-old chicks: Analysis with flagellar mutants', *Applied and Environmental Microbiology*, 59(5), pp. 1269–1273. Available at: https://doi.org/10.1128/aem.59.5.1269-1273.1993.

Nanto-Hara, F., Kikusato, M., Ohwada, S. and Toyomizu, M. (2020) 'Heat stress directly affects intestinal integrity in broiler chickens', *Journal of Poultry Science*, 57(4), pp. 284–290. Available at: https://doi.org/10.2141/jpsa.0190004.

Näther, G., Alter, T., Martin, A. and Ellerbroek, L. (2009) 'Analysis of risk factors for Campylobacter species infection in broiler flocks', *Poultry Science*, 88(6), pp. 1299–1305. Available at: https://doi.org/10.3382/ps.2008-00389.

National Archives (2017) *Commission Regulation (EU) 2017/1495*, *legislation.gov.uk*. Available at: https://www.legislation.gov.uk/eur/2017/1495 (Accessed: 12 May 2022).

National Geographic (2022) *Season*. Available at: https://education.nationalgeographic.org/resource/season (Accessed: 28 May 2022).

NCBI (1988) *National Center for Biotechnology Information*, *National Library of Medicine (US)*. Available at: https://www.ncbi.nlm.nih.gov/ (Accessed: 28 May 2022).

Newell, D.G. (2001) 'Animal models of Campylobacter jejuni colonization and disease and the lessons to be learned from similar Helicobacter pylori models', *Journal of Applied Microbiology Symposium Supplement*, 90(30), pp. 57–67. Available at: https://doi.org/10.1046/j.1365-2672.2001.01354.x.

Newell, D.G. (2002) 'The ecology of Campylobacter jejuni in avian and human hosts and in the environment', *International Journal of Infectious Diseases*, 6(Suppl. 3), pp. 3S16-3S21. Available at: https://doi.org/10.1016/S1201-9712(02)90179-7.

Newell, D.G., Elvers, K.T., Dopfer, D., Hansson, I., Jones, P., James, S., Gittins, J., Stern, N.J., Davies, R., Connerton, I., Pearson, D., Salvat, G.S. and Allen, V.M. (2011) 'Biosecurity-based interventions and strategies to reduce Campylobacter spp. on poultry farms', *Applied and Environmental Microbiology*, 77(24), pp. 8605–8614. Available at: https://doi.org/10.1128/AEM.01090-10.

Newell, D.G. and Fearnley, C. (2003) 'Sources of Campylobacter colonization in broiler chickens', *Applied and Environmental Microbiology*, pp. 4343–4351. Available at: https://doi.org/10.1128/AEM.69.8.4343-4351.2003.

Nichols, G.L., Richardson, J.F., Sheppard, S.K., Lane, C. and Sarran, C. (2012) 'Campylobacter epidemiology: A descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011', *BMJ Open*, 2, p. e001179. Available at: https://doi.org/10.1136/bmjopen-2012-001179.

Nylen, G., Dunstan, F., Palmer, S.R., Andersson, Y., Bager, F., Cowden, J., Feierl, G., Galloway, Y., Kapperud, G., Megraud, F., Molbak, K., Petersen, L.R. and Ruutu, P. (2002) 'The seasonal distribution of campylobacter infection in nine European countries and New Zealand', *Epidemiology and Infection*, 128(3), pp. 383–390. Available at: https://doi.org/10.1017/S0950268802006830.

Oakley, B.B., Vasconcelos, E.J.R., Diniz, P.P.V.P., Calloway, K.N., Richardson, E., Meinersmann, R.J., Cox, N.A. and Berrang, M.E. (2018) 'The cecal microbiome of commercial broiler chickens varies significantly by season', *Poultry Science*, p. pey214. Available at: https://doi.org/10.3382/ps/pey214.

Ohnishi, T. and Hara-Kudo, Y. (2021) 'Presence and quantification of pathogenic Arcobacter and Campylobacter species in retail meats available in Japan', *Letters in Applied Microbiology*, 73(1), pp. 81–87. Available at: https://doi.org/10.1111/lam.13478.

Olsen, K.N., Lund, M., Skov, J., Christensen, L.S. and Hoorfar, J. (2009) 'Detection of Campylobacter bacteria in air samples for continuous real-time monitoring of Campylobacter colonization in broiler flocks', *Applied and Environmental Microbiology*, 75(7), pp. 2074–2078. Available at: https://doi.org/10.1128/AEM.02182-08.

On, S.L.W., Miller, W.G., Houf, K., Fox, J.G. and Vandamme, P. (2017) 'Minimal standards for describing new species belonging to the families Campylobacteraceae and Helicobacteraceae: Campylobacter, Arcobacter, Helicobacter and Wolinella spp', *International Journal of Systematic and Evolutionary Microbiology*, 67(12), pp. 5296–5311. Available at: https://doi.org/10.1099/ijsem.0.002255.

OptiGene (2016) *OptiGene Campylobacter Detection from Boot Swab (Liquid Reaction Mix) Standard Operating Procedure (Issue 4.1)*. Horsham: OptiGene Ltd. Available at: http://www.optigene.co.uk/wp-content/uploads/2016/06/OptiGene-Campylobacter-SOP-4.1-boot-swab-liquid-reaction-mix.pdf.

Ouchi, Y., Chowdhury, V.S., Cockrem, J.F. and Bungo, T. (2021) 'Effects of Thermal Conditioning on Changes in Hepatic and Muscular Tissue Associated With Reduced Heat Production and Body Temperature in Young Chickens', *Frontiers in Veterinary Science*, 7(January), pp. 1–9. Available at: https://doi.org/10.3389/fvets.2020.610319.

Palyada, K., Threadgill, D. and Stintzi, A. (2004) 'Iron acquisition and regulation in Campylobacter jejuni', *Journal of Bacteriology*, 186(14), pp. 4714–4729. Available at: https://doi.org/10.1128/JB.186.14.4714-4729.2004.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Van Vliet, A.H.M., Whitehead, S. and Barrell, B.G. (2000) 'The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences', *Nature*, 403(6770), pp. 665–668. Available at: https://doi.org/10.1038/35001088.

Patrick, M.E., Christiansen, L.E., Wainø, M., Ethelberg, S., Madsen, H. and Wegener, H.C. (2004) 'Effects of climate on incidence of Campylobacter spp. in humans and prevalence in broiler flocks in Denmark', *Applied and Environmental Microbiology*,

70(12), pp. 7474–7480. Available at: https://doi.org/10.1128/AEM.70.12.7474-7480.2004.

Pearson, K. (1900) 'On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling', *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, 50(302), pp. 157–175. Available at: https://doi.org/10.1080/14786440009463897.

Pielsticker, C., Glünder, G., Aung, Y.H. and Rautenschlein, S. (2016) 'Colonization pattern of C. jejuni isolates of human and avian origin and differences in the induction of immune responses in chicken', *Veterinary Immunology and Immunopathology*, 169, pp. 1–9. Available at: https://doi.org/10.1016/j.vetimm.2015.11.005.

Pitter, J.G., Vokó, Z., Józwiak, A. and Berkics, A. (2018) 'Campylobacter control measures in indoor broiler chicken: Critical re-assessment of cost-utility and putative barriers to implementation', *Epidemiology and Infection*, 146(11), pp. 1433–1444. Available at: https://doi.org/10.1017/S0950268818001528.

Pollard, S. (2012) *Review of Air Quality Impacts Resulting from Particle Emissions from Poultry Farms*. Available at: https://uk-air.defra.gov.uk/assets/documents/reports/cat07/1511251444_AQ0926_Report_PM_Emissions_from_Poultry_Farms_Final_BV_AECOM_Nov_2012.pdf.

Poultry World (2015) *Cargill: SonoSteam technology to reduce Campylobacter levels*. Available at: https://www.poultryworld.net/poultry/cargill-sonosteam-technology-to-reduce-campylobacter-levels/ (Accessed: 24 September 2023).

Prachantasena, S., Charununtakorn, P., Muangnoicharoen, S., Hankla, L., Techawal, N., Chaveerach, P., Tuitemwong, P., Chokesajjawatee, N., Williams, N., Humphrey, T. and Luangtongkum, T. (2017) 'Climatic factors and prevalence of Campylobacter in commercial broiler flocks in Thailand', *Poultry Science*, 96(4), pp. 980–985. Available at: https://doi.org/10.3382/ps/pew364.

Public Health England (2015) *Year 1 of a UK-wide survey of campylobacter contamination on fresh chickens at retail (February 2014 to February 2015)*, *food.gov.uk*. Available at: https://www.food.gov.uk/other/year-1-of-a-uk-wide-survey-of-campylobacter-contamination-on-fresh-chickens-at-retail-february-2014-to-february-2015.

Public Health England (2018) 'Routine reports of foodborne illness in humans, England and Wales: weeks 22-26, 2018', 12(26), p. 5. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/740959/hpr3318_GIs.pdf.

Public Health England (2019a) *Routine reports of gastrointestinal infections in humans, England and Wales: May and June 2019*, *Health Protection Report*.

Public Health England (2019b) *Routine reports of gastrointestinal infections in humans, England and Wales: November to December, 2018*, *Health Protection Report*.

Puth, M.T., Neuhäuser, M. and Ruxton, G.D. (2015) 'Effective use of Spearman's and Kendall's correlation coefficients forassociation between two measured traits', *Animal Behaviour*, 102, pp. 77–84. Available at: https://doi.org/10.1016/j.anbehav.2015.01.010.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O. (2013) 'The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools', *Nucleic Acids Research*, 41(D1), pp. 590–596. Available at: https://doi.org/10.1093/nar/gks1219.

Quinteiro-Filho, W.M., Gomes, A.V.S., Pinheiro, M.L., Ribeiro, A., Ferraz-de-Paula, V., Astolfi-Ferreira, C.S., Ferreira, A.J.P. and Palermo-Neto, J. (2012) 'Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with Salmonella Enteritidis', *Avian Pathology*, 41(5), pp. 421–427. Available at: https://doi.org/10.1080/03079457.2012.709315.

R Core Team (2020) ‘R: A language and environment for statistical computing.’ Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.r-project.org/.

Red Tractor (2019) *Chicken Standards: Broilers and Poussin*. 4.2. London: Assured Food Standards.

Refrigerated And Frozen Foods (2015) *Faccenda Foods Implements SonoSteam Technology*. Available at: https://www.refrigeratedfrozenfood.com/articles/89550-faccenda-foods-implements-sonosteam-technology (Accessed: 24 September 2015).

Ridley, A., Morris, V., Gittins, J., Cawthraw, S., Harris, J., Edge, S. and Allen, V. (2011) ‘Potential sources of Campylobacter infection on chicken farms: Contamination and control of broiler-harvesting equipment, vehicles and personnel’, *Journal of Applied Microbiology*, 111(1), pp. 233–244. Available at: https://doi.org/10.1111/j.1365-2672.2011.05038.x.

Robyn, J., Rasschaert, G., Pasmans, F. and Heyndrickx, M. (2015) ‘Thermotolerant Campylobacter during broiler rearing: Risk factors and intervention’, *Comprehensive Reviews in Food Science and Food Safety*, 14(2), pp. 81–105. Available at: https://doi.org/10.1111/1541-4337.12124.

Rogers, D.J. and Sedda, L. (2012) ‘Statistical models for spatially explicit biological data’, *Parasitology*, 139(14), pp. 1852–1869. Available at: https://doi.org/10.1017/S0031182012001345.

Rollins, D.M. and Colwell, R.R. (1986) ‘Viable but nonculturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment’, *Applied and Environmental Microbiology*, 52(3), pp. 531–538. Available at: https://doi.org/10.1128/aem.52.3.531-538.1986.

Rosner, B.M., Schielke, A., Didelot, X., Kops, F., Breidenbach, J., Willrich, N., Gölz, G., Alter, T., Stingl, K., Josenhans, C., Suerbaum, S. and Stark, K. (2017) ‘A combined case-control and molecular source attribution study of human Campylobacter

infections in Germany, 2011-2014', *Scientific Reports*, 7, p. 5139. Available at: https://doi.org/10.1038/s41598-017-05227-x.

Rostagno, M.H. (2009) 'Can Stress in Farm Animals Increase Food Safety Risk?', *Foodborne Pathogens and Disease*, 6(7), pp. 767–776. Available at: https://doi.org/10.1089/fpd.2009.0315.

Rostagno, M.H. (2020) 'Effects of heat stress on the gut health of poultry', *Journal of Animal Science*, 98(4), pp. 1–9. Available at: https://doi.org/10.1093/jas/skaa090.

Royden, A., Wedley, A., Merga, J.Y., Rushton, S., Hald, B., Humphrey, T. and Williams, N.J. (2016) 'A role for flies (Diptera) in the transmission of Campylobacter to broilers?', *Epidemiology and Infection*, 144(15), pp. 3326–3334. Available at: https://doi.org/10.1017/S0950268816001539.

Ruff, J., Barros, T.L., Tellez, G., Blankenship, J., Lester, H., Graham, B.D., Selby, C.A.M., Vuong, C.N., Dridi, S., Greene, E.S., Hernandez-Velasco, X., Hargis, B.M. and Tellez-Isaias, G. (2020) 'Research Note: Evaluation of a heat stress model to induce gastrointestinal leakage in broiler chickens', *Poultry science*, 99(3), pp. 1687–1692. Available at: https://doi.org/10.1016/j.psj.2019.10.075.

Rushton, S.P., Humphrey, T.J., Shirley, M.D.F., Bull, S. and Jorgensen, F. (2009) 'Campylobacter in housed broiler chickens: a longitudinal study of risk factors.', *Epidemiology and infection*, 137(8), pp. 1099–1110. Available at: https://doi.org/10.1017/S095026880800188X.

Ruxton, G.D. (2006) 'The unequal variance t-test is an underused alternative to Student's t-test and the Mann-Whitney U test', *Behavioral Ecology*, 17(4), pp. 688–690. Available at: https://doi.org/10.1093/beheco/ark016.

Rychlik, I. (2020) 'Composition and Function of Chicken Gut Microbiota', *Animals*, 10(1), p. 103. Available at: https://doi.org/10.3390/ani10010103.

Sahin, O., Kassem, I.I., Shen, Z., Lin, J., Rajashekara, G. and Zhang, Q. (2015) 'Campylobacter in Poultry: Ecology and Potential Interventions', *Avian Diseases*, 59(2), pp. 185–200. Available at: https://doi.org/10.1637/11072-032315-Review.

Sahin, O., Luo, N., Huang, S. and Zhang, Q. (2003) 'Effect of Campylobacter-Specific Maternal Antibodies on Campylobacter jejuni Colonization in Young Chickens', *Applied and Environmental Microbiology*, 69(9), pp. 5372–5379. Available at: https://doi.org/10.1128/AEM.69.9.5372.

Sahin, O., Morishita, T.Y. and Zhang, Q. (2002) 'Campylobacter colonization in poultry: sources of infection and modes of transmission', *Animal Health Research Reviews*, 3(2), pp. 95–105. Available at: https://doi.org/10.1079/AHRR200244.

Saif, Y.M. (2020) *Diseases of Poultry*. 14th edn. Edited by D.E. Swayne, M. Boulianne, C.M. Logue, L.R. McDougald, V. Nair, D.L. Suarez, S. Wit, T. Grimes, D. Johnson, M. Kromm, T.Y. Prajitno, I. Rubinoff, and G. Zavala. Wiley. Available at: https://doi.org/10.1002/9781119371199.

Sakaridis, I., Ellis, R.J., Cawthraw, S.A., van Vliet, A.H.M., Stekel, D.J., Penell, J., Chambers, M., La Ragione, R.M. and Cook, A.J. (2018) 'Investigating the Association Between the Caecal Microbiomes of Broilers and Campylobacter Burden', *Frontiers in Microbiology*, 9, p. 927. Available at: https://www.frontiersin.org/article/10.3389/fmicb.2018.00927.

Sanderson, R.A., Maas, J.A., Blain, A.P., Gorton, R., Ward, J., O'Brien, S.J., Hunter, P.R. and Rushton, S.P. (2018) 'Spatio-temporal models to determine association between Campylobacter cases and environment', *International Journal of Epidemiology*, 47(1), pp. 202–216. Available at: https://doi.org/10.1093/ije/dyx217.

Sandilands, V., Whyte, F., Williams, L.K., Wilkinson, T.S., Sparks, N.H.C. and Humphrey, T.J. (2018) 'Reliably colonising broiler chickens with Campylobacter spp. using a litter-based method', *British Poultry Science*, 59(6), pp. 698–702. Available at: https://doi.org/10.1080/00071668.2018.1523538.

Sari Kovats, R., Edwards, S.J., Charron, D., Cowden, J., D'Souza, R.M., Ebi, K.L., Gauci, C., Gerner-Smidt, P., Hajat, S., Hales, S., Hernández Pezzi, G., Kriz, B., Kutsar, K., McKeown, P., Mellou, K., Menne, B., O'Brien, S., Van Pelt, W. and Schmid, H. (2005) 'Climate variability and campylobacter infection: An international study', *International*

Journal of Biometeorology, 49(4), pp. 207–214. Available at: https://doi.org/10.1007/s00484-004-0241-3.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.-A., Roy, S.L., Jones, J.L. and Griffin, P.M. (2011) 'Foodborne Illness Acquired in the United States - Major Pathogens', *Emerging Infectious Diseases*, 17(1), pp. 7–15. Available at: https://doi.org/10.3201/eid1701.P11101.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J. and Weber, C.F. (2009) 'Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities', *Applied and Environmental Microbiology*, 75(23), pp. 7537–7541. Available at: https://doi.org/10.1128/AEM.01541-09.

Sebald, M. and Véron, M. (1963) 'Teneur en bases de l'ADN et classification des vibrions.', *Annales de l'Institut Pasteur*, 105, pp. 897–910.

Sergeant, M.J., Constantinidou, C., Cogan, T.A., Bedford, M.R., Penn, C.W. and Pallen, M.J. (2014) 'Extensive microbial and functional diversity within the chicken cecal microbiome', *PLoS ONE*, 9(3), p. e91941. Available at: https://doi.org/10.1371/journal.pone.0091941.

Shannon, C.E. (1948) 'A Mathematical Theory of Communication', *Bell System Technical Journal*, 27(3), pp. 379–423. Available at: https://doi.org/10.1002/j.1538-7305.1948.tb01338.x.

Shapiro, S.S. and Wilk, M.B. (1965) 'An Analysis of Variance Test for Normality (Complete Samples)', *Biometrika*, 52(3/4), p. 591. Available at: https://doi.org/10.2307/2333709.

Sheppard, S.K., Dallas, J.F., Strachan, N.J.C., MacRae, M., McCarthy, N.D., Wilson, D.J., Gormley, F.J., Falush, D., Ogden, I.D., Maiden, M.C.J. and Forbes, K.J. (2009) 'Campylobacter Genotyping to Determine the Source of Human Infection', *Clinical*

Infectious Diseases, 48(8), pp. 1072–1078. Available at: https://doi.org/10.1086/597402.

Shi, D., Bai, L., Qu, Q., Zhou, S., Yang, M., Guo, S., Li, Q. and Liu, C. (2019) 'Impact of gut microbiota structure in heat-stressed broilers', *Poultry Science*, 98(6), pp. 2405–2413. Available at: https://doi.org/10.3382/ps/pez026.

Shreeve, J.E., Toszeghy, M., Ridley, A. and Newell, D.G. (2002) 'The carry-over of Campylobacter isolates between sequential poultry flocks', *Avian Diseases*, 46(2), pp. 378–385. Available at: https://doi.org/10.1637/0005-2086(2002)046[0378:TCOOCI]2.0.CO;2.

Sibanda, N., McKenna, A., Richmond, A., Ricke, S.C., Callaway, T., Stratakos, A.Ch., Gundogdu, O. and Corcionivoschi, N. (2018) 'A Review of the Effect of Management Practices on Campylobacter Prevalence in Poultry Farms', *Frontiers in Microbiology*, 9, p. 2002. Available at: https://doi.org/10.3389/fmicb.2018.02002.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A. and Teixeira, P. (2011) 'Campylobacter spp. As a foodborne pathogen: A review', *Frontiers in Microbiology*, 2, pp. 1–12. Available at: https://doi.org/10.3389/fmicb.2011.00200.

Skarp, C.P.A., Hänninen, M.L. and Rautelin, H.I.K. (2016) 'Campylobacteriosis: The role of poultry meat', *Clinical Microbiology and Infection*, 22(2), pp. 103–109. Available at: https://doi.org/10.1016/j.cmi.2015.11.019.

Skirrow, M.B. (1977) 'Campylobacter enteritis: a "new" disease.', *British Medical Journal*, 2(6078), pp. 9–11. Available at: https://doi.org/10.1136/bmj.2.6078.9.

Smith, C.K., Kaiser, P., Rothwell, L., Humphrey, T., Barrow, P.A. and Jones, M.A. (2005) 'Campylobacter jejuni-Induced cytokine responses in avian cells', *Infection and Immunity*, 73(4), pp. 2094–2100. Available at: https://doi.org/Doi 10.1128/Iai.73.4.2094-2100.2005.

Smith, T. and Taylor, M.S. (1919) 'Some morphological and biological characters of Spirilla (Vibrio foetus n.sp.) associated with disease of fetal membranes in cattle', *Journal of Experimental Medicine*, 4(30), pp. 299–311.

Sofka, D., Pfeifer, A., Gleiss, B., Paulsen, P. and Hilbert, F. (2015) 'Changes within the intestinal flora of broilers by colonisation with Campylobacter jejuni', *Berliner und Münchener tierärztliche Wochenschrift*, 128(3–4), pp. 104–110. Available at: https://doi.org/10.2376/0005-9366-128-104.

Sommer, H.M., Nauta, M.J. and Rosenquist, H. (2016) 'Translation of risk factor estimates into on-farm interventions and their effect on Campylobacter broiler flock prevalence', *Microbial Risk Analysis*, 2–3, pp. 27–37. Available at: https://doi.org/10.1016/j.mran.2016.06.001.

Søndergaard, M.S.R., Josefsem, M.H., Löfström, C., Christensen, L.S., Wieczorek, K., Osek, J. and Hoorfar, J. (2014) 'Low-Cost Monitoring of Campylobacter in Poultry Houses by Air Sampling and Quantitative PCR', *Journal of Food Protection*, 77(2), pp. 325–330. Available at: https://doi.org/10.4315/0362-028X.JFP-13-268.

Stahl, M., Butcher, J. and Stintzi, A. (2012) 'Nutrient acquisition and metabolism by Campylobacter jejuni.', *Frontiers in cellular and infection microbiology*, 2(February), p. 5. Available at: https://doi.org/10.3389/fcimb.2012.00005.

Stahl, M. and Stintzi, A. (2011) 'Identification of essential genes in C. jejuni genome highlights hyper-variable plasticity regions', *Functional and Integrative Genomics*, 11(2), pp. 241–257. Available at: https://doi.org/10.1007/s10142-011-0214-7.

Stern, N.J., Fedorka-Cray, P., Bailey, J.S., Cox, N.A., Craven, S.E., Hiett, K.L., Musgrove, M.T., Ladely, S., Cosby, D. and Mead, G.C. (2001) 'Distribution of Campylobacter spp. in selected U.S. Poultry production and processing operations', *Journal of Food Protection*, 64(11), pp. 1705–1710. Available at: https://doi.org/10.4315/0362-028X-64.11.1705.

Stojanov, S., Berlec, A. and Štrukelj, B. (2020) 'The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease', *Microorganisms*, 8(11), pp. 1–16. Available at: https://doi.org/10.3390/microorganisms8111715.

Strachan, N.J.C., Rotariu, O., Smith-Palmer, A., Cowden, J., Sheppard, S.K., O'Brien, S.J., Maiden, M.C.J., MacRae, M., Bessell, P.R., Matthews, L., Reid, S.W.J., Innocent, G.T., Ogden, I.D. and Forbes, K.J. (2013) 'Identifying the seasonal origins of human campylobacteriosis', *Epidemiology and Infection*, 141(6), pp. 1267–1275. Available at: https://doi.org/10.1017/S0950268812002063.

Sunkara, L.T., Achanta, M., Schreiber, N.B., Bommineni, Y.R., Dai, G., Jiang, W., Lamont, S., Lillehoj, H.S., Beker, A., Teeter, R.G. and Zhang, G. (2011) 'Butyrate Enhances Disease Resistance of Chickens by Inducing Antimicrobial Host Defense Peptide Gene Expression', *PLoS ONE*. Edited by Z. Liu, 6(11), p. e27225. Available at: https://doi.org/10.1371/journal.pone.0027225.

Thakur, S., Brake, J., Keelara, S., Zou, M. and Susick, E. (2013) 'Farm and environmental distribution of Campylobacter and Salmonella in broiler flocks', *Research in Veterinary Science*, 94, pp. 33–42. Available at: https://doi.org/10.1016/j.rvsc.2012.07.014.

Therneau, T.M. and Grambsch, P.M. (2000) *Modeling Survival Data: Extending the Cox Model*. New York, NY: Springer New York (Statistics for Biology and Health). Available at: https://doi.org/10.1007/978-1-4757-3294-8.

Thibodeau, A., Fravalo, P., Yergeau, E., Arsenault, J., Lahaye, L. and Letellier, A. (2015) 'Chicken Caecal Microbiome modifications induced by campylobacter Jejuni colonization and by a non-antibiotic feed additive', *PLoS ONE*, 10(7), p. e131978. Available at: https://doi.org/10.1371/journal.pone.0131978.

Torok, V.A., Hughes, R.J., Mikkelsen, L.L., Perez-Maldonado, R., Balding, K., MacAlpine, R., Percy, N.J. and Ophel-Keller, K. (2011) 'Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials', *Applied and Environmental Microbiology*, 77(17), pp. 5868–5878. Available at: https://doi.org/10.1128/AEM.00165-11.

Tukey, J.W. (1949) 'Comparing Individual Means in the Analysis of Variance', *Biometrics*, 5(2), pp. 99–114. Available at: https://doi.org/10.2307/3001913.

Umaraw, P., Prajapati, A., Verma, A.K., Pathak, V. and Singh, V.P. (2017) 'Control of campylobacter in poultry industry from farm to poultry processing unit: A review', *Critical Reviews in Food Science and Nutrition*, 57(4), pp. 659–665. Available at: https://doi.org/10.1080/10408398.2014.935847.

University of Kentucky (2022) *Poultry Production Manual*. Available at: https://afs.ca.uky.edu/poultry/production-manual (Accessed: 19 December 2022).

Valero-Mora, P.M. (2010) 'ggplot2: Elegant Graphics for Data Analysis', *Journal of Statistical Software*, 35(Book Review 1). Available at: https://doi.org/10.18637/jss.v035.b01.

Van, T.T.H., Elshagmani, E., Gor, M.C., Anwar, A., Scott, P.C. and Moore, R.J. (2017) 'Induction of spotty liver disease in layer hens by infection with Campylobacter hepaticus', *Veterinary Microbiology*, 199, pp. 85–90. Available at: https://doi.org/10.1016/j.vetmic.2016.12.033.

Van, T.T.H., Elshagmani, E., Gor, M.C., Scott, P.C. and Moore, R.J. (2016) 'Campylobacter hepaticus sp. nov., isolated from chickens with spotty liver disease', *International Journal of Systematic and Evolutionary Microbiology*, 66(11), pp. 4518–4524. Available at: https://doi.org/10.1099/ijsem.0.001383.

Van, T.T.H., Gor, M.C., Anwar, A., Scott, P.C. and Moore, R.J. (2017) 'Campylobacter hepaticus, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds', *Veterinary Microbiology*, 207(May), pp. 226–230. Available at: https://doi.org/10.1016/j.vetmic.2017.06.022.

Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) 'Emendation of Generic Descriptions and Proposal of Arcobacter gen . nov .', *International Journal of Systematic Bacteriology*, 41(1), pp. 88–103.

Varasteh, S., Braber, S., Akbari, P., Garssen, J. and Fink-Gremmels, J. (2015) 'Differences in susceptibility to heat stress along the chicken intestine and the protective effects of galacto- oligosaccharides', *PLoS ONE*, 10(9), pp. 1–18. Available at: https://doi.org/10.1371/journal.pone.0138975.

Verbrugghe, E., Boyen, F., Gaastra, W., Bekhuis, L., Leyman, B., Van Parys, A., Haesebrouck, F. and Pasmans, F. (2012) 'The complex interplay between stress and bacterial infections in animals', *Veterinary Microbiology*, 155(2–4), pp. 115–127. Available at: https://doi.org/10.1016/j.vetmic.2011.09.012.

Véron, M. and Chatelain, R. (1973) 'Taxonomic Study of the Genus Campylobacter Sebald and Veron and Designation of the Neotype Strain for the Type Species, Campylobacter fetus (Smith and Taylor) Sebald and Veron', *International Journal of Systematic Bacteriology*, 23(2), pp. 122–134. Available at: https://doi.org/10.1099/00207713-23-2-122.

Vidal, A.B., Rodgers, J., Arnold, M. and Clifton-Hadley, F. (2013) 'Comparison of different sampling strategies and laboratory methods for the detection of C. jejuni and C. coli from broiler flocks at primary production', *Zoonoses and Public Health*, 60(6), pp. 412–425. Available at: https://doi.org/10.1111/zph.12009.

Wagenaar, J.A., French, N.P. and Havelaar, A.H. (2013) 'Preventing campylobacter at the source: Why is it so difficult?', *Clinical Infectious Diseases*, 57(11), pp. 1600–1606. Available at: https://doi.org/10.1093/cid/cit555.

Waldenstrom, J., Broman, T., Carlsson, I., Hasselquist, D., Achterberg, R.P., Wagenaar, J.A. and Olsen, B. (2002) 'Prevalence of Campylobacter jejuni, Campylobacter lari, and Campylobacter coli in Different Ecological Guilds and Taxa of Migrating Birds', *Applied and Environmental Microbiology*, 68(12), pp. 5911–5917. Available at: https://doi.org/10.1128/AEM.68.12.5911-5917.2002.

Walker, L.J., Wallace, R.L., Smith, J.J., Graham, T., Saputra, T., Symes, S., Stylianopoulos, A., Polkinghorne, B.G., Kirk, M.D. and Glass, K. (2019) 'Prevalence of Campylobacter coli and Campylobacter jejuni in Retail Chicken, Beef, Lamb, and Pork Products in Three Australian States', *Journal of Food Protection*, 82(12), pp. 2126–2134. Available at: https://doi.org/10.4315/0362-028X.JFP-19-146.

Wassenaar, T.M. (1997) 'Toxin production by Campylobacter spp.', *Clinical Microbiology Reviews*, 10(3), pp. 466–476. Available at: https://doi.org/10.1128/cmr.10.3.466.

Weber, R., Auerbach, M., Jung, A. and Glünder, G. (2014) 'Campylobacter infections in four poultry species in respect of frequency, onset of infection and seasonality', *Berliner und Münchener tierärztliche Wochenschrift*, 127(7–8), pp. 257–266. Available at: https://doi.org/10.2376/0005-9366-127-257.

Welker, C., Faiola, N., Davis, S., Maffatore, I. and Batt, C.A. (1997) 'Bacterial retention and cleanability of plastic and wood cutting boards with commercial food service maintenance practices', *Journal of Food Protection*, 60(4), pp. 407–413. Available at: https://doi.org/10.4315/0362-028X-60.4.407.

Wheeler, W.E. and Borchers, J. (1961) 'Vibrionic Enteritis in Infants', *American Journal of Diseases of Children*, 101, pp. 60–66. Available at: https://doi.org/10.1001/archpedi.1961.04020020062010.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag.

Wigley, P. (2015) 'Blurred Lines: Pathogens, Commensals, and the Healthy Gut', *Frontiers in Veterinary Science*, 2(October), pp. 10–13. Available at: https://doi.org/10.3389/fvets.2015.00040.

Wilcoxon, F. (1945) 'Individual Comparisons by Ranking Methods', *Biometrics Bulletin*, 1(6), p. 80. Available at: https://doi.org/10.2307/3001968.

Williams, L.K., Sait, L.C., Cogan, T.A., Jørgensen, F., Grogono-Thomas, R. and Humphrey, T.J. (2012) 'Enrichment culture can bias the isolation of Campylobacter subtypes', *Epidemiology and Infection*, 140(7), pp. 1227–1235. Available at: https://doi.org/10.1017/S0950268811001877.

Williams, L.K., Sait, L.C., Trantham, E.K., Cogan, T. a and Humphrey, T.J. (2013) 'Campylobacter infection has different outcomes in fast- and slow-growing broiler chickens.', *Avian Diseases*, 57(17), pp. 238–241. Available at: https://doi.org/10.1637/10442-110212-Reg.1.

Wilson, D.J., Gabriel, E., Leatherbarrow, A.J.H., Cheesbrough, J., Gee, S., Bolton, E., Fox, A., Fearnhead, P., Hart, C.A. and Diggle, P.J. (2008) 'Tracing the source of

campylobacteriosis', *PLoS Genetics*, 4(9), p. e1000203. Available at: https://doi.org/10.1371/journal.pgen.1000203.

Wilson, M. and Wilson, P.J.K. (2021) 'Gastroenteritis Due to Campylobacter', in *Close Encounters of the Microbial Kind: Everything You Need to Know About Common Infections*. Cham: Springer International Publishing, pp. 439–450. Available at: https://doi.org/10.1007/978-3-030-56978-5_32.

Wood, S.N. (2011) 'Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models', *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, 73(1), pp. 3–36. Available at: https://doi.org/10.1111/j.1467-9868.2010.00749.x.

Woodcock, D.J., Krusche, P., Strachan, N.J.C., Forbes, K.J., Cohan, F.M., Méric, G. and Sheppard, S.K. (2017) 'Genomic plasticity and rapid host switching can promote the evolution of generalism: A case study in the zoonotic pathogen Campylobacter', *Scientific Reports*, 7(9650). Available at: https://doi.org/10.1038/s41598-017-09483-9.

World Medical Association (2013) 'Declaration of Helsinki', *Journal of the American Medical Association*, 310(20), p. 2191. Available at: https://doi.org/10.1001/jama.2013.281053.

Wreford, A. and Topp, C.F.E. (2020) 'Impacts of climate change on livestock and possible adaptations: A case study of the United Kingdom', *Agricultural Systems*, 178(October 2019), p. 102737. Available at: https://doi.org/10.1016/j.agsy.2019.102737.

Wright, J.A., Grant, A.J., Hurd, D., Harrison, M., Guccione, E.J., Kelly, D.J. and Maskell, D.J. (2009) 'Metabolite and transcriptome analysis of Campylobacter jejuni in vitro growth reveals a stationary-phase physiological switch', *Microbiology*, 155(1), pp. 80–94. Available at: https://doi.org/10.1099/mic.0.021790-0.

Xiao, S.-S., Mi, J.-D., Mei, L., Liang, J., Feng, K.-X., Wu, Y.-B., Liao, X.-D. and Wang, Y. (2021) 'Microbial Diversity and Community Variation in the Intestines of Layer Chickens', *Animals*, 11(3), p. 840. Available at: https://doi.org/10.3390/ani11030840.

Xu, Y., Yang, H., Zhang, L., Su, Y., Shi, D., Xiao, H. and Tian, Y. (2016) 'High-throughput sequencing technology to reveal the composition and function of cecal microbiota in Dagu chicken', *BMC Microbiology*, 16(1), pp. 1–9. Available at: https://doi.org/10.1186/s12866-016-0877-2.

Yates, F. (1934) 'Contingency Tables Involving Small Numbers and the χ^2 Test', *Supplement to the Journal of the Royal Statistical Society*, 1(2), p. 217. Available at: https://doi.org/10.2307/2983604.

Yin, C., Xia, B., Tang, S., Cao, A., Liu, L., Zhong, R., Chen, L. and Zhang, H. (2021) 'The Effect of Exogenous Bile Acids on Antioxidant Status and Gut Microbiota in Heat-Stressed Broiler Chickens', *Frontiers in Nutrition*, 8(November), pp. 1–17. Available at: https://doi.org/10.3389/fnut.2021.747136.

Young, G., Turner, S., Davies, J.K., Sundqvist, G. and Figdor, D. (2007) 'Bacterial DNA Persists for Extended Periods after Cell Death', *Journal of Endodontics*, 33(12), pp. 1417–1420. Available at: https://doi.org/10.1016/j.joen.2007.09.002.

Young, K.T., Davis, L.M. and DiRita, V.J. (2007) 'Campylobacter jejuni: Molecular biology and pathogenesis', *Nature Reviews Microbiology*, 5(9), pp. 665–679. Available at: https://doi.org/10.1038/nrmicro1718.

Yuki, N., Susuki, K., Koga, M., Nishimoto, Y., Odaka, M., Hirata, K., Taguchi, K., Miyatake, T., Furukawa, K., Kobata, T. and Yamada, M. (2004) 'Carbohydrate mimicry between human ganglioside GM1 and Campylobacter jejuni lipooligosaccharide causes Guillain-Barré syndrome', *Proceedings of the National Academy of Sciences of the United States of America*, 101(31), pp. 11404–11409. Available at: https://doi.org/10.1073/pnas.0402391101.

Zhou, Z.Y., Packialakshmi, B., Makkar, S.K., Dridi, S. and Rath, N.C. (2014) 'Effect of butyrate on immune response of a chicken macrophage cell line', *Veterinary Immunology and Immunopathology*, 162(1–2), pp. 24–32. Available at: https://doi.org/10.1016/j.vetimm.2014.09.002.

Zilbauer, M., Dorrell, N., Wren, B.W. and Bajaj-Elliott, M. (2008) 'Campylobacter jejuni-mediated disease pathogenesis: an update', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102(2), pp. 123–129. Available at: https://doi.org/10.1016/j.trstmh.2007.09.019.

Ziprin, R.L., Young, C.R., Stanker, L.H., Hume, M.E. and Konkel, M.E. (1999) 'The Absence of Cecal Colonization of Chicks by a Mutant of Campylobacter jejuni Not Expressing Bacterial Fibronectin-Binding Protein', *Avian Diseases*, 43(3), pp. 586–589.

Zweifel, C., Scheu, K.D., Keel, M., Renggli, F. and Stephan, R. (2008) 'Occurrence and genotypes of Campylobacter in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms', *International Journal of Food Microbiology*, 125(2), pp. 182–187. Available at: https://doi.org/10.1016/j.ijfoodmicro.2008.03.038.