Controlling the Public and Animal Health Threat from Campylobacter Infection in Broiler Chickens by Improving Gut Health and Reducing Inflammation

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Abstract

Campylobacter is a Gram-negative bacterium that causes disease in both humans and avian species. Poultry products present a major public health risk due to high levels of *Campylobacter* contamination which is a major cause of human bacterial gastroenteritis. Chickens are a primary reservoir for *Campylobacter* and there are no effective measures in place to inhibit flock colonisation and the extraintestinal spread of pathogenic strains. A large degree of variation is observed within the two *Campylobacter* species, *C. jejuni* and *C. coli* at both the genomic and phenotypic level. This has led to inconsistent findings when investigating the mechanisms by which *Campylobacter* spreads from the chicken gastrointestinal tract to edible tissues using *in vitro* and *in vivo* models. Feed additives are an increasingly popular alternative to antibiotic use in poultry farming; they present low risk of increasing antibiotic resistance and can be administered easily through food and/or water. The aim of this study was to determine the potential of three feed additives and four probiotic species as preventative measures for *Campylobacter* extraintestinal spread in poultry production. In chapter 3, a collection of *Campylobacter* isolates were sequenced and evaluated for their genotypic differences before being assessed in an avian and human cell line for their invasive capacity *in vitro*. Three isolates were selected based on their consistent *in vitro* invasive spectrum. In chapter 4, the selected *Campylobacter* isolates were challenged directly with feed additives to assess the impact on bacterial growth and motility. A significant reduction in *Campylobacter* growth was observed when challenged with 1.0% and 1.4% sodium butyrate over 24h. In chapter 5, human and avian cell lines treated with feed additives and exposed to *Campylobacter* isolates and focused on determining the cytotoxicity and any protective effects of the additives against transcellular invasion and cytokine production. Pre-treatment of epithelial cell monolayers did not significantly affect transcellular invasion of the bacterium. Chromium propionate significantly increased oxygen consumption in epithelial cells. Sodium butyrate at 0.6% increased epithelial cell production of inflammatory cytokines CXCLi1 and CXCLi2 . This thesis has; i) confirmed the diversity of *Campylobacter* species; ii) identified the direct inhibitory effects of feed additives on *Campylobacter* growth; iii) identified a novel mechanism of modified oxygen consumption by chromium propionate on epithelial cells; iv) identified the ability of sodium butyrate to induce CXCLi1/2 chemokines in avian epithelial cells. This work supports the growing evidence that feed additives are important alternatives for controlling *Campylobacter* in the chicken gut.

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 14/03/2023

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed [redacted]

Date 14/03/2023

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.

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The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

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I would like to dedicate this work to my Nanny Bruno. I know you would have believed in me the most, and I hope I am making you proud.

Contents

List of Figures

List of Tables

List of Abbreviations

AGPs	Antibiotic-Based Growth Promoters
AMPs	Antimicrobial Proteins
APCs	Antigen Presenting Cells
AvBD8	Avian β-defensin 8
BF	Bursa of Fabricus
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
CaCo-2	Heterogeneous human epithelial colorectal adenocarcinoma cell line
CC	Clonal Complexes
CCDA	Charcoal cefoperazone deoxycholate agar
CCV	*Campylobacter*-containing Vacuoles
CFU	Colony Forming Units
Cia	*Campylobacter* Invasion Antigens
COI	Cost of Illness
Cr	Chromium
CrMe	Chromium Methionine
CrOx	Chromium Oxide
CT	Caecal Tonsils
DNA	Deoxyribonucleic Acid
Dpi	Days Post Infection
EC	European Commission
EFSA	European Food Standards Agency
FCR	Feed Conversion Ratio
FERG	Foodborne Disease Burden Epidemiology Reference Group
FlpA	Fibronectin like Protein A
Fn	Fibronectin

FSA	Food Standards Agency
GALT	Gut Associated Lymphoid Tissue
GBS	Guillain-Barre Syndrome
GI	Gastrointestinal
GPA	Gentamicin Protection Assay
GRAS	Generally Recognised as Safe
GTP	Guanosine Triphosphate
HMP	Human Microbiome Project
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IECs	Intestinal Epithelial Cells
IEL	Intra-epithelial Lymphocytes
IFNγ	Interferon Gamma
IL	Interleukin
LOS	Lipooligosaccharides
LPS	Lipopolysaccharide
MALT	Mucosa-Associated Lymphoid Tissue
mCCDA	Modified charcoal cefoperazone deoxycholate agar
MCFA	Medium Chain Fatty Acid
MCPs	Methyl Accepting Chemotaxis Proteins
MeOPN	*O*-methyl phosphoramidate
MHBA	Mueller Hinton Broth Agar
MLST	Multi Locus Sequence Typing
MPN	Most Probable Number
NK	Natural Killer Cell
PAMPs	Pattern Associated Molecular Patterns
PP	Payers Patch
PRRs	Pattern Recognition Receptors

RA	Reactive Arthritis
rDNA	ribosomal DNA
RNA	Ribonucleic Acid
SCFA	Short Chain Fatty Acid
SOD	Superoxide Dismutase
STs	Sequence Types
T3SS	Type-III-secretion System
Tlps	Transducer-Like Proteins
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
VFA	Volatile Fatty Acids
WHO	World Health Organisation

Chapter 1: Introduction

The leading cause of human gastroenteritis is the Gram-negative bacterial genus *Campylobacter*. Poultry products present a public health risk as they are the primary reservoir for this bacterium and human consumption of contaminated poultry products is considered the number one cause of human infection (Suzuki and Yamamoto, 2009). To prevent human infection with *Campylobacter* it is vital that controlling the spread begins at the source and contamination during rearing and processing of poultry is reduced/prevented. Current biosecurity interventions are not effective at controlling *Campylobacter* (Lu et al., 2021) and therefore it is vital that an economically viable product is developed that successfully reduces *Campylobacter* load within chickens/on poultry products at retail sale to levels that do not present such a risk to human health.

This project investigates the potential of feed additives and probiotic additives to be used as chicken feed additives to control *Campylobacter* using *in-vitro* experimentation. The direct effects on *Campylobacter* growth, and indirect effects on epithelial cell invasion and avian cell immune response has been investigated.

This literature review summarises what is known to date about *Campylobacter* as a species, including virulence mechanisms, pathogenesis, and genomic variation. In addition, the avian and human gastrointestinal systems and immune defence mechanisms will be introduced. Current on-farm and production level *Campylobacter* targeted biosecurity measures will be discussed, and their efficacy assessed; finally, the products investigated within this study will be introduced and what is known about their mechanisms of action that lead to their selection for this study.

1.1 Campylobacter species

1.1.1 History and Discovery of *Campylobacter* spp.

In 1886, *Vibrio*-like organisms were observed by Theodor Escherich in the stool samples of infants suffering with intestinal symptoms now associated with *Campylobacter* infection, however at the time the disease was called ‘cholera infantum’ (Kist, 1986). In the early 1900s, *Vibrio*-like organisms were reported to cause bovine and ovine abortion (McFadyean & Stockman, 1913; Tresse et al., 2017) and these were subsequently classified as *Vibrio fetus* (Acheson & Allos, 2001; T. Smith & Taylor, 1919), and were later reclassified as *Campylobacter fetus* (Doyle, 1981). Jones, Orcutt and Little (1931)

reproduced the symptoms of winter dysentery in healthy cattle by inoculation with a pure culture of *Vibrio*-bacterium isolated from diseased cattle; it was observed that the first site to be infected within the intestinal tract was the jejunum, leading to the proposal of *Vibrio jejuni* in 1931 (Doyle, 1981). A third *Vibrio* organism was isolated microaerobically into pure culture by Doyle in 1944 and reported to cause swine dysentery; this species was classified as *Vibrio coli* (Andress & Barnum, 1968; Doyle, 1981). The first official report of human infection with *Vibrio* spp. was in 1947, *V. fetus* was present in two blood cultures from a pregnant woman with symptoms of influenza; a still born infant was delivered with a placenta infected with *V. fetus* (Doyle, 1981). These *Vibrio* species have since been reclassified as *Campylobacter* species based on differences from true vibrios in GC content (Peterson, 1994).

1.1.2 The Genus *Campylobacter*

The genus *Campylobacter* was first proposed in 1963 by Sebald and Veron, due to specific microaerobic growth conditions, morphology and DNA composition that deviated from "true" *Vibrio* species (O'Loughlin et al., 2015; Sebald & Veron, 1963; Silva et al., 2011). Dekeyser *et al.* (1972) were the first to isolate *Campylobacter* from human blood and stool samples, leading to the first reports of human gastroenteritis caused by *Campylobacter* spp. (Acheson & Allos, 2001; S. F. Park, 2002; Sheppard & Maiden, 2015; Snelling et al., 2005).

The family *Campylobacteraceae* is a highly diverse bacterial family formed of three genera: *Arcobacter*, *Sulfurospirillum* and *Campylobacter* (Lastovica et al., 2014; Robyn et al., 2015; Snelling et al., 2005). The genus *Campylobacter* currently has 32 species and nine subspecies of spiral, microaerophilic, Gram-negative, rod-shaped bacteria that are 0.2-3.5 μm long by 0.2-0.4 μm wide (Costa & Iraola, 2019; Hoepers et al., 2016; Robyn et al., 2015; Smibert, 1978). *Campylobacter* spp. are typically motile and show a characteristic corkscrew motility facilitated by a single (and sometimes multiple) flagellum(a), that is two to three times the length of the cell body and present at one or both ends of the bacterium (Hoepers et al., 2016; Smibert, 1978; Snelling et al., 2005). *C. jejuni*, *Campylobacter coli* (*C. coli*) and *Campylobacter lari* (*C. lari*) are thermotolerant species meaning they can grow at temperatures between 30 and 45˚C, with an optimum growth temperature of 42 ˚C (Robyn et al., 2015).

1.1.3 Isolating and culturing *Campylobacter* spp.

Campylobacter spp. can be isolated from a range of sources, such as food, human faeces, the environment, and animal faeces by direct plating onto selective agar or by enrichment culture (Jennings et al., 2011; Kim et al., 2016). Obtaining a pure culture of *Campylobacter* spp. from mixed samples requires a microaerobic environment (5% O_2; 10% CO_2; 85% N_2) and temperature between 37-42˚C due to the slow growing and fastidious nature of these bacteria (Gorkiewicz et al., 2003; Kim et al., 2016). These temperatures also replicate the internal conditions of the human and avian gastrointestinal tract encountered by *Campylobacter* (37 and 42 ˚C, respectively) (Bolzani et al., 1979; Cuevas-Ferrando et al., 2020).

Standard protocols for isolating and culturing *Campylobacter* spp. define optimum growth conditions for a microaerophilic bacterium as an atmosphere with low oxygen tension (5% O_2, 10% CO_2, 85% N_2) (Myintzaw et al., 2021; Robyn et al., 2015; Soto-Beltrán et al., 2022; Sukted et al., 2017). Optimal temperature for the growth of thermotolerant *Campylobacter* spp. (*C. jejuni*, *C. lari*, *C. coli* and *C. upsaliensis*) is between 37-42˚C; incubating at higher temperatures in this range may prevent growth of unwanted microorganisms within the sample, and aid selection (Gharst et al., 2013; Soto-Beltrán et al., 2022). Other *Campylobacter* spp. (*C. hyointestinalis* and *C. fetus*) are described as non-thermotolerant and grow optimally at 37˚C but are unable to grow at higher temperatures (Dehao Chen et al., 2021; Soto-Beltrán et al., 2022).

Typically, when recovering *Campylobacter* spp. from food or environmental samples, the first step is to use an enrichment culture, to enhance recovery from damaged bacterial cells or samples with low bacterial numbers (Soto-Beltrán et al., 2022; L K Williams et al., 2012). Enrichment culture works by resuscitating bacteria within the sample that have been exposed to stress and growth inhibitors (Soto-Beltrán et al., 2022). For *Campylobacter* isolation there are recommended enrichment culture methods including the use of Preston (Hayashi et al., 2013), Bolton (Baylis et al., 2000) and modified Exeter liquid media (Mattick, 2003; Soto-Beltrán et al., 2022). Williams *et al.* (2012) showed that the enrichment culture method used can bias the *Campylobacter* subtype recovered, therefore it is important to consider this when choosing an enrichment broth for selective recovery. In addition, the original sample should be considered when choosing enrichment broths (F. J. Bolton & Robertson, 1982). A final consideration for the

enrichment step is the promotion of unfavourable microflora in the sample, and so growth conditions should be optimised for the isolation of the specific *Campylobacter* species being targeted (Soto-Beltrán et al., 2022).

There are several types of selective plating media for the isolation of *Campylobacter* spp. Charcoal cefoperazone deoxycholate agar (CCDA) is the most common plating media for isolation from mixed samples including faeces; it consists of a basal medium modified with amphotericin B (anti-fungal) and cefoperazone (antimicrobial) (mCCDA) (Gharst et al., 2013; Soto-Beltrán et al., 2022) and often results in significantly higher yields of *Campylobacter* than alternative agars (Acke et al., 2009). Soto-Beltrán *et al.* (2022) highlight the use of synthetic chromogenic enzyme substrates for the identification of the targeted *Campylobacter* isolate based on enzymatic activity. To reduce the rate of detecting false positives in recovery from poultry, the addition of rifampicin is suitable (Soto-Beltrán et al., 2022). However, the addition of numerous antibiotics during isolation may result in selection of isolates that have antibiotic resistance, therefore biasing datasets (Y. Jo et al., 2017; Soto-Beltrán et al., 2022).

Standard protocols for qualitative isolation of *Campylobacter* spp. directly from a sample involves streaking the sample (which may, or may not, have been recovered in a selective enrichment broth) directly onto selective agar, and incubating at 37-42 ˚C under microaerobic conditions (5% O_2; 10% CO_2; 85% N_2) (Line *et al.*, 2001; Acke *et al.*, 2009; Sukted *et al.*, 2017). The further modification of mCCDA media with Polymyxin B has improved the isolation rate, with decreased growth of competing microflora and increased sensitivity to *Campylobacter* compared to regular CCDA (Jung-Whan Chon et al., 2012).

There are several distinguishing morphological traits that enable the identification of *Campylobacter* colonies on mCCDA, including grey colouring with a shiny appearance (Figure 1.1; Al-Edany, Khudor and Radhi, 2015). Blood agar plates are also used for culturing *Campylobacter* from frozen stocks or from a colony pick; Columbia blood agar (basal medium) supplemented with cefoperazone, and lysed horse blood are most widely used (Gharst et al., 2013). The major drawback of mCCDA is its very dark appearance which makes it difficult to count translucent *Campylobacter* colonies (LINE et al., 2001). Mueller Hinton Broth Agar (MHBA) and Campy-cefex agar were compared to mCCDA with regards to this characteristic and better facilitate the enumeration of *Campylobacter*,

specifically when quantifying directly from a sample (J.-W. Chon et al., 2012; Jung-Whan Chon et al., 2012; LINE et al., 2001).

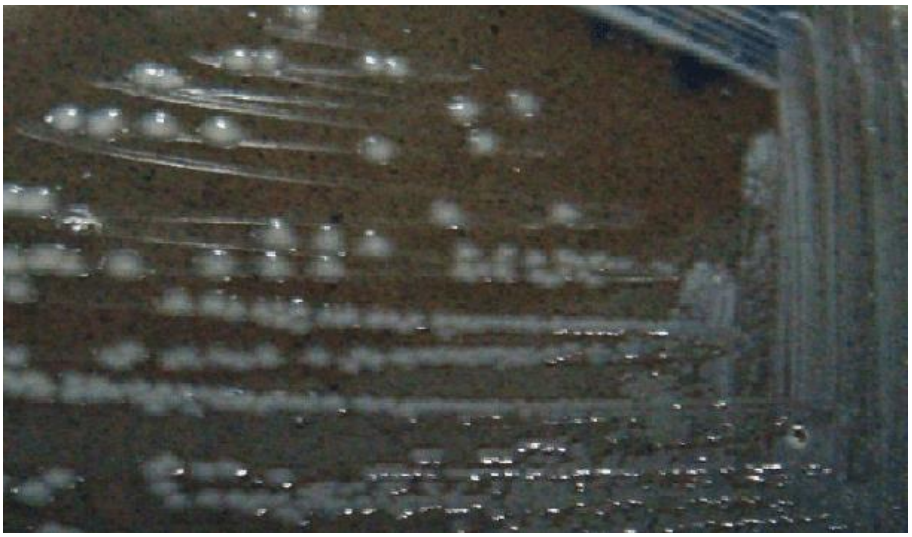

Figure 1.1 Plating of *C. jejuni* on mCCDA yields shiny grey colonies (Al-Edany, Khudor and Radhi, 2015).

Incubation time for the successful isolation and culture of *Campylobacter* spp. varies between 24-48 hours and is dependent on the incubation temperature and how microaerobic conditions are generated. Williams *et al.* (2009) found incubation for 24- and 48-hour periods yielded successful isolation of *Campylobacter* spp. with little advantage of the longer time point.

1.1.4 *Campylobacter* Genomics and Phylogeny

Genetic analysis of *Campylobacter* isolates is fundamental to the study of campylobacteriosis, with molecular epidemiology being of particular importance (Sheppard et al., 2012). *Campylobacter* spp. has an unusually high rate of genetic recombination. Multi Locus Sequence Typing (MLST) analysis has shown that horizontal gene transfer in *Campylobacter* spp. occurs at a higher rate compared to *Escherichia coli* and *Salmonella Typhimurium*, which generates two times more genetic diversity than de novo mutations (Epping et al., 2021; D. John, 2018).

C. jejuni and *C. coli* are the two most clinically relevant species to humans. These two distinct species share an 86.5% nucleotide sequence similarity within their housekeeping genes and show evidence of genetic exchange (Bull et al., 2006; Epping et al., 2021; Sheppard et al., 2009). These species are predicted to have diverged into clusters of related lineages 6,580 years ago with divergence into distinct clades 400 years ago (*C. jejuni*) and 1,000-1,700 years ago (*C. coli*) (Epping et al., 2021) (Figure 1.2).

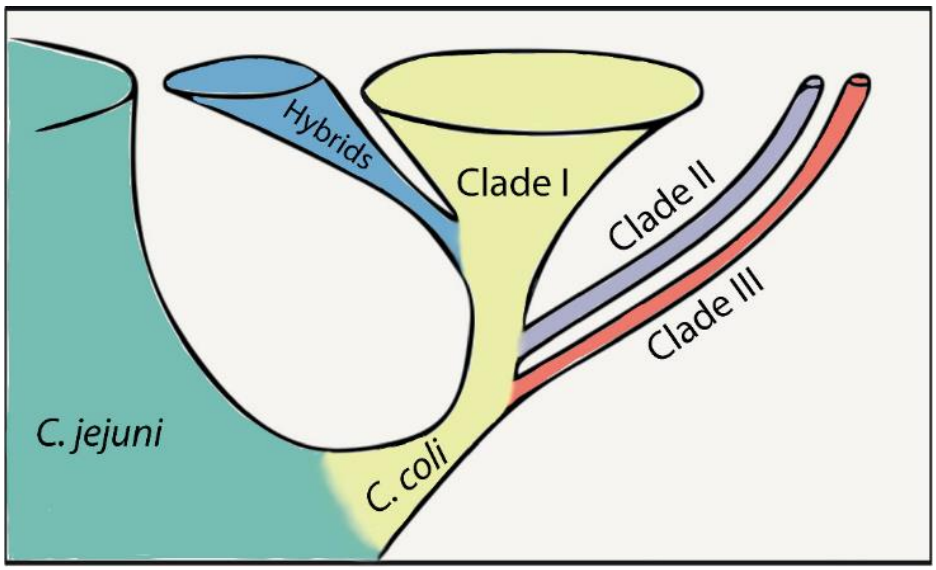

Figure 1.2 Evolutionary divergence of *C. jejuni* and *C. coli* into two distinct species and further into three clades due to ecological niches. *C. coli* hybrid strains developed from recombination between clade I *C. coli* strains and *C. jejuni*. (Taken from: Epping, Antão & Semmler, 2021).

C. jejuni NCTC 11168 was the first strain to be genome sequenced in 2000 (Parkhill et al., 2000). The genome of this specific *C. jejuni* strain was shown to have a total of 1,642,481 base pairs (Parkhill et al., 2000). This development revolutionised technology for postgenomic investigations, which included, but was not limited to, the identification of colonisation factors that are specific to *C. jejuni* and revealed hypervariable regions which may serve a role in survival (Gaynor et al., 2004; D. John, 2018; Parkhill et al., 2000). To date, over 7,000 *Campylobacter* sequence types have been described (Skarp et al., 2016). MLST data has shown that large clonal complexes (CC) have clusters of closely related *Campylobacter* genotypes that can colonize a range of hosts (D. John, 2018). The CC are formed of sequence types (STs) that share alleles at one or more MLST loci of seven housekeeping genes (Colles et al., 2003; Dingle et al., 2002; Panzenhagen et al., 2021).

1.1.5 *Campylobacter* Species Diversity

Since its discovery, the number of confirmed species within the genus *Campylobacter* has risen to 32 species with nine subspecies that can be grouped into five distinct clades – *C. jejuni, C. coli, C. lari, C. concisus* and *C. fetus* groups (Figure 1.3; Costa and Iraola, 2019).

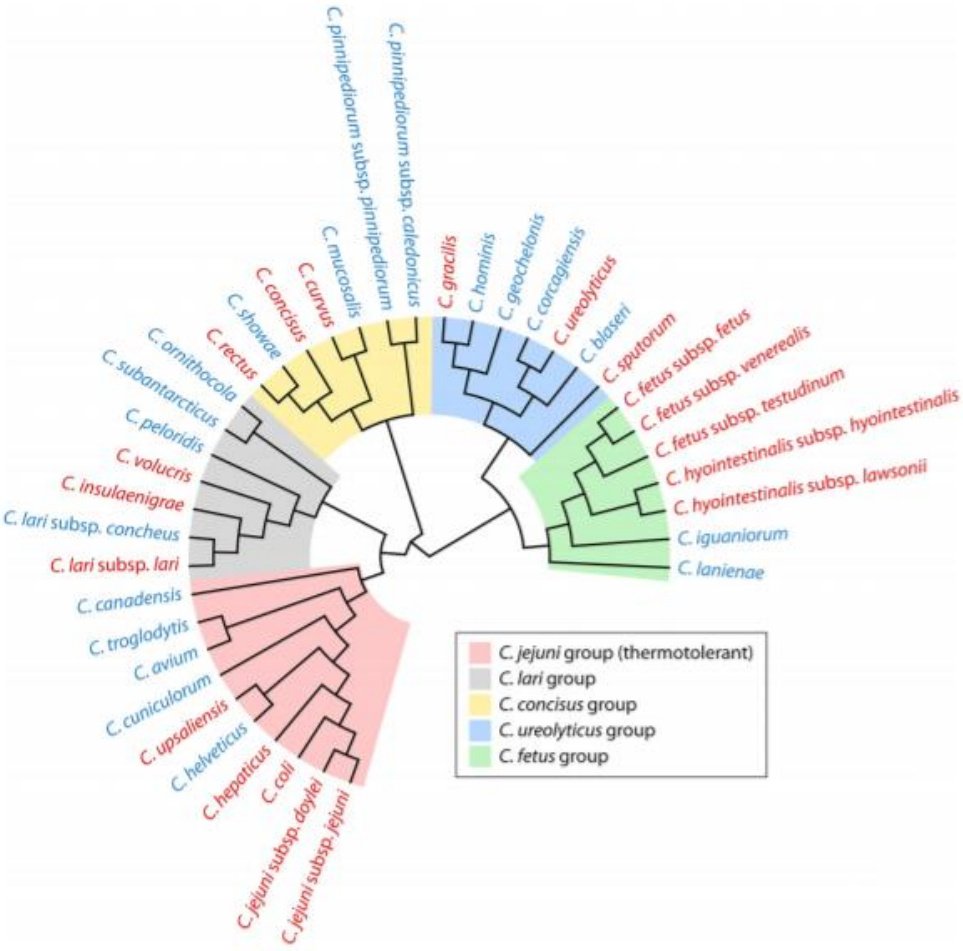

Figure 1.3 Phylogenetic tree of the genus *Campylobacter* with species divided into five distinct clades. Red tip labels correspond to species that are known to be pathogenic in humans/animals, blue tip labels correspond to non-pathogenic species (Costa & Iraola, 2019).

However, when investigating the literature, the number of reported species can vary (Chlebicz & Śliżewska, 2018). By 1988 the number of *Campylobacter* species, subspecies and proposed groups totalled 16 (Barrett et al., 1988; Butzler et al., 1973), this rose to 15 in 2002, 12 of which were pathogenic to humans (S. F. Park, 2002). The rapid increase in the number of confirmed species over the last 20 years is due to the revelation that species previously thought to be similar based on phenotypic properties, are genetically dissimilar (Gorkiewicz et al., 2003). Current knowledge of *Campylobacter* is based upon isolates taken from human disease, the agricultural environment, and the food

chain, therefore the number of species and subspecies recognised are not representative of the natural *Campylobacter* population that exists in all environments (Sheppard & Maiden, 2015).

1.2 *Campylobacter* and Disease

1.2.1 Campylobacteriosis

The collective term given for infectious, foodborne diseases caused by members of the *Campylobacter* genus is campylobacteriosis (Coker, 2002; Sarkar et al., 2014). Of the 32 named and validated *Campylobacter* species, *C. jejuni* and *C. coli* are the major causative agents of gastrointestinal infections within humans (Coker, 2002; Skarp et al., 2016). The disease campylobacteriosis, primarily caused by *C. jejuni* and *C. coli,* is increasing in prevalence across the world making it of major public health importance (Coker, 2002; Sarkar et al., 2014).

1.2.2 *Campylobacter* infection in humans

In 2020, the Foodborne Disease Burden Epidemiology Reference Group (FERG) within the World Health Organisation (WHO) estimated that globally there are 550 million cases of bacterial foodborne illness every year, with *Campylobacter* being one of the four major causes of gastrointestinal disease surpassing the number of cases caused by *E. coli, Listeria* and *Salmonella* spp. (Chlebicz & Śliżewska, 2018; Myintzaw et al., 2021; Sheppard & Maiden, 2015). The number of cases of campylobacteriosis worldwide can be difficult to estimate due to many cases going unreported, therefore the true incidence is unknown, specifically within developing countries (Ingrid Hansson et al., 2016; Myintzaw et al., 2021).

Human infection occurs primarily through handling and consumption of raw or undercooked poultry products contaminated with *Campylobacter*. However, ingestion of contaminated water or raw food products and direct contact with animals can also result in bacterial infection and colonisation of the human intestinal epithelium (Hoepers et al., 2016; S. F. Park, 2002; Sheppard & Maiden, 2015; Wagenaar et al., 2006). It has been estimated that up to 80% of campylobacteriosis cases have a foodborne origin, however there is a large variation in these estimates from different researchers (Mughini Gras et al., 2012; Whiley et al., 2013).

The infective dose of *C. jejuni* for the development of campylobacteriosis has been reported to be as low as 500 to 800 CFU (colony forming units) (Black et al., 1988; Robinson, 1981). However, Hara-Kudo and Takatori (2011) used a most probable number (MPN) method to calculate an infective dose of 360 CFU for this species (Kaakoush et al., 2015).

Human campylobacteriosis is rarely fatal in developed countries, however, it is rarely asymptomatic because humans are not a primary host for this bacterium (Sheppard & Maiden, 2015). Symptoms of this bacterial disease develop after an average incubation period of 24-72 hours but can take up to 5 days for symptoms to present (Hoepers et al., 2016; Horn & Lake, 2013; Sheppard & Maiden, 2015; Wagenaar et al., 2006). The clinical manifestation is highly diverse, ranging from a self-limiting and non-fatal gastrointestinal illness to death, however death primarily occurs in immunocompromised individuals (Ruiz-Palacios, 2007; Snelling et al., 2005; Teunis et al., 2018).

Approximately 10% of human campylobacteriosis cases are admitted to hospital, and post infection sequelae associated with certain serotypes of *C. jejuni* such as reactive arthritis and Guillain-Barre syndrome (GBS) presents in ~66% of hospitalised cases but other *Campylobacter*-related sequelae have associated risk/prevalence, these are outlined in Table 1.1 (Allos, 1997; Ruiz-Palacios, 2007; Sheppard & Maiden, 2015; Snelling et al., 2005; Strachan & Forbes, 2010; Trudy M. Wassenaar & Blaser, 1999).

Table 1.1 Sequalae associated with human campylobacteriosis and incidence rates/risk worldwide.

Sequelae	Incidence Rate(s)/Risk	Reference(s)

Reactive Arthritis (RA)	▪ 5% of campylobacteriosis cases develop RA ▪ Lower incidence per 1000 cases than *Salmonella* and *Shigella*	Pope *et al*., 2007; Ajene, Walker & Black, 2013; Myintaw, Jaiswal & Jaiswal, 2021
Guillain-Barre Syndrome (GBS)	▪ 31,700 GBS cases per year worldwide ▪ 1 in 1000 cases of *C. jejuni* infection develops GBS worldwide. ▪ Scandinavian countries; <10 GBS cases per 100,000 campylobacteriosis cases ▪ Spain and Poland; >100 GBS cases per 100,000 campylobacteriosis cases	Allos, 1997; Whitley *et al.*, 2013; Mangen *et al.*, 2016; Myintaw, Jaiswal & Jaiswal, 2021
Irritable Bowel Syndrome (IBS)	▪ 1 in 14 (7.1%) subjects in a case study developed IBS after confirmed bacterial gastroenteritis – increased risk for females. ▪ Risk calculated of contracting IBS because of *Campylobacter* infection = 2.8 (95% CI 1.9-41)	BMJ, 1997; Tam & O'Brien, 2016; O'Brien, 2017
Inflammatory Bowel Disease (IBD)	▪ Increased risk of IBD associated primarily with *C. concisus* and *C. showae* ▪ Three-fold increase in risk of IBD following *Campylobacter* infection ▪ IBD (first time diagnosis) reported in 107/13,148 *Campylobacter* exposed individuals (1.2%)	Gradel *et al*., 2009; O'Brien, 2017

In low- and middle-income countries, campylobacteriosis is a significant cause of morbidity and mortality in children under the age of 5 years (T. N. Clarke et al., 2021). However, it is hard to determine the epidemiology of human infection due to differences in the methodologies used to isolate *Campylobacter* spp. and cases that go unreported (Ingrid Hansson et al., 2016; Sheppard & Maiden, 2015). It has been estimated that for every reported case of campylobacteriosis, 9.3 cases are unreported (Sheerin et al., 2014).

1.2.3 Economic impact of human *Campylobacter* infection

Campylobacter imposes a significant burden on the economy (Table 1.2). There are high costs associated with personal loss to individuals suffering with acute campylobacteriosis and associated sequalae, death of individuals, a strain on the public health care sector, and a financial burden to the food industry (Roberts *et al.*, 2003; Hansson *et al.*, 2016; Devleesschauwer *et al.*, 2017; Myintzaw, Jaiswal and Jaiswal, 2021).

The global cost of illness due to *Campylobacter* is yet to be calculated. Lack of data in low- and middle-income countries and variation in data collection methods makes this task very difficult, therefore studies have focussed on estimating the cost of illness per country using mathematical models (Chlebicz & Śliżewska, 2018; Ingrid Hansson et al.,

2016; Heimesaat et al., 2021; Myintzaw et al., 2021). Most economic impact assessments on *Campylobacter* have been generated over the last 40 years within high income countries and focus on the cost of human illness and death to society (Table 1.2) (Devleesschauwer et al., 2017). The results of cost of illness (COI) models and cost analysis data from various high-income countries (Table 1.2) as both total cost to countries and cost per case; COI models enable health care and food industries to design strategies and policies to reduce the economic impact of a disease in a way that is economically viable (C. Jo, 2014).

Trends in COI are valuable for determining the urgency of intervention and the impact of interventions already put in place. Reduced-COI models can be formulated to show the potential impact of intervention strategies and to ensure that the reduction in COI outweighs the intervention cost.

Table 1.2 Economic burden of *Campylobacter* infection in high income countries – costs were converted to GBP using average conversion rates from 20-05-2022.

Country	Year	Total Annual Economic Burden of *Campylobacter*	Estimated cost per case	Reference(s)
United Kingdom	1993-1995 1994-1995 2008-2009	£70 million* £69.6 million* £49.4 – £50.3 million* £1.26 million**	 £85	Roberts *et al.*, 2003; Tam and O'Brien, 2016; O'Brien, 2017
USA	2010 2011 2013	$1,560 million (£1,273 million) $1.7 billion (£1.4 billion) $1.9 billion (£1.55 billion)	 $2283 (£1866)	Hoffmann, Batz and Morris, 2012; Scharff, 2012; Hoffmann, Maculloch and Batz, 2015; O'Brien, 2017
Netherlands	2011	€76 million (£65 million)	€706 (£601)	Mangen *et al.*, 2015; O'Brien, 2017
Sweden	2012-2016	€54.5 million (£46.3 million) average per year	€979 (£834)	Sundström, 2018
Belgium	Not specified	€27million (£23million)	n/a	Viaene, Gellynck and Messens, 2007
Switzerland	2012-2014	€29-45 million (£25-38 million)	€63-95 (£54-81)	Schmutz *et al.*, 2016; O'Brien, 2017

*Societal cost only – health service and patient costs

**Cost of *Campylobacter*-related GBS

There are multiple factors that contribute to the COI model development beyond the direct costs to the healthcare system (doctor consultations, hospitalisation, and rehabilitation, etc.). The COI also considers the direct non-healthcare costs such as patient travel costs, and the indirect non-healthcare costs such as loss of earnings and productivity losses (M. J. J. Mangen et al., 2004). It is important for accurate COI models that all illness related costs are included in model development. For example, the economic burden of *Campylobacter* related GBS totalled £1.26 million in 2008 (Table 1.2); research into COI model development reported that not including *Campylobacter* related sequalae resulted in high underestimations for the burden of *Campylobacter* infections (M.-J.J. Mangen et al., 2016).

Despite the differences between COI estimates due to study design, and the type of healthcare system within the country of interest, one thing is abundantly clear, *Campylobacter* infection poses a huge economic burden.

1.2.4 *Campylobacter* infection in chickens

Infection of broiler flocks with *Campylobacter* is usually detected at an age of 2-3 weeks (Conlan et al., 2007; Neill et al., 1984; Orhan Sahin et al., 2002b; Van Deun, Pasmans, Ducatelle, et al., 2008). During the first 2-3 weeks of life, *Campylobacter* load within the chick may be too low to be detected therefore giving the illusion of being "*Campylobacter* free" (Cox *et al.*, 2012). Newly hatched chicks are reported to be typically *Campylobacter* free because horizontal transmission (from environmental sources) is thought to be the primary route for initial infection and vertical transmission (egg-borne) is believed to be less likely (O. Sahin et al., 2003). However, egg-borne transmission does not have to be from hen to the chick (transovarian), fecal matter surrounding the egg can contaminate the shell properties and be ingested by the chick during hatching (Cox *et al.*, 2012).

Following initial infection, *Campylobacter* can spread rapidly through a broiler flock horizontally through faecal shedding and coprophagic behaviour (Conlan et al., 2007; Newell & Fearnley, 2003). *Campylobacter* colonises the lower gastrointestinal (GI) tract of the bird, predominantly the cecum and cloaca, however extra-intestinal spread of the bacterium leads to colonization of liver, muscle tissues, spleen and gall bladder (Orhan Sahin et al., 2002b). Infected individuals within a flock (typically the majority) remain colonised throughout until slaughter, with the result that the remaining birds are subject to cross-contamination at processing plants (Orhan Sahin et al., 2002b). This cross-contamination at processing is another route for the bacterium to spread to chicken products at retail level for consumer purchase.

In commercial chicken flocks there is a high prevalence of asymptomatic *Campylobacter* carriage reported, which led the scientific community to agree that *Campylobacter* existed commensally within chickens (W. A. Awad et al., 2015; Suzanne Humphrey et al., 2014; C. Pielsticker et al., 2012). Indeed, the immune response to colonisation of the GI tract by *Campylobacter* was previously believed to be tolerogenic. However, it is now known that there is a change to intestinal barrier function, and an initiation of inflammatory responses. In addition to this, systemic infections can be caused, and clinical

manifestations of the disease are documented (W. A. Awad et al., 2015; David Hermans et al., 2011b; Suzanne Humphrey et al., 2014; L. K. Williams et al., 2013).

It is now well documented that some strains of *Campylobacter* are pathogenic in chickens and affect bird welfare by reducing gut health (W. A. Awad et al., 2018; Suzanne Humphrey et al., 2014; T. Humphrey, 2006). *Campylobacter* interacts with the chicken gut epithelium in multiple ways to aid extra-intestinal spread, consequently damaging the gut (W. A. Awad et al., 2018). A crucial part of normal intestinal function is an intact epithelium, the surface area is made up of villi and crypts (W. A. Awad et al., 2018; Kovanda et al., 2019). When experimentally infected with *Campylobacter* spp., histomorphological changes within the chicken intestines have been reported (W. A. Awad et al., 2015). At 12 days post infection (dpi) Humphrey *et al.* (2014) documented villus thickening, shortening and fusion within the ileum, and at 14 dpi, Lamb-Rosteski *et al.* (2008) documented villus atrophy (W. A. Awad et al., 2018). The changes elicited by *Campylobacter* infection decrease the absorptive function within the small and large intestines, with a reduction in absorption of key nutrients (Na^+, amino acids, glucose) (W. A. Awad et al., 2015, 2018).

Hock marks and pododermatitis (Figure 1.4) are well-documented problems within modern poultry breeds and are associated with poor quality litter and/or poor gut health and are a direct effect of *Campylobacter* on bird welfare (L. K. Williams et al., 2013). As previously mentioned, *Campylobacter* is a direct contributor to poor gut health leading to more frequent defecation which saturates the litter within the chicken house, leading to a high ammonia content that causes these physical symptoms by burning the legs and feet of the bird (Suzanne Humphrey et al., 2014).

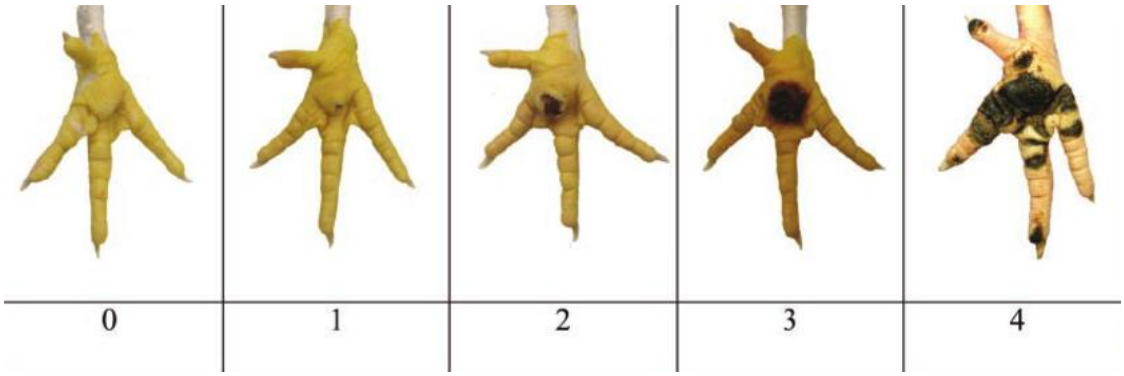

Figure 1.4 Pododermatitis scoring scale for broiler chickens based on visual assessment. Score of 0 shows no pododermatitis and a score of 4 is for severe pododermatitis (Taken from Rushen, Butterworth & Swanson, 2011).

1.2.5 Economic burden of chicken infection

When *Campylobacter* is present within a flock there are added costs that are not immediately obvious, resulting in COI models being the primary method used to estimate the economic burden of foodborne disease (Devleesschauwer et al., 2017). *Campylobacter* poses a bacterial threat to the poultry industry covering all aspects of poultry processing from farm to fork (Umaraw et al., 2017).

Legislation within high income countries enforces safe handling and processing of poultry products to monitor and reduce *Campylobacter* prevalence at retail level to reduce the health burden this bacterium poses. In the UK and Europe there are requirements outlined in legislation (EC) 852/2004 that ensure production of food is safe and hygienic (FSA, 2021). Standard regulatory activities are therefore in place throughout the poultry processing chain to prevent *Campylobacter* outbreaks because of contamination of poultry meat at retail level (Devleesschauwer et al., 2017; W. Jacobs-Reitsma et al., 2014). These regulatory activities cost the industry through employing individuals to undertake testing activities and potential loss in product if a breach of protocol or risk of outbreak is identified. However, despite monitoring during processing, 89.1% of chicken products at retail level in the UK are reported to be contaminated with *Campylobacter* (J. Smith, 2013; Wong et al., 2007).

1.3 Pathogenesis of *Campylobacter*

Campylobacter relies on important virulence factors during its pathogenesis (Panzenhagen *et al*., 2021) where the key steps involved include i) motility and chemotaxis; ii) adhesion; iii) invasion; iv) metabolic flexibility and v) serum resistance.

1.3.1 Motility of *Campylobacter*

Virulence factors for motility and chemotaxis (Table 1.3) are essential functions for *Campylobacter* survival within, and colonisation of the GI tract, in both human and avian hosts (D. J. Bolton, 2015). During infection *Campylobacter* reside predominantly within the mucus that lines the intestinal epithelium and must migrate to favourable environments for growth and invasion into other tissues (Dasti et al., 2010; Lertsethtakarn et al., 2011).

Table 1.3 Virulence factors and encoding genes involved in *Campylobacter* motility and chemotaxis (Bolton, 2015).

Virulence factor(s)	Encoding gene(s)
Major flagellin protein	*flaA*
Major flagellin protein	*flaB*
Chemotaxis proteins	*cheA, cheB, cheR, cheV, cheW & che Z*
Protein required for persistence in the cecum	*acfB*

The flagella of *Campylobacter* spp. are organised into three distinct subunits – the basal body complex (components within the bacterial cell membrane), the hook, and the flagellar filament, for which approximately 25-30 proteins are required for structure (Figure 1.5) (Lertsethtakarn et al., 2011; Lopes et al., 2021). The filament is encoded for by two genes *flaA* and *flaB* (Table 1.3) and are expressed from independent promoters (van Vliet & Ketley, 2001; T. M. Wassenaar et al., 1993). Typically, there is higher expression of *flaA* than *flaB* and therefore the flagellum in motile strains is usually dominated by the flaA protein; however, motility is affected by mutational changes resulting in abnormal expression levels of *flaA* and *flaB* (Lertsethtakarn et al., 2011). Wassenaar *et al.*, (1994), found that flagella filament dominant in the flaB protein (*flaA flaB*+) resulted in immobile *Campylobacter* strains, both over and under expression of *flaB* hinders motility; highlighting the importance of flaB protein in motility, invasion, and pathogenesis (van Vliet & Ketley, 2001).

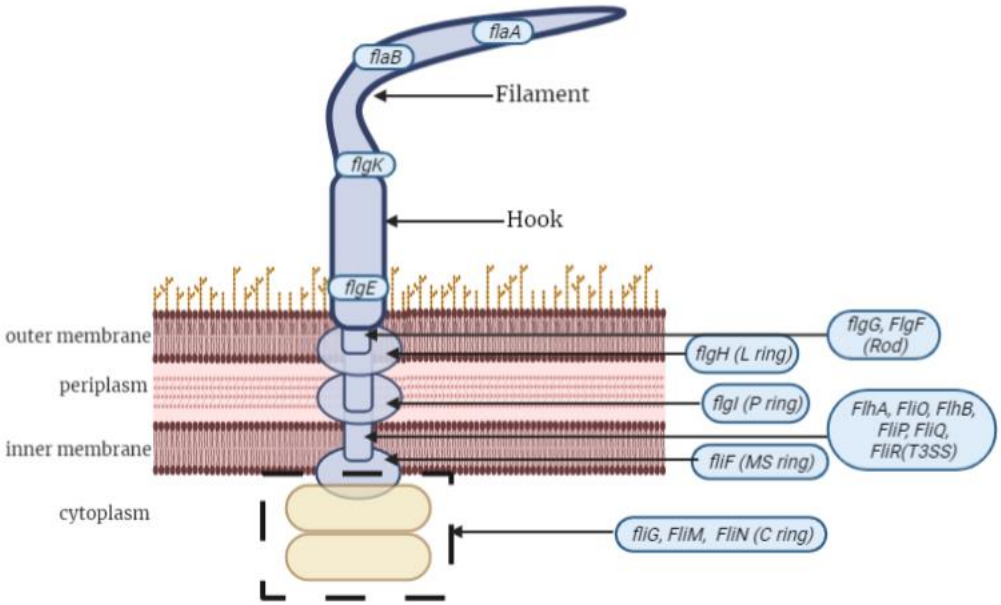

Figure 1.5 *Campylobacter* flagella structure showing major subunits and encoding genes (adapted from: Lertsethtakarn, Ottemann & Hendrixson, 2011; Lopes et al., 2021). Created in biorender.com

Chicken TLR5 (Toll like receptor) recognises and binds flagellin proteins and this initiates the immune response within the gut, however there are two glycosylation systems that can post-translationally modify flagellin, changing its structure and therefore enable evasion of host defences (Lopes et al., 2021; van Vliet & Ketley, 2001; Wigley, 2013).

Campylobacter colonisation and pathogenicity also depends on the ability to detect, respond to, and move along chemical gradients, this is known as chemotaxis (Victoria Korolik, 2019; van Vliet & Ketley, 2001). *Campylobacter* detects chemical ligands in the environment by chemosensory receptors (methyl accepting chemotaxis proteins – MCPs, also known as transducer-like proteins – Tlps) (Victoria Korolik, 2019; Lopes et al., 2021; van Vliet & Ketley, 2001). Tlps in *C. jejuni* have been classified into three groups, A, B and C, dependent on structural homology to other organisms (Victoria Korolik, 2019; Lopes et al., 2021; van Vliet & Ketley, 2001). Chandrashekhar *et al.* (2015) found that, in addition to their chemotaxis role, group B and C Tlps are important for invasion of both human and avian intestinal epithelial cells. Following extracellular stimulation of MCP/Tlps, a signal transduction cascade is initiated that results in flagellar movement either toward, or away from the chemical stimulus (Lopes et al., 2021). *C. jejuni* is chemotactically attracted to mucins, L-serine and L-fucose, however is repelled by bile acids (van Vliet & Ketley, 2001). By avoiding unfavourable environments and being able

to move directionally in response to external chemical ligands, *Campylobacter* spp. can survive within the GI tract.

1.3.2 Adhesion of *Campylobacter* to epithelial cells

Adhesion of *Campylobacter* to host epithelial cells is a prerequisite for successful colonisation, and is a multifactorial process mediated by several adhesins on the cells surface; adhesion to intestinal epithelial cells prevents clearance of *Campylobacter* from the gastrointestinal tract via mucosal shedding, peristalsis, and fluid flow (D. J. Bolton, 2015; Konkel et al., 2010; Lopes et al., 2021). Isolates of *C. jejuni* from symptomatic patients showed greater levels of adhesion to cells *in vitro* than isolates from asymptomatic individuals, showing the importance of this virulence mechanism in pathogenesis and disease development (Konkel et al., 2010). *In vitro* and *in vivo* studies have identified several key proteins and carbohydrates as key contributors to successful adherence of *Campylobacter* to host cells (Table 1.4) (Lugert et al., 2015).

Table 1.4 Virulence factors and encoding genes involved in *Campylobacter* adherence (Bolton, 2015).

Virulence factor(s)	Encoding gene(s)
Campylobacter adhesion protein A	*capA*
Phospholipase A	*pldA*
Chaperone playing role in exporting proteins to outer membrane	*peb4*
42-kDa lipoprotein, role in adhesion to Hep-2 cells	*jlpA*
Campylobacter adhesion to fibronectin protein	*cadF*
Fibronectin like protein A	*flpA*
Major outer membrane protein	*MOMP*

Campylobacter adhesins are exposed on the bacterial cell surface and facilitate the binding to host cell receptors such as fibronectin (Fn) (Konkel et al., 2010), a structural glycoprotein of host extracellular matrix containing domains with bacterial binding sites (Konkel et al., 2010; Labat-Robert, 2012; Zheng et al., 2006a). CadF (encoded by the highly conserved *cadF* gene) is a *Campylobacter* surface exposed protein that also

facilitates binding to Fn (D. J. Bolton, 2015; Konkel et al., 2010; Lugert et al., 2015). Once CadF has bound to Fn a signalling process is triggered, leading to activation of Rho family GTPases, and results in *Campylobacter* cell internalisation (D. J. Bolton, 2015; Konkel et al., 2010; Lugert et al., 2015).

Fibronectin like protein A (FlpA) (encoded by the *flpA* gene) (Table 1.4) is another *Campylobacter* surface exposed protein which promotes attachment to host cells by attaching to Fn; it has been proposed the FlpA and CadF proteins work in conjunction with each other for maximum adherence and invasion of host epithelial cells (D. J. Bolton, 2015; Konkel et al., 2010; Lopes et al., 2021). An *in vivo* study by Larson *et al* (2013) showed that for *C. jejuni* to cause severe disease in germ-free mice the presence of the FlpA protein was necessary (Lopes et al., 2021).

CapA is a surface exposed autotransporter lipoprotein (Table 1.4) (D. J. Bolton, 2015; Lugert et al., 2015). For adhesion to human colon adenocarcinoma epithelial cells (CaCo-2; ECACC, Cat number 86010202), *Campylobacter* adhesion protein CapA (encoded by the *capA* gene) was shown to be an essential mediator (Lopes et al., 2021). In addition, mutations in the *capA* gene resulted in decreased adherence to chicken cells (Chicken LMH hepatocellular carcinoma cells - ATCC CRL-2117), unlike FlpA and CadF proteins, changes in the CapA protein did not alter the colonisation potential of *Campylobacter* (Flanagan et al., 2009; Rubinchik et al., 2012).

1.3.3 Invasion of epithelial cells by *Campylobacter*

An important mechanism that *Campylobacter* employs is its ability to successfully invade intestinal epithelial cells, consequently causing colon damage, perturbing the intestines absorptive capacity, and inducing diarrheal symptoms via loss of cellular function (Lugert et al., 2015; Trudy M. Wassenaar & Blaser, 1999; Wooldridge & Ketley, 1997). It is recognised that not all *Campylobacter* strains are capable of invading and that there is a strain-dependency regarding the degree of isolate invasiveness (D. J. Bolton, 2015; Lopes et al., 2021; Wooldridge & Ketley, 1997). Commonly used cell lines for *in-vitro* work with *Campylobacter* include human CaCo-2 which were used within this study. An avian intestinal epithelial cell line was also used (MM-CHiC clone: 8E11; Tentamedix GmbH; formerly Micromol, Germany) (D. John, 2018; Russell & Blake, 1994).

Successful cellular invasion requires previous participation of *Campylobacter* in adhesion, motility, chemotaxis, and cell surface macromolecule expression (D. J. Bolton,

2015; Lopes et al., 2021). To internalise bacteria, host cell signalling must be stimulated, and the host cell cytoskeleton must be rearranged (Biswas et al., 2003; Buelow et al., 2011). However, *Campylobacter* does not possess a type-III-secretion system (T3SS) like other enteropathogenic bacteria which directly injects effector proteins into the host cells (Christensen et al., 2009; Gabbert et al., 2023). Despite this, there is a component of the flagella that is a homologue to the T3SS which serves this purpose (Table 1.5) (D. J. Bolton, 2015; Lugert et al., 2015). The proteins delivered into the host cell cytoplasm via this homologue system are called the *Campylobacter* invasion antigens (Cia) (D. J. Bolton, 2015; Lopes et al., 2021). CiaB is considered of primary importance for effective cell entry, it exhibits homology to the molecules secreted by the T3SS in other enteropathogens.*In vitro* analysis has shown that *ciaB* mutant strains exhibit a marked reduction of invasive capabilities into INT-407 cells (Dasti *et al.*, 2010; Haddad *et al.*, 2010; Lopes *et al.*, 2021). Experimental investigation has shown that *ciaB* is not essential for *Campylobacter* invasion, however it is required for host cell internalisation of the bacterium (Haddad *et al.*, 2010).

Table 1.5 Virulence factors and encoding genes involved in *Campylobacter* invasion (Bolton, 2015)

Virulence factor(s)	Encoding gene(s)
Components of flagellar export apparatus (T3SS)	*lhA, flhB, fliQ, fliP, fliO & fliR*
73-kDa protein, involved in invasion	*ciaB*
Protection against antimicrobial proteins	*virK*
Role in apoptosis	*fspA*
Intracellular survival	*ciaI*
Iron acquisition	*ceuE*

Once within the host cell, *Campylobacter* multiply within *Campylobacter*-containing vacuoles (CCV) which they alter to avoid fusion with lysosomes by deviating from the canonical endocytic pathway (Buelow et al., 2011; Watson & Galán, 2008). Indeed, CiaI has been shown to aid survival within the CCV by preventing delivery of the CCV to lysosomes (Buelow et al., 2011; Lugert et al., 2015). Translocation of *Campylobacter* from intestinal lumen to within host cells requires microfilament reorganization and

microtubules in addition to endocytosis mechanisms (e.g., lipid rafts) (Lopes et al., 2021; Monteville et al., 2003). This multifactorial invasion process is still being extensively studied and evaluated experimentally, to gain a full understanding of the mechanisms that control invasion (Lopes et al., 2021).

1.3.4 Metabolic flexibility of *Campylobacter*

To successfully colonise and survive within numerous different hosts, and compete within the gut microbiota of varying compositions, *Campylobacter* must be able to acquire nutrients from the environment in sufficient amounts (Stahl et al., 2012). During pathogenesis, *Campylobacter* must survive a variety of different environments with varying nutrient availability. For instance, in water, which is nutrient poor, and in the chicken GI tract which is nutrient rich in free amino- and keto- acids, which is the preferential nutrient substrate for this pathogen (Bronowski et al., 2014; Hofreuter et al., 2008).

Campylobacter does not possess the glycolytic enzyme phosphofructokinase, therefore making it unable to source carbon from sugars (Bronowski et al., 2014; Hofreuter et al., 2008). Serine, aspartate, glutamate, and proline have been identified as the four preferential amino acid substrates for *Campylobacter*, however successful colonisation of the avian intestinal tract has been shown to specifically require L-serine catabolism (Hofreuter et al., 2008; Velayudhan et al., 2004). It is also suggested that *in vivo*, *C. jejuni* uses peptides as an amino acid source despite the mechanism of uptake being unknown (Rasmussen et al., 2013). When existing in water, which is generally nutrient poor, *C. jejuni* upregulates an immunogenic protein Cj0917, which is homologous to the *E. coli* protein CstA (carbon starvation protein A), and heavily involved in survival of starvation and peptide uptake (Rasmussen et al., 2013). The gene encoding protein Cj0917 (*cj0917*), is therefore, crucial for environmental survival and spread to host animals.

In addition to phosphofructokinase, *Campylobacter* lacks several enzymes that are essential within the glycolytic pathway, pentose phosphate pathway, and Entner-Doudoroff pathway that metabolise carbohydrates and are used by other enteropathogens, therefore *Campylobacter* is often described as assaccharolytic (Christine M. Szymanski & Gaynor, 2012). However, some strains of *C. jejuni* upregulate a gene operon (*cj0481-cj0490* in *C. jejuni* NCTC 11168) when in the presence of L-fucose, which can be used as a substrate for growth (Stahl et al., 2011; Christine M. Szymanski & Gaynor, 2012).

The carboxylation of pyruvate to oxaloacetate, and further into phosphoenolpyruvate is a key contributor to the generation of glucose from non-carbohydrate substrates (gluconeogenesis) (Mohammed et al., 2004; Velayudhan & Kelly, 2002). Mendz, Ball and Meek (1997) also demonstrated the role of pyruvate as an essential intermediate metabolite used by *Campylobacter* to form succinate (potentially utilising the pyruvate carbon skeleton), acetate, and formate via a mixed acid fermentation pathway.

In addition to metabolic flexibility with regards to nutrient availability, *Campylobacter* must also be able to survive varying oxygen levels (Bronowski et al., 2014). *Campylobacter* is a microaerobic bacterium, and exposure to oxygen within the host and the environment, can cause oxidative damage to protein, nucleic acid and membrane, leading to cell death (Atack & Kelly, 2009; Bronowski et al., 2014). *Campylobacter* can withstand oxidative stresses by employing defence mechanisms, namely superoxide dismutase (SOD) and catalase (Atack & Kelly, 2009; Bronowski et al., 2014). SOD is a metalloenzyme that protects from reactive oxygen derivatives by catalysing the conversion of oxygen radicals to hydrogen peroxide and dioxygen (Atack & Kelly, 2009; Purdy et al., 1999). Purdy *et al.* (1999) also demonstrated the importance of the *sodB* gene of *C. coli* in resistance to oxygen exposure when grown on model food products, indicating a crucial role in SOD production for survival on poultry products at retail sale, a main source of human campylobacteriosis.

C. jejuni possesses a single catalase enzyme (*KatA*) which removes H_2O_2 from cells, the upregulation of *KatA*is induced by H_2O_2 and O_2^- (Atack & Kelly, 2009). Grant & Park (1995) demonstrated the requirement for *C. coli* to possess the *katA* gene to survive reactive oxygen derivatives; however, pyruvate supplementation *in vitro* has been reported to reduce the need for catalase induction under oxidative stress and allow *Campylobacter* growth and proliferation in a fully aerobic atmosphere (Atack & Kelly, 2009). Whilst catalase and SOD are defence mechanisms for *Campylobacter* under oxidative stress, pyruvate availability in the environment has been demonstrated as a substrate that can enable *Campylobacter* to survive environmental conditions that expose the bacterium to both oxidative and nutritional stressors.

1.3.5 *Campylobacter* serum resistance

To successfully leave the gut and colonise edible tissues (termed extraintestinal spread), *Campylobacter* must first overcome complement-mediated bactericidal activity in

mammalian serum. Failure to overcome this first line of defence by the body confines the pathogen to the mucosal surface (Blaser et al., 1985, 1987). The complement system comprises over 30 proteins (within plasma and membrane bound) which play a vital role in the innate immune system (pathogen killing, clearance of apoptotic cells, immune complex clearance etc.). Complement is triggered by presence of either bacteria, bacterial products/surfaces or immune complexes which results in an enzymatic cascade (Ross, 1986; Shariat *et al.*, 2021). *Campylobacter* that are isolated from faecal matter are typically serum sensitive, therefore are maintained within the GI tract. In contrast, isolates from systemic sites are typically serum resistant (Blaser et al., 1987). Guerry *et al.* (2000) showed that sialylation of the *Campylobacter* lipooligosaccharide (LOS) core contributes to increased resistance to the bactericidal effects of human serum, avoiding killing by complement mediated activity, which prolongs the presence of the bacteria within the host and increases the likelihood of systemic infection being established (Mortensen et al., 2009). Another capsular structure produced by *Campylobacter* to evade the bactericidal effects of host serum is the *O*-methyl phosphoramidate (MeOPN) expressed by the majority of *C. jejuni* isolates, and is a common element shared by several species of *Campylobacter* (van Alphen et al., 2014). However, whilst the MeOPN structure increases resistance to human serum, it results in a loss of invasion into human epithelial cells. Despite this, van Alphen *et al.* (2014) concluded that the MeOPN structure is beneficial overall to *Campylobacter* pathogenesis and virulence.

1.4 The Gastrointestinal Tract in Humans and Chickens

The digestive system is made up of multiple components, the primary ones being the GI tract and digestive accessory organs (salivary glands, liver, gall bladder, exocrine pancreas). The function of the digestive system is to ingest and digest food entering an animal and convert it into the energy and nutrients for use by the organism, and then expel the remaining waste product (Barboza et al., 2010; Ogobuiro et al., 2021; Reed & Wickham, 2009; Svihus, 2014). A second, but critical role of the digestive system is in immune surveillance via gut-associated lymphoid tissue (GALT) (Reed & Wickham, 2009). Bacterial, viral, and parasitic enteral antigens are sampled via inductive sites within the intestinal wall, and as a result an immune response is mounted (Bar-Shira et al., 2003; Mörbe et al., 2021).

Despite the major goal of the digestive system being similar for humans and chickens, the anatomical features of both systems have also evolved unique features (Figure 1.6). The human digestive system begins at the oral cavity where food is broken down through chewing and saliva. Oral digestive enzymes breakdown food, for example, amylase converts starch and glycogen into simple sugars, and lingual lipase which breaks down short chain fatty acids (Butterworth et al., 2011; Jolitz & Foster, Louis, 2011; Margit & Scow, 1973; Ogobuiro et al., 2021; Reed & Wickham, 2009). In comparison, chickens swallow food without any processing which enters the crop where it is stored and slowly moistens to aid digestion further down the digestive tract (Jolitz & Foster, Louis, 2011; Svihus, 2014).

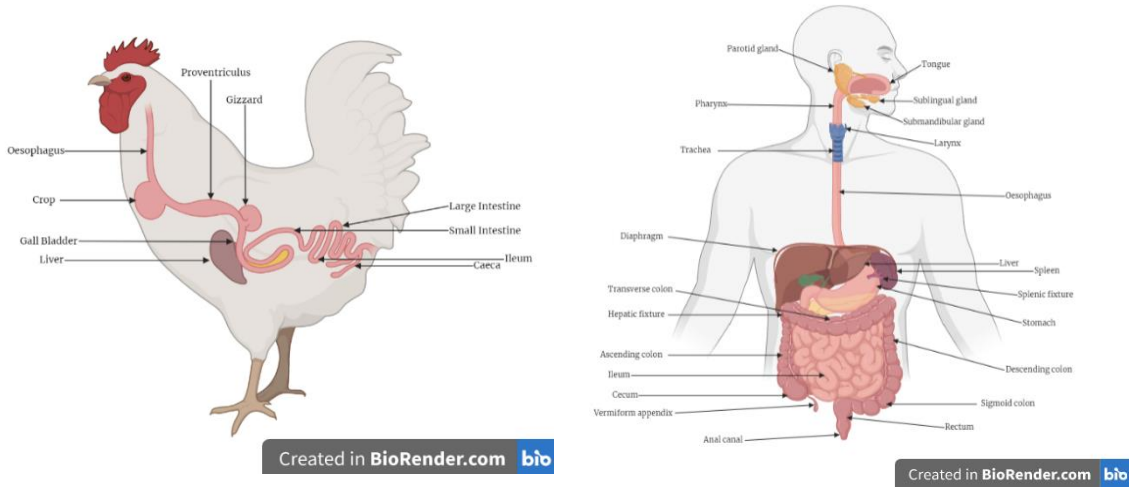

Figure 1.6 Chicken and human digestive system components including GI tract and digestive accessory organs. Created with biorender.com

Food then enters the stomach which lies below the diaphragm (Figure 1.6). The stomach is a J-shaped organ that is split into three divisions (fundus, body and antrum), and expands to many times its volume allowing it to digest large volumes of food when given access by a lower oesophageal sphincter (Jolitz & Foster, Louis, 2011; Reed & Wickham, 2009). The human stomach chemically and mechanically begins to break down the partially digested food by mixing it with water and gastric juices. Pepsin and hydrochloric acid are released in the stomach which begins protein digestion. Additionally hydrochloric acid serves a bactericidal role and aids vitamin B12 absorption (Jolitz & Foster, Louis, 2011; Ogobuiro et al., 2021; Reed & Wickham, 2009). In comparison to the human stomach, chickens have two compartments that serve a similar function: the proventriculus and the gizzard (Figure 1.6). Upon entering the proventriculus, hydrochloric acid and pepsinogen (precursor to pepsin) are secreted prior to mixing with

food in the gizzard (sometimes referred to as the ventriculus) (Jolitz & Foster, Louis, 2011; Svihus, 2014). The gizzard also serves a purpose (like that of the human oral cavity), to grind feed material using the stones that are present throughout the strongly myelinated muscles and kolin layer (Jolitz & Foster, Louis, 2011; Svihus, 2014).

Both humans and chickens have a small intestine which is comprised of three segments: the duodenum, jejunum, and ileum (Jolitz & Foster, Louis, 2011; Ogobuiro et al., 2021; Reed & Wickham, 2009; Svihus, 2014). In both humans and chickens, the small intestine is where most of the nutrient absorption occurs. A combination of intestinal and pancreatic enzymes enters the duodenal loop and break down its contents which is held very briefly here before moving into the jejunum (Ogobuiro et al., 2021; Reed & Wickham, 2009; Svihus, 2014). The jejunum is the part of the small intestine where major nutrients (lipids, sugars, electrolytes, potassium) are digested and absorbed, and finally the ileum which serves primarily as a site of water and mineral absorption (Jackson & McLaughlin, 2009; Lema et al., 2020; Svihus, 2014; Walter & Ley, 2011).

Upon reaching the large intestine most nutrients have been absorbed. In humans, the large intestine consists of the appendix, colon, rectum, and anal canal (Ogobuiro et al., 2021; Reed & Wickham, 2009). Within the colon, bacterial fermentation occurs and faecal matter (food with water removed and nutrients absorbed) enters the rectum until it can be expelled as waste (Jolitz & Foster, Louis, 2011; Ogobuiro et al., 2021). In contrast, chickens have a pair of ceca which are located at the ileal-colonic junction which serves as a site of fatty acid formation and cellulose breakdown which contribute to energy metabolism (Jolitz & Foster, Louis, 2011; Svihus, 2014). Humans have separate orifices for digestive, urinary, and reproductive tracts, whereas the chicken digestive and reproductive tracts are joined at the vent (Jolitz & Foster, Louis, 2011).

The pH of the digestive system plays a vital role in the digestibility of nutrients and the ability of microorganisms to survive and contribute to overall host health. Within Ross 308 birds (commonly farmed broiler chicken breed), the pH of the digestive tract ranges between 3.5 to 6.6 (Mabelebele et al., 2013), in comparison, the intraluminal pH of the human digestive tract is less acidic and ranges from 5.7 to 7.4 (Fallingborg, 1999). The gizzard is the digestive organ with the lowest pH (3.5) in chickens, however the human caecum typically has the lowest pH value (5.7) in humans (Fallingborg, 1999; Mabelebele et al., 2013). Interestingly, in Ross 308 birds, the caeca display the highest pH value

throughout the entire digestive tract (7.4), whereas in humans the organ with the highest intraluminal pH (7.4) is the small intestine (Fallingborg, 1999; Mabelebele et al., 2013). In addition to relatively low pH throughout the avian digestive tract, the oxygen availability is minimal to none, with studies reporting strict anaerobic metabolism of microorganisms found within the GI tract of avian species (Dunkley et al., 2009). However, in humans, whilst the intestinal organs are predominantly anaerobic, there is evidence of both aerobic and microaerobic bacteria within these environments that regulate and maintain low oxygen availability (Albenberg et al., 2014).

1.4.1 Human gut microbiome

The human gut is a multifaceted ecosystem of microorganisms whose genes contribute significantly to metabolism, human health, and disease by adding non-host-encoded enzymatic proteins to the digestive tract (Cani, 2018; Heintz-Buschart & Wilmes, 2018; Kho & Lal, 2018). To reduce any competition between host and the approximately 40 trillion gut microbes, for dietary substrates, the digestion of food and absorption of nutrients occurs in the stomach and small intestine, separated from most gut microbes that occur further down the GI tract in the large intestine (Dave et al., 2012; Walter & Ley, 2011; Xu & Knight, 2015). Microbes within a microbiota are differentiated by their genes, which are collectively known as the microbiome (Dave et al., 2012; Mörbe et al., 2021; Siezen & Kleerebezem, 2011; Xu & Knight, 2015).

Analysis of the 16S ribosomal DNA (rDNA) has concluded that over 90% of the bacterial phylotypes that are present in healthy human intestinal microbiota are from one of three enterotypes: *Bacteroidetes, Firmicutes,* and *Actinobacteria* (Arumugam et al., 2011; Dave et al., 2012; Siezen & Kleerebezem, 2011; Xu & Knight, 2015). A healthy gut microbiota will have a unique and stable balance of the three enterotypes which synthesise vitamins, aid digestion of indigestible components, support the detoxification of bile acids and metabolite generation (Carding et al., 2015; Mörbe et al., 2021; Siezen & Kleerebezem, 2011).

The intestinal microbiota has been found to vary significantly between individuals, with no "normal" human gut microbiota defined. This is due to multiple intrinsic and extrinsic factors including diet, age and even nationality (Figure 1.7) (Dave et al., 2012; Schmidt et al., 2018). However, the human microbiome project (HMP - https://www.hmpdacc.org/), and other research has led to the identification of key microbes within the gut microbiota responsible for providing key benefits to human health. In contrast, an imbalance (dysbiosis) of the three bacterial enterotypes may lead to pathogenesis of the intestines and there is an association with extra-intestinal disorders (Carding et al., 2015; Mörbe et al., 2021; Xu & Knight, 2015).

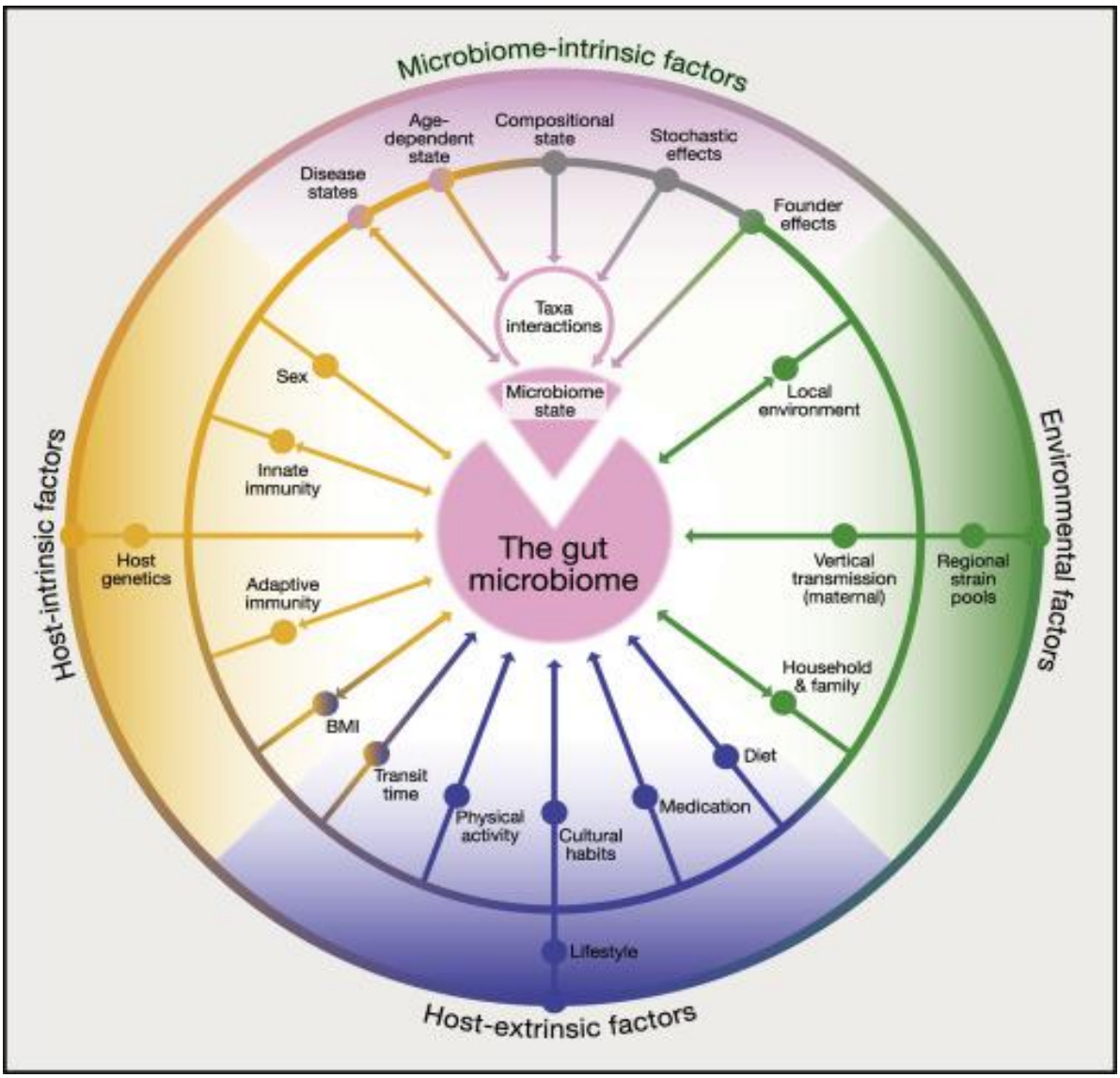

Figure 1.7 Factors affecting the composition of human microbiome (image taken from: Schmidt, Raes & Bork, 2018).

1.4.2 Human gut immunity

The immune system must be in a constant but delicate balance whereby it is able to expel and eliminate pathogenic microbes while avoiding autoimmunity (Kosiewicz et al., 2011; Wu & Wu, 2012). The human (and mammalian) intestinal tract is an important immune organ and must work in conjunction with the immune system to protect the host (Carding et al., 2015; Cheng et al., 2020; Vijay-Kumar et al., 2014; Wu & Wu, 2012). The intestinal epithelium is a single layer of cells that acts as a physical barrier between the gut lumen where harmful pathogens and microbes may exist (Allaire et al., 2019; Zhang et al., 2015). Throughout the gastrointestinal tract there is a variation in the intestinal structure and composition of cells (both of non-hemopoietic and hemopoietic origin), specifically between the small to large intestine and this is due to their functional differences (Allaire et al., 2019; Vijay-Kumar et al., 2014). Homeostasis of the intestine requires balanced and efficient interactions between commensal microbes, intestinal epithelial cells (IECs), and immune cells within the gut mucosa (Muniz et al., 2012). In addition to the single cell layer, a 200 μm thick mucosal layer is present in humans which acts as a source of nutrients for commensal microbes, it is continually shed into the gut lumen which limits the number of pathogenic microbes reaching the epithelial cell layer (Zhang et al., 2015). The main component of this lubricating and trapping barrier are glycoproteins called mucins which have a direct role in the coordination of immune responses to infection; deficiency of these molecules (specifically lack of MUC2) leads to gut inflammation (Vijay-Kumar et al., 2014).

Cells that line the human small and large intestines are referred to as IECs, of which the majority are enterocytes which serve an absorptive function and support transport of nutrients, electrolytes, and water (Vijay-Kumar et al., 2014; Wu & Wu, 2012). Between enterocytes there are several junctional protein structures (tight junctions, adherens junctions and desmosomes) which provide stability, regulate integrity of the barrier and control paracellular permeability (Kho & Lal, 2018; Vijay-Kumar et al., 2014). If disruption to these junctional proteins occur then there is increased permeability to microbes within the gut lumen, which leads to immune responses that can result in intestinal inflammation (Kho & Lal, 2018).

There are a range of IEC subtypes that have other key functions involved in immune surveillance and protection from pathogens beyond those attributed to their structural role (Al-Banna et al., 2018). Some subtypes of IECs can secrete antimicrobial peptides

(AMPs), cytokines and chemokines (Muniz et al., 2012; Wu & Wu, 2012). AMPs are also known as host defence peptides, and within the intestinal tract include bioactive molecules such as defensins, cathelicidin, regenerating proteins and lysozymes which function as effector molecules within the innate immune system and are usually located on the IEC surface (Gong et al., 2021; Gubatan et al., 2021; Muniz et al., 2012). AMPs can destroy microbial cells by disrupting the integrity of the cell membrane, and inhibiting DNA, RNA, and protein synthesis, by electrostatically or hydrophobically interacting with intracellular targets (Gong et al., 2021). Chemokines are chemoattractant cytokines that are secreted by IECs and stimulated in response to IECs interaction with components in most Gram-negative bacteria, for example peptidoglycan of *Campylobacter* (Al-Banna et al., 2018; Hughes & Nibbs, 2018). This group of molecules stimulate the migration of antigen-presenting cells (APCs) such as monocytes, dendritic cells, and lymphocytes and induce cell movement by chemotaxis, to control recruitment of populations of innate and adaptive immune cells within the intestinal mucosa (Hughes & Nibbs, 2018; Oldham, n.d.; Vijay-Kumar et al., 2014). Cytokine production and response by IECs is critical for innate immune responses and regulating immune function within the gut (Table 1.6) (Onyiah & Colgan, 2016).

Table 1.6 Regulatory cytokines that interact with IECs and their relative immune functions (adapted from Onyiah & Colgan, 2016).

Regulatory cytokine	Function
Tumor necrosis factor (TNF-α)	Pro-inflammatory, barrier function
Interferon gamma (IFNγ)	Antigen presentation, pro-inflammatory, barrier function
IL-8	Pro-inflammatory, Leukocyte recruitment
IL-10	Barrier function, homeostasis, pro-inflammatory

1.4.3 Chicken gut microbiome

The microbial community within the chicken gut is vital for absorption of nutrients, immunity, and resistance to disease causing pathogens and therefore has a direct relationship with animal productivity (Diaz Carrasco et al., 2019; Kogut et al., 2020; Shang et al., 2018). The development of the avian intestinal epithelium, which forms a

physical barrier to pathogens, is strongly influenced by the structure of the intestinal microbiota (Diaz Carrasco et al., 2019).

In contrast to mammalian species where gut microbiota (and other microbial communities) are transmitted from mother to infant via the placenta, uterus, and vagina, in avian species this process is isolated from the mother and initial colonization is largely influenced by the environment (Diaz Carrasco et al., 2019; Ding et al., 2017). Prior to hatching, microorganisms may pass through pores of the eggshell, and post-hatching is when newly hatched chicks acquire most of their intestinal microbiota (Diaz Carrasco et al., 2019; Rychlik, 2020). High demand for poultry products has led to modern-day intensive production of birds which has dramatically changed the living environment in comparison to their wild bird ancestors (Rychlik, 2020). Modern-day birds are hatched and raised in facilities that adhere to strict hygiene practices to avoid colonisation with pathogenic bacteria, resulting in acquisition of gut microbiota from an artificial environment, rather than through an organic environment like that of the red jungle fowl from which broiler chickens were domesticized (Diaz Carrasco et al., 2019; Ding et al., 2017; Kubasova et al., 2019; Oakley et al., 2014). While this sterile environment may reduce the likelihood of colonisation with pathogenic microorganisms and therefore reduce risk to the public from contaminated poultry products, newly hatched chicks fail to benefit from microbiota from a maternal source due to eggs being removed and artificially incubated instead of within a nest and in intimate contact with the mother for 21 days (Ding et al., 2017; Kubasova et al., 2019; Rychlik, 2020).

The definition of a core microbiota for the chicken gut must be defined from adult chickens, due to the microbiota within newly hatched and young chickens being highly variable (Rychlik, 2020; Shang et al., 2018). In the first week of life, the caecum of commercial chicks is firstly colonised by *Enterobacteriaceae*, followed by Firmucutes such as *Lachnospiraceae* and *Ruminococcaceae* between 7 to 14 days This colonisation is primarily from contact with microorganisms within the hatchery environment, food, and water (Diaz Carrasco et al., 2019; Kubasova et al., 2019). As the bird grows, species richness and complexity of the gut microbiota increases in the caecum and colon. In other areas of the GI tract lower concentrations of bacteria may be found due to more extreme pH or dilution with bile (Diaz Carrasco et al., 2019; Oakley et al., 2014; Rychlik, 2020; Shang et al., 2018).

In healthy, adult chickens 95% of the gastrointestinal microbiota is represented by two phyla, *Firmicutes* and *Bacteroidetes*, the remaining 5% is primarily *Proteobacteria* and *Actinobacteria*, with a combination of minority phyla such as *Fusobacteria* and *Synergistetes* which are common but poorly represented numerically (Ding et al., 2017; Kubasova et al., 2019; Rychlik, 2020).

The microbiota, while influencing the development of the intestinal structures, also directly forms a protective barrier against colonization by pathogenic bacteria through attaching to enterocyte epithelial walls resulting in a competitive exclusion of potential pathogens (Shang et al., 2018). Vitamins, short chain fatty acids (SCFAs) and other exogenous molecules are produced by the gut microbiota and contribute to animal nutrition and health (Oakley et al., 2014; Shang et al., 2018).

1.4.4 Chicken gut immunity

The chicken intestinal tract is an active organ that plays a significant role in immune homeostasis in response to microbiological, physiological, and physical exposures. The intestine can differentiate immune responses to invading pathogens, whilst tolerating self-antigens (Kogut et al., 2020). The mucosa-associated lymphoid tissues (MALT) acts as a first line of defence on mucosal surfaces throughout the whole gastrointestinal tract of chickens (and mammals) (Casteleyn et al., 2010; Lillehoj & Trout, 1994). The intestine specific MALT is the gut associated lymphoid tissue (GALT) which includes lymphoid structures including caecal tonsils (CT), bursa of Fabricius (BF), lymphoid cells within the lamina propria and Peyer's patch (PP) (Figure 1.8) (Casteleyn et al., 2010; Lillehoj & Trout, 1994; A. L. Smith et al., 2014). The GALT comprises more immune cells than any other tissue and is a site for a large amount of cellular traffic between immune structures and infection sites, in addition the structures within the GALT assist in the induction of immune responses (A. L. Smith et al., 2014).

Chickens do not possess highly structured lymph nodes like those seen in the mammalian immune system, instead they have lymphoid aggregates that line the gut and sample the lumen contents delivering it to macrophages and dendritic cells (A. L. Smith et al., 2014). The most studied structures of the avian GALT are the CT, which are a cluster of aggregated lymphoid tissue, forming nodules located near the ileocolonic junction (Casteleyn et al., 2010; Clench & Mathias, 1995; Lillehoj & Trout, 1994; A. L. Smith et al., 2014). The CT appear in the late embryonic stage of development, but do not fully develop until after hatching when B and T lymphocytes are formed in germinal centres (Casteleyn et al., 2010). In addition, plasma cells are present which secrete surface immunoglobulins M, Y and A which are crucial for protection from pathogens, and preliminary stages of the immune response (Lillehoj & Trout, 1994).

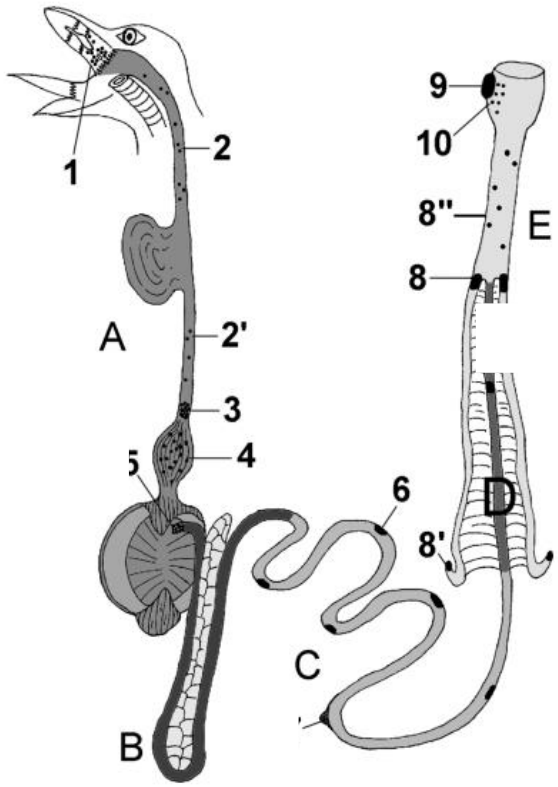

Figure 1.8 Schematic diagram of the chicken intestinal tract highlighting the GALT locations: 1, pharyngeal tonsil; 2 and 2', lymphoid tissue in oesophagus; 3, oesophageal tonsil; 4, lymphoid tissue of proventriculus; 5, pyloric tonsil; 6, Peyer's patch; 7, vitelline diverticulum; 8, caecal tonsils; 8', lymphoid tissue within caecum; 8'', lymphoid tissue in rectum; 9, bursa of Fabricius; 10, lymphoid tissue in proctodeum. (Taken from Casteleyn et al., 2010).

There are up to six PPs located within the chicken jejunum and may be referred to as intestinal tonsils. They consist of 40% B lymphocytes, 40% T regulatory cells, 5-9% macrophages, and minority cells such as dendritic and phagocytic cells (Casteleyn et al., 2010; Lillehoj & Trout, 1994). They function as an inductive site for IgA responses to

pathogenic microbes, for example differentiation of T regulatory cells within PP is driven by microbiota antigens carried by dendritic cells, that triggers IgA- producing B plasma cells to differentiate and increase secretion of IgA (Kogut et al., 2020).

The gut immune system is comprised of three layers, all of which are contained within the GALT; barrier, innate and adaptive immunity (Yoo et al., 2020)**.** Similarly, to mammals, IECs constitute a physical barrier to invading microbes (both pathogenic and commensal in nature) and secrete mucins that may be used by the host microbiota to inhibit unfavourable bacteria from reaching the underlying lamina propria (Abreu, 2010; Brisbin et al., 2008). In addition, mucus can trap invading bacterial pathogens and facilitates removal via luminal flow (Broom & Kogut, 2018). Chicken mucus, when compared to human mucus, was shown to decrease *C. jejuni* virulence *in vitro*; potentially owing to the differences in chicken mucin structure, glycosylation, and charge in comparison to human mucins (Brisbin et al., 2008; Byrne et al., 2007). Tight junctions between intestinal epithelial cells also play a role in barrier function, limiting the nutrients, minerals and microbes that can pass transcellularly into the blood stream (Wigley, 2013).

There are a range of AMPs that have an immunomodulatory role within the chicken gut and are secreted by Paneth cells (Abreu, 2010; Brisbin et al., 2008; Diaz Carrasco et al., 2019; Y. Hong et al., 2020). β-defensins are a sub-family of highly conserved AMPs that are expressed in the gut during infection. They are released in response to LPS detection by avian TLR-4 and cause bacterial cell lysis by permeabilization of the cell membrane (Diaz Carrasco et al., 2019; Wigley, 2013). Higher levels of defensin expression are also seen in birds with higher levels of intestinal inflammation; Avian β-defensin 8 (AvBD8), for example, has been shown to not only exhibit direct anti-microbial effects but stimulate expression of pro-inflammatory cytokines such as IL-1β, and IFNγ (Y. Hong et al., 2020; Wigley, 2013).

Pattern recognition receptors (PRRs) present on epithelial cells and immune cells of the lamina propria recognise pathogen-associated-molecular-patterns (PAMPs). More importantly TLRs are a class of PRR crucial for the initiation of the innate immune response due to their role in recognising PAMPs (Abreu, 2010; Jiao et al., 2019; Kogut et al., 2020). This interaction with host PRRs triggers cellular defence mechanisms including cytokine secretion, immune cell recruitment, and direct anti-microbial

mechanisms which are an important aspect of innate immunity (Kogut et al., 2020). Birds have 10 known TLRs and recognise specific components of a pathogen, for example, TLR-4 binds LPS (lipopolysaccharide) and TLR-5 recognises flagellin, however *Campylobacter* can evade this via glycosylation of its flagella (Wigley, 2013).

Intra-epithelial lymphocytes (IEL) are highly specialised and can be found within the lamina propria and represent components of the adaptive immune response (Beal et al., 2006). The major subsets of these cells include natural killer (NK) and T cells with varying forms of the T cell receptor (γδ or αβ), followed by a smaller subset of B cells the proportions of these cells within the epithelium vary dependent on bird age, genetics, and environmental factors (A. L. Smith et al., 2014). These cells function by producing cytokines, interferons, inducing cytotoxic reactions, and immunoregulation (A. L. Smith et al., 2014). The first evidence for functional differences in lymphocyte populations was demonstrated within the chicken model, showing that B cells are derived from the bursa of Fabricus, and T cells develop in the thymus (Beal et al., 2006). Avian T cells, when activated via T cell receptor (TCR), can destroy target cells via cytotoxic effects (cytotoxic T cells) and can assist B cells to differentiate (helper T cells) (Sharma & Tizard, 1984). B cells are activated by specific antigens, sometimes with assistance from $CD4^+$ cells; activated B cells differentiate into plasma cells which secrete antibodies (IgM, IgG/IgY or IgA), and repeat exposure to the same specific antigen induces class switching of antibodies.

There are three classes of chicken antibodies that have been characterised (IgM, IgY/IgG and IgA) (Carlander et al., 1999). Initial challenge by pathogenic bacteria stimulates B cells to produce IgM which is a B cell surface expressed antibody; IgY (homologue of human IgG) is mainly active during prolonged infections, class switching of IgM to IgY is initiated after extended exposure to a specific antigen (Beal et al., 2006). IgA is predominantly expressed by plasma cells, after B cells differentiation, within the intestines, it inhibits adhesion to epithelial cells and neutralises toxins (Beal et al., 2006; Curran, n.d.).

1.5 Limiting *Campylobacter* within Chickens

The chicken reservoir is responsible for between 50% to 80% of human *Campylobacter*iosis according to the European Food Standards Agency (EFSA) in 2008 (Koutsoumanis et al., 2020). Therefore, reducing or eliminating *Campylobacter* spp.

within broiler chicken flocks is essential for controlling the number of human Campylobacteriosis cases (Lin, 2009). Quantitative risk assessments have been conducted for targeting *Campylobacter* counts with varying results. A 2-log reduction in *Campylobacter* on the broiler chicken carcass could reduce the number of human Campylobacteriosis cases 30 times and reducing the number of caecal *Campylobacter* levels by 3-log has the potential to reduce the public health risk by 90% (Koutsoumanis et al., 2020; Rosenquist et al., 2003).

It is still largely unknown how *Campylobacter* initially infects chickens. However, it is understood that transmission is likely a combination of environmental sources, introduced by nearby livestock or due to poor biosecurity. There are also reports of vertical transmission from laying hens to chicks (Orhan Sahin et al., 2002b). Once infected, however, the bacterium spreads rapidly through flocks via horizontal transmission (Sibanda et al., 2018). There are multiple strategies employed throughout the supply chain to reduce the public health risk from *Campylobacter* (Sibanda et al., 2018). Within poultry farms the aim is to prevent *Campylobacter* entering houses, resulting in more negative flocks. Biosecurity measures such as overshoe use, vehicle disinfection, and rodent/insect control, are employed by poultry farms to reduce environmental exposure (Lin, 2009; Meunier et al., 2016b). In addition, commercial competitive exclusion cultures (such as Broilact) have shown to reduce *Campylobacter* load in the chicken gut (Lin, 2009; Schneitz & Hakkinen, 2016; Szott et al., 2022). There are also novel interventions that have been investigated and are being directed toward on-farm usage including antimicrobials such as bacteriophages, bacteriocins (Lin, 2009) and feed additives (Connerton et al., 2011; David Hermans et al., 2011b). Despite multiple strategies in place to limit the spread of *Campylobacter* within flocks there is not a fully effective, reliable strategy that is both economically and practically viable (Guyard-Nicodème et al., 2017).

1.5.1 Reducing Environmental *Campylobacter* Exposure in Chickens (Biosecurity)

Natural infection of commercial broiler flocks with *Campylobacter* is age dependent. Typically, birds are *Campylobacter* free until 2-3 weeks of age. However, infection can be detected as early as 10 days old (Newell & Fearnley, 2003). The period whereby newly hatched chicks may be infected, but load is undetectable is known as the lag phase; the cause of the lag phase is unclear and is seen in both commercial and free-range flocks

(Newell, 2001). At primary production there are several individual biosecurity practices that have been named by the European Food Standards Agency (EFSA) to limit the entry of *Campylobacter* into broiler houses and have been described as the only effective intervention if adhered to strictly (Facciolà, Avventuroso, et al., 2017; I Hansson et al., 2010; Koutsoumanis et al., 2020).

Minimising the footfall and number of staff entering any one specific house is a vital step that should be taken to reduce the risk of flock contamination (Koutsoumanis et al., 2020; Refrégier-Petton et al., 2001). Refrégier-Petton *et al.*, (2001) compared flock infection when tended by one or two members of staff and demonstrated that the number of *Campylobacter* positive flocks on-farm could be reduced by up to 15.8% when only one staff member was allowed entry to the house. In addition to movement between houses on the same farm, movement between multiple farms has also shown to be a key risk factor for *Campylobacter* exposure (Koutsoumanis et al., 2020; Lyngstad et al., 2008). Employment of animal caretakers from agencies should be avoided as this significantly increases the likelihood of flock infection via cross contamination (Lyngstad et al., 2008).

The term 'hygiene barrier' refers to a process that prevents bacterial contamination of an end-product with an infective dose capable of causing disease to consumers (SFHT, 2009). With regards to pathways into the broiler house which may be exploited by *Campylobacter* there are several hygiene barriers that have been identified.

Hygienic anterooms are common in European poultry farms, and these are located between the house door and the entrance to the broiler house (Koutsoumanis et al., 2020). The purpose of the anteroom is to set a clear, physical boundary between contaminated and clean area (closest to the birds) (Figure 1.9) (Soon & Baines, 2013). There is a

Figure 1.9 Schematic of hygienic anteroom in broiler houses

dedicated changing zone within the anteroom to provide an area for staff to change into footwear and overalls dedicated to that house (Sibanda et al., 2018); not only are there a separate set of boots and overalls for each house but there should be a basin for handwashing and a footbath for disinfection of boots when entering and leaving the anteroom and house (Hermans *et al*., 2011).

A study in the Netherlands investigated the effect of these parameters on reducing *Campylobacter* prevalence in 23 flock's broiler farms (Table 1.7) and found that the use of designated boots and handwashing prior to entering the house significantly decreased the risk of a flock becoming infected ($P < 0.05$) (Van de Giessen *et al*., 1996). In this study, boot disinfection did not reduce the overall risk of flock infection, however, boot dips have been shown to significantly increase the time taken for a flock to become infected (Evans & Sayers, 2000).

Table 1.7 Analysis of biosecurity practices across farms in the Netherlands in their efficacy of reducing the risk of flock infection with *Campylobacter* (Van de Giessen et al., 1996).

Biosecurity Practice	Number of positive flocks (%)	Number of negative flocks (%)	*P* value
Changing into house specific boots	10/23 (43.5)	12/15 (80.0)	0.032
Hand washing prior to entering the flock	5/23 (21.7)	9/15 (60.0)	0.021
Footbath disinfection prior to entering the flock	14/25 (56.0)	12/15 (80.0)	0.132

Due to the highly specific growth requirements of *Campylobacter*, abiotic sources of infection within the poultry house itself (house litter, water, food) pose a considerable risk (Newell *et al*., 2011). After a house has been cleared of the previous flock the immediate external area is cleaned and disinfected and this includes the food and water distribution equipment, to reduce the risk of a *Campylobacter* cross contamination (Damjanova *et al*., 2011; Koutsoumanis *et al*., 2020). Cardinale *et al*. (2004) studied the effect of various cleaning and disinfection practices on *Campylobacter* infection being carried over into a subsequent flock (Table 1.8). They showed that the area surrounding a poultry house, when poorly maintained with regards to cleanliness, correlated with an increased risk of carry-over infection (Cardinale *et al*., 2004). This contrasts to reports in a review by Newell *et al*. (2011) where the thoroughness of cleaning and disinfection did not correlate with infection.

Table 1.8 Effect of different cleaning and disinfection practices prior to placement of broiler flocks across broiler farms in Sengal (Cardinale et al., 2004).

Cleaning and Disinfection Practice	Positive flocks (%)	Negative flocks (%)
Downtime <15 days	52.9	21.4
Downtime >15 days	1.4	15.7
Cleaning	35.7	32.9
No cleaning	27.1	4.3
Detergent used for cleaning	32.9	25.7
Detergent not used for cleaning	30.0	11.4
Disinfection	30.0	27.1
No disinfection	32.9	10.0
Second disinfection	15.7	18.6
No second disinfection	47.1	18.6
Poultry house surroundings cleaned and disinfected	14.3	22.9
Poultry house surroundings not cleaned and disinfected	48.9	14.3
Poultry house surroundings clean	21.4	22.9
Poultry house surroundings dirty	41.4	14.3

The time between flock clearing and placement of chicks is referred to as downtime and has been reported to range from between three days to two weeks (Newell *et al*., 2011; Koutsoumanis *et al*., 2020). Studies focusing on a correlation between length of downtime and risk of *Campylobacter* infection provide conflicting data (Table 1.9).

Table 1.9 Summary of studies researching length of downtime between clearing and placing of chicks, and the percentage of positive flocks.

Length of downtime	Percentage positive (%)	*P* value	Reference
≤ 21 days	44	0.022	Berndtson *et al*. 1996
> 21 days	25		
1 – 7 days	51.2	<0.001	Georgiev *et al*. 2017
8 – 14 days	54.4		
15 – 21 days	48.3		
22 - 47 days	72.9		

1.5.2 Use of antibiotics therapies for *Campylobacter* reduction in chickens

The discovery of natural and synthetic antibiotics led to success in the reduction of bacterial infections and in addition increased feed efficiency in broiler production (Mehdi

& Godbout, 2018). Antibiotic use varies considerably between country due to animal husbandry legislation, economic variables, and level of development (Roth *et al*., 2019). In France, intensively farmed poultry flocks are routinely treated with polymyxins, penicillins and tetracyclines, however in Canadian broiler production, tetracyclines dominate the class of antibiotic administered (Mehdi & Godbout, 2018; Roth *et al*., 2019). Antibiotic-based growth promoters (AGPs) have been used in the UK and are still being used in the rest of the world to increase weight gain and improve feed conversion within broilers in addition to their antimicrobial therapeutic effect (Agyare *et al*., 2018; Cardinal *et al*., 2019). AGPs (Table 1.10) were legal for use (up until 2006) by the European Commission, (EC) however antibiotic resistance led to their withdrawal from animal production.

Table 1.10 AGPs banned for use within the EC (Cardinal et al., 2019)

AGP	Banned by EC since	Antibiotic Group
Bacitracin	1999	Cyclic Peptide
Monensin	2006	Ionophore
Virginiamycin	1999	Streptogramin
Tylosin	1999	Macrolide
Spiramycin	1999	Macrolide
Avoparcin	1997	Glycopeptide
Olaquindox	1999	Qunioxaline

While antibiotics have been effective at limiting intestinal pathogens, they can have detrimental effects on the intestinal microbiota and thus also negatively affect host defence. In addition, antibiotic resistance is becoming increasingly prevalent amongst *Campylobacter* isolates from both chicken and human sources (Price *et al*., 2005; Hughes, Hermans, & Morgan, 2008; Luangtongkum *et al*., 2010; Agyare *et al*., 2018; Roth *et al*., 2019).

Antibiotic resistant strains of *Campylobacter* in both human, environmental and poultry isolates has led to the ban of several commonly used antibiotics (avoparcin, virginiamycin, bacitracin zinc, tylosin phosphate, and spiromycin) in 1999 (Table 1.10) for use in animal production. By 2006 the European Commission banned the remaining AGPs to reduce the chance of antimicrobial resistance developing and transmitting these bacterial strains to humans (Hughes, Hermans, & Morgan, 2008).

1.5.3 Use of alternative therapies for *Campylobacter* reduction in chickens

There are numerous natural antimicrobial agents that can substitute for antibiotic use, the first of which are bacteriocins (Riley, 1998; Joerger, 2003; Mehdi & Godbout, 2018; Dai et al., 2020). Bacteriocins are small, antagonistic peptides that are ribosomally synthesised by bacteria and probiotics, and function by disrupting the bacterial membrane of target cells, thus killing other bacterial cells (Dai et al., 2020; El-Hack et al., 2021). At least one bacteriocin is produced by at least 30% of bacterial species, with many being produced by commensal bacteria of the intestines and function as innate defence mechanisms (Riley, 1998; Dai et al., 2020). Addition of bacteriocins to broiler feed prior to slaughter has been experimentally effective in reducing *Campylobacter* load in poultry and reduces food chain contamination. Furthermore *in-vitro* studies have demonstrated the antagonistic properties of these peptides (El-Hack et al., 2021) (Table 1.11).

Table 1.11 Summary of different bacteriocin effects on *Campylobacter* load

Bacteriocin	Bacterial origin	Effect	Reference
OR-7	*Lactobacillus salivarius* (NRRL B-30514)	Bacteriocin added to feed for 3 days (7-10 days of age) reduced colonisation at least one-millionfold compared with *Campylobacter* levels in untreated groups.	Stern *et al*., 2006
-	*Lactobacillus salivarius* (NRRL B-30514)	8/9 chicks administered 250mg bacteriocin/kg feed had no detectable *C. jejuni* levels, 1/9 chicks had significantly reduced cecal levels of *C. jejuni*	Stern *et al.*, 2006
-	*Paenibacillus polymyxa* (NRRL B-30509)	*C. jejuni* completely eliminated from 10 chick ceca administered 250mg bacteriocin/kg feed (>one-billion-fold reduction)	Stern *et al.*, 2006
SRCAM 37	*Paenibacillus polymyxa* (NRRL B-30507)	Antagonistic activity against multiple *Campylobacter* isolates from broiler chickens	Svetoch *et al.*, 2005
SRCAM 119	*Paenibacillus polymyxa* (NRRL B-30508)	Antagonistic activity against multiple *Campylobacter* isolates from broiler chickens	Svetoch *et al.*, 2005
SRCAM 602	*Paenibacillus polymyxa* (NRRL B-30509)	Antagonistic activity against multiple *Campylobacter* isolates from broiler chickens	Svetoch *et al.*, 2005
SRCAM 1580	*Bacillus circulans* (NRRL B-30644)	Antagonistic activity against multiple *Campylobacter* isolates from broiler chickens	Svetoch *et al.*, 2005

A recent advance in natural therapies to control *Campylobacter* within poultry is via the use of bacteriophages (El-Hack *et al.*, 2020). Bacteriophages are naturally occurring and are selected to target pathogenic bacteria. Receptors for bacteriophages are present on target *Campylobacter* and binding leads to bacterial cell lysis (Carvalho et al., 2010; K. Sørensen et al., 2012). The focus of bacteriophage use is not necessarily to prevent poultry colonisation but to reduce intestinal *Campylobacter* load within birds prior to slaughter (Abd El-Hack et al., 2020). Carvalho *et al.* (2010) demonstrated the potential of a three-phage cocktail to target and inhibit *C. jejuni* and *C. coli* within broiler chickens by two administration methods (oral gavage and via feed supplementation). The phages used, phiCcoIBB35, phiCcoIBB37, and phiCcoIBB12, were originally isolated from intestinal contents of poultry (Carvalho et al., 2010). The results from the *in vivo* trial showed that the "phage cocktail" reduced *C. coli* and *C. jejuni* by 2 $\log_{10}$ cfu/g regardless of administration method. Interestingly, feed supplementation with phages did however reduce *Campylobacter* at a faster rate than oral gavage (Carvalho et al., 2010).

Phage F336 (isolated from duck intestinal contents) is a *Campylobacter* targeting bacteriophage. Phage F336 relies on the MeOPN (*O*-methyl phosporamidate) receptor of the CPS of *C. jejuni* NCTC 11168 for successful infection and inhibition of the bacteria (M. C. H. Sørensen et al., 2011). However, *C. jejuni* has been shown to easily resist infection by phage F336 due to phase variation in the MeOPN transferase, suggesting that resistance mechanisms can be employed by *C. jejuni* (M. C. H. Sørensen et al., 2011) in response to this strategy.

All organisms produce AMPs and these serve a vital role in innate immunity. They have been used at low doses in feed to control outbreaks of enteric pathogens via selective killing of intestinal pathogenic bacteria and modification of the intestinal microbiota (Joerger, 2003; Nazeer et al., 2021; S. Wang et al., 2016). There are several benefits to using AMPs over conventional antibiotic therapies; i) they do not appear to induce bacterial resistance as readily due to their mechanism of action; ii) they protect the host by alternative mechanisms in addition to directly attacking pathogenic microbes; iii) they maintain gut homeostasis, and modulate inflammatory responses (S. Wang et al., 2016). The direct mechanism of action of AMPs can be concisely described as via binding to and subsequent disruption of the bacterial membrane or via blocking intracellular functions if the AMP enters the bacterial cell (Abd El-Hack et al., 2020). However, there are limitations to the use of AMPs; the LOS of *C. jejuni* specifically, appears to aid AMP resistance as reported by van Dijk *et al.* (2012), and proteolytic enzymes within the chicken gut may lead to instability of these compounds *in vivo* (Abd El-Hack et al., 2020).

1.5.4 Chicken feed additives for *Campylobacter* reduction in chickens

Feed formulation is of vital importance in the poultry industry as it affects gut health, which directly affects welfare and productivity of broiler chickens (Ali et al., 2021; Choct, 2009). Due to the ever-growing consumer demands for poultry products, feed used in commercial farming must be high quality and economically viable, in addition they must meet nutritional requirements to ensure efficient growth, prevent disease and result in high feed conversion ratios (FCR) (Pirgozliev et al., 2019).

The EFSA recognises five type of feed additives (Table 1.12) and defines feed additives as products used in animal nutrition to improve feed quality, improve quality of meat from the animal origin, and/or improve the animals' performance and health. Furthermore, the EFSA also recognises that feed additives may be used to influence the

environment with regards to methane reduction in commercial farming (EFSA, n.d.; Hashemi & Davoodi, 2010; Pirgozliev et al., 2019). In this section a selection of the feed additive categories and their target functions on poultry nutrition and health will be summarised.

Table 1.12 Five categories of feed additives recognised by the EFSA, examples and functions (EFSA, 2022).

Feed additive category	Example	Function	Reference
Technological	Organic acids, antioxidants, pellet binders	Feed preservation, protect feed from microbial/fungal destruction, positive influence on FCR or growth performance	Paul *et al.*, 2007
Sensory	Flavours, colourants	Effect on palatability and odour	Karásková, Suchý and Straková, 2016; Rychen *et al.*, 2018
Nutritional	Vitamins, amino acids	Boost immune function, protect proteins/lipids from oxidative damage	Choct, 2009; Shakeri *et al.*, 2020
Zootechnical	Enzymes, probiotics, prebiotics, phytogenics	Improve feed digestibility, stabilise, and benefit gut microflora, prevent risk of developing antibiotic resistant pathogens	Pirgozliev, Rose and Ivanova, 2019
Coccidiostats and histomonostats	Naturally occurring polyether ionophores, synthetic coccidiostats (i.e., halofuginone)	Control of protozoan infections, enhance FCR	Clarke *et al.*, 2014

1.5.4.1 Technological feed additives

Technological feed additives are aimed at improving the longevity of feed by preserving, preventing contamination with microbes and fungus, and may also have a role in improving feed conversion ratio (FCR) (Paul et al., 2007). These additives are not targeted at improving animal health. For example, bentonite is recognised as a technological feed additive by the EFSA for many animal species, its primary function is to reduce feed contamination with the mycotoxin aflatoxin (Rychen et al., 2017). Enzyme based technological additives, such as FUMzyme® have also been developed and have been shown to degrade fumonisin mycotoxins in feed (Rychen et al., 2016).

1.5.4.2 Sensory feed additives

Addition of sensory feed additives is not targeted at improving gut health, but rather improving feed quality, and palatability.

1.5.4.3 Nutritional feed additives

Nutrition is crucial for body growth of broiler chickens; nutritional additives, known as nutraceuticals, have immunomodulatory potential and boost immune function, which consequently prevents manifestation of various diseases (Alagawany et al., 2021; Choct, 2009; Shakeri et al., 2020). Amino acids are added to poultry feed due to their role as constituents of protein which can increase broiler body mass, however there is a limit to the quantity of amino acids that can be used in replacement of protein within feed and achieve optimal growth performance (Alagawany et al., 2021; Baker, 2009). Threonine has been extensively investigated as an amino acid additive, within broilers it has shown to improve the thickness of the intestinal epithelia, promote antibody synthesis, improve FCR, and reduce quantity of intestinal cytokines in LPS-challenged birds (Al-Hayani, 2017; Alagawany et al., 2021; Azzam & El-Gogary, 2015; Y. Chen et al., 2018; Zaefarian et al., 2008).

1.5.4.4 Zootechnical feed additives

Phytogenics are considered a zootechnical additive by the EFSA, they are plant-derivatives/extracts and include herbs, spices, essential oils, and oleoresins (e.g., balsam) (Hashemi & Davoodi, 2010; Pirgozliev et al., 2019). The EFSA recognises phytogenics within the zootechnical category of feed additives in animal nutrition, however Karásková, Suchý and Straková, (2016) summarise how phytogenic additives can be classified into multiple categories. Carotenoids are derived from carrots and are commonly used as a sensory additive for laying hen feed to increase colour characteristics of the egg yolk (Karásková et al., 2016; Kotrbáček et al., 2013). In terms of being technological additives, phytogenic additives such as essential oils (oregano, anise, and citrus peel) resulted in lower ammonium concentration in the ileum of broiler chickens (J.-C. Hong et al., 2012; Karásková et al., 2016). Immunomodulation and animal product quality are the outcomes of zootechnical phytogenics such as yeast (Karásková et al., 2016). With regards to use in chicken feed, there is significant interest in using this class of feed additive as an alternative to antibiotic growth promoters, due to increasing

antibiotic resistance and subsequent limitation on the use of antibiotics in the poultry industry (Ali et al., 2021; Hashemi & Davoodi, 2010).

Pre- and probiotics are feed additives that are used to improve gut health and subsequently the immune system they are commonly used as an alternative to antibiotic growth promoters (Adhikari & Kim, 2017). Prebiotics are defined as 'a selectively fermented ingredient that results in specific changes in composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health' (Pourabedin & Zhao, 2015); competitive exclusion of pathogens by the gut microbiota of chickens fed prebiotics has been described by Callaway *et al.* (2008) and improvement of gut morphology has been reported by Pourabedin *et al.* (2014) (Pourabedin and Zhao, 2015). Prebiotics act by providing nutrition for beneficial groups of gut microflora to gut mucosa and epithelia (Adhikari & Kim, 2017). Probiotics are defined by WHO as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Adhikari & Kim, 2017). Their most common mechanism of action is competing with pathogenic microbes for a common niche within the gut for replication, this does not just refer to competition for physical attachment sites within the intestinal tract but also competition by production of antimicrobial compounds and enhancement of the host animal immune system (Abd El-Hack et al., 2020; Adhikari & Kim, 2017).

1.5.4.5 Current *Campylobacter* targeted feed additives

There are a range of commercial feed products currently used in poultry farming aimed at reducing *Campylobacter* load and prevalence within flocks. Probiotics are a class of feed additive that are currently popular within poultry farming due to their natural origin, growth promoting, and antimicrobial effect. Probiotics have been used in intensive farming since the 1960s, however scientific investigation regarding their use began in the 2000s (Santini et al., 2010). Commonly used probiotic species in commercial feed additives that target *Campylobacter* via competitive exclusion, bactericidal effect, and/or improving gut immunity include *Lactobacillus acidophillus, Enterococcus faecium, L. casei,* and *Bifidobacterium thermophilus* (Morishita et al., 1997; Santini et al., 2010; Willis & Reid, 2008). Willis and Reid (2008) investigated the effect of a commercially available probiotic mixture on broiler performance, *Campylobacter* load and organ weight in North Carolina, USA. The commercial feed additive was PrimaLac DFM and the primary probiotic species and minimum presence per g of feed was reported ($1x10^8$ cfu/g of *Lactobacillus casei, L. acidophillus, Bifidobacterium thermophilum, and*

Enterococcus faecium). The study reported that bodyweight of male chicks in the DFM fed group were significantly lower than the male chicks in the control group, as was carcass yield; however female chicks responded significantly better with regards to bodyweight on DFM feed (Willis & Reid, 2008). The DFM group in this study also showed reduced levels of *Campylobacter* but were not free of the pathogen; the study concluded that the commercial probiotic mixture was not sufficient for acceptable reduction of *Campylobacter* (Willis & Reid, 2008). When the feeding of probiotic was coupled with restricted feeding (8 h/day) *Campylobacter* colonisation was reduced to lowest levels within the study compared to the control group indicating the importance of multiple factors in administration of feed additives to achieve the best results (Abd El-Hack et al., 2020).

Mortada *et al.* (2020) investigated the efficacy of the commercial probiotic PoultryStar ME (BIOMIN America, Inc) which contains *Lactobacillus reuteri, Pediococcus acidilactici, Bifidobacterium animalis*, and *Enterococcus faecium*, on *C. coli* proliferation *in vitro* and *C. coli* cecal load *in vivo*. Interestingly, *in vitro* the supernatant from these probiotics reduced *C. coli* proliferation significantly at a 1:1 supernatant: pathogen dilution, increasing the ratio of supernatant:pathogen further inhibited proliferation of *Campylobacter* (Mortada et al., 2020). Despite this positive result *in vitro*, there was no significant reduction in caecal *C. coli* load or carcass contamination *in vivo* when birds were fed 0.5kg probiotic/ton basal feed; this study highlighted the need for understanding *Campylobacter* transmission to poultry farms and that *in vitro* experimentation does not always mimic the biological processes and responses that are seen *in vivo*.

1.5.4.6 Novel *Campylobacter* targeted feed additives for chickens

There are several bioactive substances that are being scientifically investigated as potential *Campylobacter* feed additives including probiotics, prebiotics, phytochemicals, and organic acids; their natural origins, and antimicrobial properties have made them desirable as alternatives to antibiotics.

TYPLEX® Chelate, is a novel synthetic feed additive, formed of L-tyrosine and iron (Fe (III)) (Khattak et al., 2018; Skoufos et al., 2019). Khattak *et al.* (2018) found that *C. jejuni* biofilm formation was significantly reduced *in vitro* when challenged with TYPLEX® Chelate, in addition this novel feed additive reduced *C. jejuni* carriage in the ceca of birds challenged with litter contaminated with *C. jejuni* by 2 $\log_{10}$ per gram caecal sample

(Skoufos et al., 2019). Inhibiting the ability of *Campylobacter* to form biofilms reduces environmental survival of the pathogen and its entry into the food chain, additionally biofilm formation is a key factor in persistent human infection (Khattak et al., 2018). Because human infection is typically associated with the consumption of contaminated poultry, it is vital that caecal colonisation of broilers is reduced. When this novel feed additive was administered in doses of 0.05 and 0.20 g/kg of feed the *C. jejuni* load within the ceca was significantly reduced (Khattak et al., 2018). The hypothesised mechanism of action for TYPLEX® chelate, that reduces caecal *Campylobacter*, is based on increases in volatile fatty acids (VFA) being associated with the reduction of Enterobacteriaceae in broiler chickens (Khattak et al., 2018; Kubena et al., 2001). Fermentation of probiotic bacteria results in VFA formation, and these compounds are largely attributed to the antimicrobial effects of probiotics (Olnood et al., 2015).

Organic short- chain fatty acids (C_1- C_7) (SCFA) and Medium-chain fatty acids (C_8- C_{12}) (MCFA), have been shown to alter micro-environments by acidification which is unfavourable for *Campylobacter* leading to pathogen inactivation (Jansen et al., 2014; Molnár et al., 2015)**.** Additionally, these compounds exhibit anti-*Campylobacter* activity *in vitro* (F. Solis de los Santos et al., 2009; Van Gerwe et al., 2009). Heres *et al.* (2004) demonstrated that feed supplemented with high levels of the SCFAs lactic acid and acetic acid at 5.7% and 0.7% respectively, reduced the *in vitro* growth of *Campylobacter* by 2-3 $\log_{10}$ cfu. The *in vivo* effects of this SCFA mixture were also investigated and showed that acidified feed reduced broiler chicken susceptibility to *Campylobacter* infection, however, the addition of SCFA at high concentrations has been shown to negatively impact broiler body weight (Van Gerwe et al., 2009). It has been speculated by Van Gerwe *et al.* (2009) that lowering the pH to <5.5 may be the causative factor in decreased body weight gain due to chicken intestinal pH being 5.8-6.0. At pH >5.5 the activity of SCFA ceases, however MCFA such as 1-monoglyceride of capric acid maintain activity at pH 7.0 by mixing with a buffer, feed, and tap water, which could bypass the negative effects of low pH on bodyweight gain and feed conversion (Thormar et al., 2006; Van Gerwe et al., 2009).

1.5.4.6.1 Caprylic Acid

Caprylic acid is an 8-carbon medium-chain fatty acid that is generally recognised as safe (GRAS), despite not being commercially available as a feed or water additive for broiler chickens it has shown promising results against *Campylobacter* both *in vitro* and *in vivo*,

however some studies have shown inconsistent results (Table 1.13) (Metcalf et al., 2011). The effect of MCFA, in general, still does produce contradictory results as demonstrated by Hermans *et al.* (2010), who reported a combination of caproic, caprylic and capric acid exhibited *in vitro* anti-*Campylobacter* effects *in vitro*, but these effects were not reflected *in vivo* on caecal *Campylobacter* loads.

Table 1.13 Summary of *in vitro* and *in vivo* studies using the novel feed/water additive caprylic acid against *Campylobacter*.

In vitro* or *in vivo	**Concentration(s) administered (%)**	**Effect on *Campylobacter***	**Reference(s)**
In vitro	0.1, 0.5	After coincubation for 30mins a 1 $\log_{10}$ cfu/mL reduction was seen for 0.1% caprylic acid; 0.5% caprylic acid reduced the cfu/mL by more than 7.9 $\log_{10}$ cfu/mL	Molatová *et al.*, 2010
In vivo	0.35, 0.7, 1.4	3- and 7- day feed supplementation with 0.7% caprylic acid decreased *C. jejuni* counts within the caeca by 3 and 2 logs cfu/g respectively 1.4% caprylic acid showed inconsistent effect 0.35% caprylic acid effective when fed for 7 days	de los Santos *et al.*, 2009
In vivo	0.35, 0.525, 0.7, 0.875, 1.05, 1.225, and 1.4	<1.05% caprylic acid consistently reduced caecal *Campylobacter* content 0.7% caprylic acid reduced caecal *Campylobacter* content compared to positive control	de los Santos *et al.*, 2008
In vivo	0.175, 0.35, 0.7, 1.4, 2.8	Water soluble caprylic acid administered 8 days post exposure to *Campylobacter*; in the first trial, 0.175% caprylic acid reduced caecal *Campylobacter* counts but this was not consistent in trial 2	Metcalf *et al.*, 2011

Caprylic acid presents a practical and economically viable option as a commercial feed additive targeted at decreasing *C. jejuni* carriage in broiler chickens. Due to the variable nature of this compound in reducing the enteric *C. jejuni* counts, further investigation into these compounds mechanisms of action must be undertaken to optimise the bactericidal effect and produce consistent results. Suggested mechanisms of action of caprylic acid may be like a pre- or probiotic, in that it alters the intestinal microbiota, which in turn

decreases *Campylobacter* counts, this hypothesis is supported by organic acid increasing the lactic acid bacteria count in the ileum and cecum of broiler chickens (Yadav & Jha, 2019). In addition caprylic acid may have a direct effect on *C. jejuni* colonisation by inhibiting virulence factors, however further investigation is required to confirm this hypothesis (F. Solis de los Santos et al., 2009).

1.5.4.6.2 Butyric Acid

Butyric acid is an organic 4-carbon volatile-SCFA (VSCFA) and has been identified as a crucial compound for the correct development of GALT, and therefore immune modulation, and as an energy providing substrate for the host post-absorption, for example as a prime energy source for enterocytes that line the intestinal tract (Antongiovanni et al., 2007; Fernández-Rubio et al., 2009; Józefiak et al., 2004). In addition, this compound has been recognised as an inhibiting factor for some pathogenic microbes without affecting the host intestinal microbiome (Fernández-Rubio et al., 2009; Józefiak et al., 2004). SCFAs such as butyric acid has been used in the United States during meat processing as a surface animal carcass wash to remove bacterial contamination, however this is not entirely effective as remaining microbes are able to proliferate and remain on poultry products at unacceptable levels (Beier et al., 2019). In poultry farming, butyrate supplementation has been shown to significantly reduce infection of birds with other enteric pathogens such as *Salmonella enteriditis*, leading to the interest in investigating this compound as a *Campylobacter* targeted additive (Fernández-Rubio et al., 2009). Several studies have investigated the potential of butyric acid as a new broiler feed additive targeted at *Campylobacter* reduction and improving broiler gut health (Table 1.14) and thus far have shown that butyrate does provide beneficial effects on intestinal health, protection of cells against *Campylobacter* pathogenesis, and is bactericidal against *Campylobacter* strains. However, there is variation in the efficacy of butyrate as a bactericidal compound between different bacterial strains, and some studies have highlighted that combination treatments of butyrate with probiotics may enhance anti-*Campylobacter* effects (Kovanda et al., 2019; Ocejo et al., 2017).

Table 1.14 Summary of *in vitro* and *in vivo* studies using the novel feed additive butyric acid against *Campylobacter* and its effect on gut health

In vitro* or *in vivo	**Concentration(s) administered (%) ***	**Effect on gut health**	**Effect on *Campylobacter***	**Reference(s)**
In vivo	0.1% calcium butyrate 0.1% calcium butyrate + 6% dry whey powder	Broiler villus height increased by day 28 compared to control diet (all treatments) Butyrate + whey diet had highest villus height: crypt depth compared to all other treatments	No difference in colonisation or shedding ($p > 0.05$)	Ocejo *et al.*, 2017
In vitro	0.001, 0.025, 0.05, 0.1, 0.2, 0.25, 0.3, 0.35	n/a	0.05% and 0.08% butyric acid were the MIC values against two strains of *C. jejuni*	Kovanda *et al.*, 2019
In vivo	0.05	n/a	No significant reduction in *C. jejuni* 5 dpi	Van Deun, Haesebrouck, *et al.*, 2008; Van Deun, Pasmans, *et al.*, 2008; M Meunier *et al.*, 2016
In vivo	0.1, 0.25	No effect on broiler gut immunity compared to control treatment	Significantly reduced number of viable *Campylobacter*	Ebrahimi *et al.*, 2016
In vitro	0.25mM, 0.5mM	Concentration dependent decrease in *C. jejuni* invasion into CaCo-2 monolayer	n/a	Van Deun, Pasmans, Van Immerseel, *et al.*, 2008

*If concentrations not available in %, alternate units stated

1.5.4.6.3 Chromium

Chromium (Cr) is a biologically active, essential mineral for animals and humans, it is found naturally within the body in trace amounts, and contributes to various metabolic activities (Arif, Alagawany, et al., 2019; Dębski et al., 2004). One important function is in glucose metabolism, which varies greatly between humans and poultry due to poultry species being more resistant to insulin than mammals, resulting in higher blood glucose levels and lower insulin levels (Brooks et al., 2016; Spears et al., 2019). Intensive

production of broiler chickens places several stressors on the birds, which ultimately reduces performance. One important parameter affected is heat induced stress, during which the hormones released (e.g., corticosterone) reduce sensitivity of broilers to insulin even further (Brooks et al., 2016; Spears et al., 2019). It is well documented that a function of Cr is to improve receptor sensitivity within insulin-sensitive tissues, leading to improved cell uptake of glucose, indicating the potential of Cr broiler feed supplementation to increase broiler productivity and performance (Arif, Hussain, et al., 2019; Piray & Foroutanifar, 2022; Spears et al., 2019; White & Vincent, 2019). Chromium chloride, at present, is the most common form of Cr used to supplement commercial poultry feed for nutritional purposes, however it is poorly absorbed by poultry within the GI tract. Organic forms of Cr are more efficiently absorbed and able to cross the intestinal epithelium as they are chelated with amino acids (e.g., chromium-methionine) (Safwat et al., 2020).

There are many reviews on the effect of Cr supplementation on broiler performance, however this novel compound's potential for targeting pathogenic bacteria is poorly described (Arif, Hussain, et al., 2019; Brooks et al., 2016; Omoleye et al., 2021; Spears et al., 2019; White & Vincent, 2019). Safwat *et al.* (2020) compared the effect of inorganic Cr (Chromium oxide – CrOx) versus the organic complex Cr methionine (CrMe) on total bacterial counts, and bacterial counts of *Salmonella* and *E. coli* in broiler chicks. This research found that total bacteria count was significantly reduced in both diets supplemented with either the organic or inorganic Cr complex compared to control feed; *Salmonella* and *E. coli* counts were significantly reduced in broiler chicks fed Cr supplemented diets, however the organic form (CrMe) at the highest dose reduced *Salmonella* counts significantly more than the inorganic form (CrOx) at its highest supplemented concentration (Table 1.15) (Safwat et al., 2020).

Table 1.15 Bacterial counts (number x 10^6) for broiler chicks supplemented with difference complexes and levels of chromium (taken from Safwat et al., 2020)

	Control	Chromium oxide		Chromium methionine	
Bacteria		**500ppb**	**1000ppb**	**500ppb**	**1000ppb**
Total bacterial count	3.2^{A}	2.35^{B}	2.47^{B}	2.45^{B}	2.2^{B}
Salmonella	1.15^{A}	0.7^{BC}	0.85^{B}	0.75^{BC}	0.6^{C}
E. coli	1.35^{A}	0.9^{B}	0.85^{B}	0.85^{B}	0.75^{B}

Note: Means within the same row with different letters are significantly difference ($p < 0.05$)

The reduction in pathogenic enterobacteria seen in broiler chickens administered feed supplemented with Cr suggests that addition of Cr above the nutritionally required levels increases immune status, as reported by Lee *et al.* (2003) who observed increased antibody production against infectious bronchitis in broilers supplemented with 400 ppb Cr (Safwat et al., 2020). Positive linear associations between serum IgG levels and Cr supplementation in broiler feed were also reported by Piray and Foroutanifar (2022) (Figure 1.10). In contrast, it has been reported that high doses/long term exposure to Cr may induce cytotoxic and genotoxic reactions that are detrimental to the body, this mechanism is poorly understood, however it is understood that the bioavailability, solubility of the compound, and chemical speciation of the Cr complex contributes greatly to its biological outcome (Shrivastava, 2002).

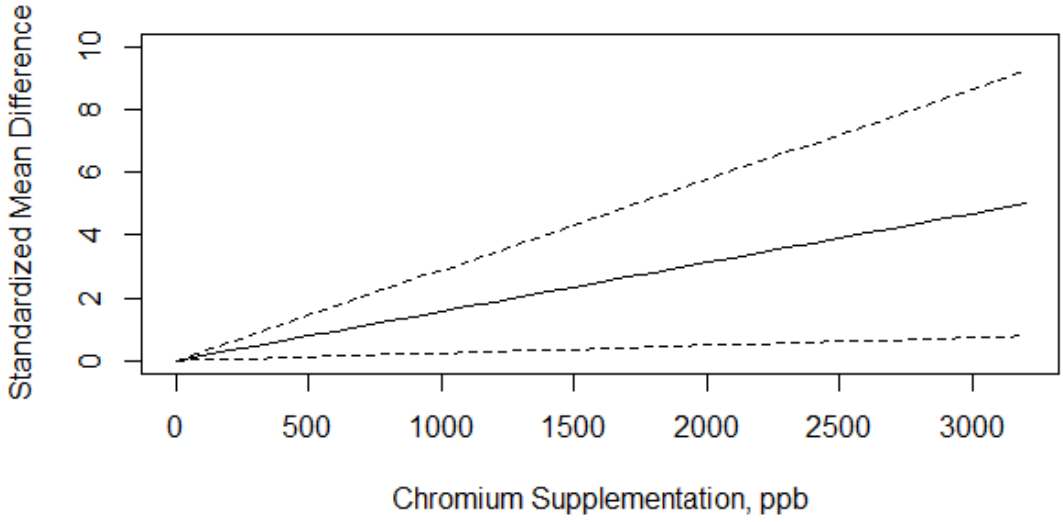

Figure 1.10 Association between chromium supplementation and serum IgG concentration. The solid line and the dashed lines represent the estimated standardized mean difference and its 95% confidence intervals. No chromium supplementation (0 µg/kg diet, ppb) was used as the control diet. (Taken from Piray & Foroutanifar, 2021)

The present study investigates the potential for butyric acid, caprylic acid and chromium propionate to be used as *Campylobacter* targeted feed additives in the poultry industry. There are significant differences in the structure and chemical formulation of the proposed additives as summarised in Table 1.16.

Table 1.16 Structure of feed additives used in this study

Common Name	IUPAC Name	Chemical Formula
Caprylic Acid	Octanoic Acid	$C_8H_{16}O_2$
Butyric Acid	Butanoic Acid	C_3H_7COOH
Chromium Propionate	Propanoic acid, Chromium (3+) salt	$C_9H_{15}CrO_6$

1.6 Limitations to current literature

When assessing the available scientific literature and studies most of the *in vitro* work for interactions of *Campylobacter* and gut epithelial cell lines is done using human cells, namely CaCo-2 (human) or HeLa, with little exploration into *in vitro* interactions with avian cell lines (such as 8E11). John *et al.* (2017) is one of few papers that explore avian host immune responses to *Campylobacter in vitro* and provide quantitative analysis on the cytokine production of these cells. The additives within this thesis will be targeted at *Campylobacter* within the avian host, as opposed to the human host, and thus it is essential that the molecular mechanisms of *Campylobacter* within avian cells (e, g, 8E11) are thoroughly investigated.

Secondly, the data surrounding the efficacy of feed additives is largely conducted during *in vivo* trials, reporting the *Campylobacter* reduction in cfu/g at colonisation sites but not investigating the underlying mechanism of action. For instance, is the compound directly bactericidal or indirectly contributing to improved host gut health and immunity? This thesis aims at exploring the potential molecular mechanisms of three organic compounds and four probiotic products to target *Campylobacter* reduction within the avian host.

Finally, *in vitro* study of *Campylobacter* is often limited to studying only one or two laboratory strains (e.g *C. jejuni* NCTC 11168); it is known that there are significant differences between strain virulence, pathogenicity, motility, invasive capacity, and metabolism. Therefore, studying a limited number of strains does not provide data that represents *Campylobacter* as a species. This thesis studies seven strains with the aim to better represent the spectrum of *Campylobacter* activity and associated characteristics.

1.7 Aims and Objectives

The main aim of this thesis was to investigate the potential of feed additives in reducing *Campylobacter* growth and invasion *in vitro* and to uncover their potential mechanisms

of action. Thus, the main focus of this thesis is: i) identify *Campylobacter* strains that represent the invasive spectrum exhibited by this bacterial species, ii) determine the direct effects of feed additives and probiotic strains on *Campylobacter*, and iii) study human and chicken host interactions with *Campylobacter*, feed additives, and probiotic strains using epithelial cell lines. The hypothesis of this thesis is **that feed additives can inhibit *Campylobacter* invasion *in vitro* through growth limiting action and by aiding host immune defences.**

Chapter specific aims were:

Chapter 3:

- Investigate genetic diversity within a collection of *Campylobacter* isolates focussing on virulence and antimicrobial resistance genes.
- Determine the differences in *Campylobacter* strain growth under varying atmospheric conditions and temperatures.
- Investigate the effect of source of isolation on invasive capabilities of *Campylobacter*.
- Identify a set of standard *Campylobacter* to use in feed additive testing during *in vitro* assays.

Chapter 4:

- Determine the direct bactericidal effect of feed additives on *Campylobacter* growth *in vitro.*
- Determine the direct bactericidal effect of media conditioned with probiotic species on *Campylobacter* growth *in vitro.*
- Investigate the ability of chromium propionate to affect the motility of *Campylobacter in vitro.*

Chapter 5:

- Determine if feed additives are cytotoxic to human and avian epithelial cell lines at proposed concentrations.
- Determine if feed additives provide a protective effect to human and avian cell lines against *Campylobacter* invasion *in vitro.*

- Quantify inflammatory cytokines expressed by avian epithelial cell lines induced by exposure to *Campylobacter* strains.
- Quantify inflammatory cytokines expressed by avian epithelial cell lines pre-incubated with feed additives and how preincubation affects host cell responses to *Campylobacter* exposure.

Chapter 2: Materials and Methods

2.1 Materials

The materials used throughout this study have been organised into specific tables. These are chemical reagents, cell culture media and media (Table 2.1), primers and probes (Table 2.2) and finally prepared solutions (Table 2.5).

Table 2.1 Chemical reagents, media and other premade solutions used throughout the study

Chemical reagent, media, antibiotics, enzymes	Includes	Supplier	Product Code
Oxoid™ *Campylobacter* liquid growth supplement (500 mL)	-	Oxoid	13295409
Columbia Agar base with 5% Defibrinated Horse Blood (pH 7.3± 0.2 at 25˚C)	Special Peptone (23.06 gm/L) Starch (1.0 gm/L) Sodium Chloride (50 gm/L) Agar (10 gm/L)	Oxoid	CM0331
Triton X-100, laboratory grade (100 mL)	-	Sigma-Aldrich	9002-93-1
Gentamicin (50 mg/mL) (10 mL)	-	ThermoFisher Scientific	15750060
AlamarBlue™ Cell Viability reagent	-	ThermoFisher Scientific	DAL1025
TrypLE express enzyme (500 mL)	No Phenol Red EDTA	Gibco	12604021
DMEM/F12 Glutamax™ Supplement (500 mL)	Glutamax™ Phenol Red High Glucose Sodium Pyruvate No HEPES	Gibco	31331093
DMEM, high, glucose + pyruvate (500 mL)	L-Glutamine Phenol Red Sodium Pyruvate High Glucose No HEPES	Gibco	41966029
MEM Non-essential Amino Acid Solution (100x) (100 mL)	-	Gibco	11140050
Phosphate Buffered Saline (PBS), pH 7.4 (for cell culture) (500 mL)	No Calcium No Magnesium No Phenol Red No Sodium Pyruvate	Gibco	10010023
PBS tablets (pH 7.3 – pH 7.5)	137 Mm Sodium Chloride 2.7 mM Potassium Chloride 10 mM Phosphate Buffer	VWR	E404-200TABS

	(Then 1 tablet dissolved in 100 mL water)		
Trypan Blue (0.4%) (100 mL)	-	Gibco	15250061
L-Glutamine (L-Glut) (220 mM) (100 mL)	No Phenol Red	Gibco	A2916801
Penicillin-Streptomycin (Pen-Strep)	10,000 units/mL penicillin 10,000 µg/mL Streptomycin	Gibco	15140122
Amphotericin B (250 µg/mL) (50 mL)	-	Gibco	15290026
Fetal Bovine Serum (FBS), qualified, Brazil (500 mL)	-	Gibco	10270106
MAC cabinet gas supply	10% Carbon Dioxide 5% Oxygen 85% Nitrogen	BOC	226971-L
Brucella Agar Powder	Agar (13 g/L). Casein Peptone (10 g/L), D (+)-Glucose (1 g/L), meat peptone (10 g/L), sodium chloride (5 g/L), yeast extract (2 g/L)	Sigma Aldrich	18795-500G
CampyGen 3.5 L sachet	-	Oxoid	CN0035A
Sodium Hydroxide			
iScript cDNA synthesis kit	5X Reverse-Transcriptase Reaction Mix, iScript reverse transcriptase, nuclease-free water	BioRad	1708891
Brilliant II qPCR MasterMix	-	Agilent	600804
Glycerol (100%)	-		
HEPES solution BIOXTRA (100 mL)	1 M, pH 7.0-7.6	Sigma (Merck Life Science UK Ltd)	H0887
RNeasy Plus Mini Kit (250)	RNeasy Mini Spin Columns, gDNA, Eliminator Spin Columns, Collection Tubes, RNase-Free Water, Buffers	Qiagen	74136
Ham's F-12 Nutrient Mix GlutaMAX™ Supplement	-	Fisher Scientific	11514436
QIAamp DNA Mini Kit	QIAamp Mini Spin Columns, QIAGEN Proteinase K, Reagents, Buffers, Collection Tubes (2mL)	Qiagen	51306
RNase A 2.5 mL (100 mg/mL)	-	Qiagen	19101
Caprylic Acid 100% (1%; 69 mM)	-	Kemin Animal Nutrition and Health	-
Butyric Acid 100% (1%; 113 mM)	-	Kemin Animal Nutrition and Health	-
Chromium Propionate (10%)	Composed of: Propionic Acid, Sodium Propionate and Propylene Glycol	Kemin Animal Nutrition and Health	-
LPS (1mg/mL)	*E. coli* 055:B5	Invivogen	

Table 2.2 Primers and probes used throughout the study; the probes used in this study have been modified from the reference publication to have different fluorescent labels

cDNA	Probe Name	Probe Sequence 5'-3'	Primer Sequence 5'-3'	Reference
Chicken 28S	SK28S	[**FAM**] AGGACCGCTACGG ACCTCCACCA [**TAM**]	**(F)** GGCGAAGCCAGAGG AAACT **(R)** ACGACCGATTTGCAC GTC	(Shini & Kaiser, 2009)
Chicken CXCLi1	CXCLi1	[**ROX**] TCGCTGAACGTGCT TGAGCCATACCTT [**BHQ_2**]	**(F)** TGGCTCTTCTCCTGA TCTCAATG **(R)** GCACTGGCATCGGA GTTCA	(Shini & Kaiser, 2009)
Chicken CXCLi2	CXCLi2	[**HEX**] TCTTTACCAGCGTC CTACCTTGCGACA [**BHQ_1**]	**(F)** GCCCTCCTCCTGGTT TCAG **(R)** TGGCACCGCAGCTC ATT	(Shini & Kaiser, 2009)
Chicken TGFβ	TGFβ4	[**FAM**] ACCCAAAGGTTAT ATGGCCAACTTCTG CAT [**TAM**]	**(F)** AGGATCTGCAGTGG AAGTGGAT **(R)** CCCCGGGTTGTGTTG GT	(Shini & Kaiser, 2009)

Primers and probes used throughout the study have specific efficiencies for analysis annealing temperatures for qPCR (Table 2.3 and 2.4 respectively). Primer efficiencies were determined previously by Dr Heather Chick at Swansea University (unpublished work) according to the following method: complementary DNA (cDNA) was diluted 1:10 in a 10 series, a qPCR reaction was conducted, and a standard curve was plotted. qPCR reactions were started at a temperature of 55˚C (Table 2.4) and increased by 1˚C until maximum efficiency was reached. The slope of the regression was used to calculate primer efficiency using the following equation:

$$Efficiency\ (\%) = (10^{\frac{-1}{slope}} - 1) \times 100$$

The primer efficiency output using this equation varied between 105-123% (Table 2.3). The primer efficiency (%) was converted to be used in the pfaffl equation.

Table 2.3 Primer efficiency and converted primer efficiency for probes used in the study

Primer/Probe set	Primer Efficiency (%)	Converted Primer Efficiency
28S	111.6	2.116
CXCLi1	122.1	2.2211
CXCLi2	112.57	2.1257
TGFβ	105.89	2.0559

Table 2.4 probe specific annealing temperatures for qPCR reactions

Primer/Probe set	Annealing temperature (˚C)
28S	55
CXCLi1	57
CXCLi2	55
TGFβ	59

Table 2.5 Solutions prepared and used throughout the study

Solution	Includes
Glycerol stock for bacterial preservation	50 mL sterile H_2O + 50 mL 100% glycerol
8E11 cell culture medium	500 mL DMEM/F12 Glutamax™ Supplement + 5 mL Penicillin-Streptomycin (Pen-Strep) (10.000 U/ml, 10,000 µg/mL) + 5 mL L-Glutamine (L-Glut) (220 mM) + 5 mL Amphotericin B (250 µg/mL) + 50 ml Fetal Bovine Serum (FBS)
8E11 antibiotic free cell culture medium	500 mL DMEM/F12 Glutamax™ Supplement + 5 mL L-Glutamine (L-Glut) (220 mM) + 50 ml Fetal Bovine Serum (FBS)
CaCo-2 cell medium	500 mL DMEM, high, glucose + pyruvate + 5 mL Penicillin-Streptomycin (Pen-Strep) (10.000 U/ml, 10,000 µg/mL) + 5 mL L-Glutamine (L-Glut) (220 mM) + 5 mL MEM Non-essential Amino Acid Solution + 50 mL Fetal Bovine Serum (FBS)
CaCo-2 antibiotic free cell culture medium	500 mL DMEM, high, glucose + pyruvate + 5 mL L-Glutamine (L-Glut) (220 mM) + 5 mL MEM Non-essential Amino Acid Solution + 50 mL Fetal Bovine Serum (FBS)
0.1% Triton X-100 in PBS	0.5 mL 100% Triton X-100 + 499.5 mL 100% PBS (5 tablets dissolved in 500 mL)
Ethanol (known %)	100% ethanol (variable volume) + sterile H_2O (variable volume)
Caprylic Acid 10% stock solutions (693.4 mM)	1 mL 100% Butyric Acid + 9 mL sterile H_2O
Caprylic Acid 2X working concentrations	0.5%, 1.5%, 2.5% 3.5% achieved by diluting variable volume of 10% working solution in variable volumes of sterile H_2O or media (protocol specific); HEPES buffer (1M) added in variable volumes to make solution pH 7.0
Caprylic Acid 1X working concentrations	0.25% (17.3mM), 0.75% (51.9mM),1.25% (86.5mM), 1.75% (121.1mM)

Butyric Acid 10% stock solutions (1134.9 mM)	10 mL Butyric acid pH 7.0 (variable mL Sodium Hydroxide (5.0 M) + variable mL 100% Butyric Acid – added using pH probe until solution pH = 7.0) + mL sterile H_2O
Butyric Acid 2X working concentrations	0.4% (45.4mM), 1.2% (136.2mM), 2.0% (227.0mM), 2.8% (317.8mM) achieved by diluting variable volume of 10% working solution in variable volumes of sterile H_2O or media (protocol specific)
Butyric Acid 1X working concentrations	0.2% (22.7mM), 0.6% (681.mM), 1.0% (113.5mM), 1.4% (158.9mM)
Chromium Propionate 0.1% stock solutions	0.2 mL 10% Chromium Propionate + 19.8 mL sterile H_2O
Chromium Propionate 2X working concentrations	0.00004%, 0.00012%, 0.0002%, 0.00028% achieved by diluting variable volume of 0.1% working solution in variable volumes of sterile H_2O or media (protocol specific)
Chromium Propionate 1X working concentrations	0.00002%, 0.00006%, 0.0001%, 0.00014%
LPS working concentration (500 ng/mL)	10 µl LPS (1mg/mL) diluted in 90 µl sterile H_2O to reach a working concentration of 500ng/mL)

Molarity of feed additives is presented in Table 2.5, to determine the molarity of caprylic acid and butyric acid used in experiments from the % v/v the following conversion formulas can be used:

$$n = \frac{mass}{molecular\ weight}$$

$$C = \frac{n}{volume}$$

The components of the above formulas should be inputted in the following units: n (number of moles), mass (g), molecular weight (g/mol), C (concentration in mol/L) and volume (L). The molecular weight of caprylic acid is 144.214 g/mol and butyric acid is 88.106 g/mol.

For instance, using these formulas, butyric acid with a molecular weight of 88.106 g/mol a 10 % solution would equate to 1135 mM.

The total volumes of reagents used for cDNA conversion and PCR reactions are described below (table 2.6 and 2.7, respectively).

Table 2.6 Volumes of reagents used in one reaction of RNA to cDNA conversion.

Reagent	Volume per reaction (µl)
5x iScript Reaction Mix	4
iScript RT	1
Nuclease free water	Variable (calculated by subtracting the volume of RNA template, iScript reaction mix and iScript RT from the total reaction volume)
RNA template	Variable (1µg RNA = 1000/ (RNA concentration))

Table 2.7 PCR reagents and volumes

Reagent	Volume (µl)
Brilliant II qPCR MasterMix	12.5
Forward Primer	0.63
Reverse Primer	0.63
Probe	0.63
Nuclease free water	8.63

2.2 *In vitro* Microbiology

2.2.1 Bacterial isolates

The *Campylobacter* isolates used in this study were archived strains within the Microbiology and Infectious Disease (MID) group within the Swansea University Medical School. The *Campylobacter* isolates were sub-cultures of strains collected from naturally infected free-range chickens (John, 2018). The isolates were stored in a glycerol stock (prepared as in Table 2.3) at -80˚C in a 1:1 ratio (500 µl of 50% glycerol: 500 µl bacterial liquid culture). A 10 µl sterile loop was used to streak the glycerol stock onto a Columbia blood agar (CBA) plate with 5% defibrinated horse blood (Oxoid, Basingstoke, UK) and incubated at 37 ˚C or 42˚C (human and avian internal temperature respectively) under microaerobic conditions (5% O_2; 10% CO_2; 85% N_2) using a CampyGen 3.5 L sachet (Oxoid) in an airtight container or in a Don Whitley M85 Workstation (MAC cabinet) (DW Scientific, West Yorkshire, UK). Incubator on screen temperature was validated using a manual thermometer. A single colony was picked using a 1 µl sterile loop and was placed into a tube with cryopreservation beads, the tube was agitated by hand for 10 s and stored at -80˚C for use in this study. A full list of all *Campylobacter* strains used in this study, their species, source, and clonal complexes are shown below (Table 2.4).

Table 2.8 List of all *Campylobacter* strains used throughout this project; a "-" indicates inconclusive results on PCR analysis for the specific strain however 28S analysis did conclude the strain was *Campylobacter* species.

Isolate ID	Species	Clonal complex	Source	Reference
M1	*C. jejuni*	45 (ST-137)	Human	(Friis et al., 2010)
NCTC 11168	*C. jejuni*	ST-21	Human	(Parkhill et al., 2000)
C7-2	*C. jejuni*	828	Chicken Caeca	BBSRC LifeEnd BB/M009610/1

C11-1	*C. coli/C. jejuni*	828/464	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C13-2	*C. jejuni*	-	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C15-3	*C. coli*	828	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C18-2	*C. coli*	828	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C20-2	*C. jejuni*	-	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C23-2	*C. jejuni*	828	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C24-2	*C. jejuni*	353	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
C26-3	-	-	Chicken Caeca	BBSRC LifeEnd BB/M009610/1
G7-1	*C. coli*	464	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G14	*C. jejuni*	353	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G18	*C. jejuni*	353	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G20-2	*C. jejuni*	828	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G23-2	*C. jejuni*	828	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G24-1	*C. jejuni*	353	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G25-2	*C. jejuni*	353	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G28-2	*C. coli*	828	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
G29-3	*C. coli*	828	Chicken Ileum	BBSRC LifeEnd BB/M009610/1
L7-2	*C. jejuni*	5136	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L11-1	*C. jejuni*	464	Chicken Liver	BBSRC LifeEnd BB/M009610/1

L13-2	*C. jejuni*	-	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L15-2	*C. jejuni*	464	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L18	-	-	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L23-1	*C. jejuni*	464	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L24	*C. coli*	828	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L25	*C. jejuni*	353	Chicken Liver	BBSRC LifeEnd BB/M009610/1
L29-1	*C. coli*	828	Chicken Liver	BBSRC LifeEnd BB/M009610/1

Bacillus spp. used throughout this study were *Bacillus subtilis* PB6, *Bacillus subtilis* F*A, *Bacillus subtilis* BA2.2 and *Bacillus licheniformis*; were all supplied in powder form by Kemin Animal Health and Nutrition Ltd (Herentals, Belgium) under a material transfer agreement.

2.2.2 Bacterial culture conditions

2.2.2.1 *Campylobacter* culture

Working *Campylobacter* cultures were obtained from cryopreservation beads streaked onto Columbia blood agar (CBA) plates with 5% defibrinated horse blood (Oxoid, Basingstoke, UK) and incubated at 37˚C or 42˚C (human and avian internal temperature respectively) under microaerobic conditions (5% O_2; 10% CO_2; 85% N_2) using a CampyGen 3.5 L sachet (Oxoid) in an airtight container or in a Don Whitley M85 Workstation (MAC cabinet) (DW Scientific, West Yorkshire, UK). For liquid culture, a single colony was picked from a CBA plate and inoculated into Brucella agar with added *Campylobacter* growth supplement (CGS; Oxoid, Table 2.1) and incubated at 37˚C or 42˚C for 24 h under microaerobic conditions. Prior to all experiments, *Campylobacter* suspensions were standardised to an OD_{600} of either 0.05 or 0.1 to ensure consistency. 1 mL of overnight culture was measured using a mini spectrophotometer (Jenway 7200, Staffordshire UK or BMG SPECTROstar nano, Ortenberg, Germany), the solution was diluted with fresh broth and absorbance was measured again until the desired optical density was reached. The dilution of the bacterial suspension within specific experiments

was taken into consideration when deciding on the OD to standardise to. An optical density of 0.1 and 600 nm was equivalent to approximately $1x10^7$ cfu/mL as calculated by colony counts and previous research (John, 2018).

2.2.2.2 Culture of *Bacillus* spp. and preparation of conditioned media

Freeze-dried *Bacillus* powder was reconstituted in sterile water to reach a 0.175% (w/v) concentration. Then, 100 µl of the reconstituted *Bacillus* was spread evenly over a CBA plate and incubated at 37˚C in an aerobic atmosphere overnight. After incubation, a single colony was picked using a 10 µl sterile loop and suspended in 4 mL of either Brucella broth, Mueller Hinton Broth (MB), antibiotic free 8E11 media or antibiotic free CaCo-2 media (Table 2.3). Suspensions were incubated for 24 h at 37˚C under aerobic conditions.

For the conditioned media, suspensions of *Bacillus* strains were prepared by adding 0.2 g of dried *Bacillus* powder into a 50 mL polypropylene tube containing 20 mL of pre-warmed 8E11 or CaCo-2 antibiotic free media (Table 2.3) resulting in a 0.1% (w/v) suspension. The solution was mixed thoroughly for 10 s using an IKA Vortex genius 3 (Oxford, England). The solution was incubated under a range of specific conditions and time periods (Table 8.1 and Table 8.2). After incubation, the tube was agitated by hand to mix contents and the optical density (600 nm) of 1 mL of the solution was recorded. The remaining 19 mL of solution was centrifuged at 1902 xg for 20 mins in an Eppendorf 5810R centrifuge (Stevenage, UK). The centrifuged solution was carefully filter sterilised through a 0.22 mm pore syringe (Merck) and stored as 1 mL aliquots in a microcentrifuge tube at -20˚C for future use.

2.2.3 Bacterial growth curves

2.2.3.1 *Campylobacter* growth curves

Growth of *Campylobacter* isolates was determined by measuring changes in optical density over 24 h. *Campylobacter* strains were incubated overnight at 37˚C / 42˚C under microaerobic conditions (as described in section 2.2.2.1) in brucella broth with CGS (Oxoid). The overnight culture was standardised by diluting using fresh brucella broth (+ CGS) to 0.05 OD_{600}, and 200 µl was added to triplicate wells in 10 different Nunc 96-well tissue culture plates, with one plate being prepared for each time point. Plate one was placed in a BMG Omega plate reader (BMG Omega, Bucks, UK) and OD_{600} was recorded (T=0), this plate was also used for T=1; the 10 plates were placed in a MAC cabinet at 37 or 42˚C under microaerobic conditions. At various time points (0, 1, 2, 3, 4,

6, 8, 10, 15, 20 and 24 h) the OD_{600} was measured in the BMG Omega Plate Reader. Each plate was discarded after measurement. The mean of at least two replicates was calculated for spectrophotometric measurement of growth.

2.2.3.2 *Bacillus* growth curve

The optical density (600 nm) of the *Bacillus* overnight culture (section 2.2.2.2) was measured using a mini spectrophotometer and the solution was standardised to 0.1 OD_{600} using fresh media/broth (using the same methodology described in section 2.2.2.1). To triplicate wells in a 96-well tissue culture plate, 100 µl of the diluted overnight cultures was added. The plate was placed in a BMG Omega Plate Reader and set to run for 24 h with an optical density reading (600 nm) taken for each well taken every hour, the plate was agitated by the plate reader for 15 s prior to each reading to mix the wells and eliminate air bubbles. A mean of triplicate wells was calculated to determine mean optical density.

2.2.4 *Campylobacter* growth challenged with feed additives and conditioned media

Campylobacter spp. were cultured and the OD_{600} was standardised to 0.1 (tolerance 0.7-1.3) (approximately 1 x 10^7 cell/mL) as described previously (section 2.2.2.1) using fresh 2X brucella broth (Table 2.3). This was to ensure that the same number of bacterial cells was used in each experiment. The standardised culture was serially diluted in fresh brucella broth containing CGS and plated out in 10 µl volumes onto CBA plates for enumeration of *Campylobacter* (CFU/mL).

Working concentrations of caprylate, butyrate, and chromium propionate (Table 2.3) were diluted in antibiotic free media to 2X desired concentrations (Table 2.3) and 100 µl was added to triplicate wells of a 96-well plate. Then to each well, 100 µl of standardised *Campylobacter* liquid culture (0.1 OD_{600}) was also added.

For *Campylobacter* growth challenged with *Bacillus* conditioned media, 1 mL aliquots from section 2.2.2.2 were thawed and diluted, using fresh antibiotic free media (CaCo-2 or 8E11, Table 2.3) to 1/10, 1/100 and 1/1000 dilutions. In a 96-well tissue culture plate, 100 µl of *Campylobacter* liquid culture (0.1 OD_{600}) and 100 µl of conditioned media were combined and repeated in triplicate wells. At 0 h the optical density (600 nm) was recorded in a BMG plate reader.

For both *Bacillus* conditioned media and feed additive treatments the plate was placed in a MAC cabinet for 24 h at 42˚C under microaerobic conditions. After 24 h, the optical density (600 nm) was recorded using in a mini spectrophotometer. Then, a mean of the three wells (experimental triplicate) was calculated for time point 0 and 24. To calculate the percentage change in optical density (600 nm) the following equation was used:

Percentage change in optical density (OD) = $\left(\frac{OD\ timepoint\ 24 - OD\ timepoint\ 0}{OD\ timepoint\ 0}\right) \times 100$

2.2.5 Bacterial motility assay

All *Campylobacter* strains were grown for 48 h on CBA at 42˚C under microaerobic conditions in a MAC cabinet. A single colony was inoculated into 5 mL of brucella broth containing CGS and incubated overnight at 42˚C under microaerobic conditions in a MAC cabinet. The OD_{600} of each liquid culture was measured and diluted to 0.1 OD_{600} (2 mL suspension) using a mini spectrophotometer. Two ml of brucella broth medium supplemented with 0.3% agar was aliquoted into each well of a 6-well plate and allowed to solidify for 20 min. The *Campylobacter* suspension (2 mL) was mixed for 5 s using an IKA Vortex Genius 3 before the suspension was added to the centre of triplicate wells by stabbing with a sterile pipette tip that had been dipped into the suspension. The plates were incubated at 37˚C for 24 h under microaerobic conditions in a MAC cabinet. Motility of each bacterial strain was determined by measuring from the centre of the well to the furthest point of the migration zone with a ruler (mm). The mean of triplicate wells was used to calculate motility.

2.2.6 *Campylobacter* DNA extraction for sequencing

Campylobacter strains were cultured (section 2.2.2.1) and incubated overnight in brucella broth containing CGS at 37˚C under microaerobic conditions using a MAC cabinet. Briefly, 1 mL of overnight bacterial suspension was centrifuged at 5534 xg for 5 minutes, the supernatant was discarded; the process of adding 1 mL of overnight suspension and centrifugation was repeated until a visual pellet formed within the tube, following which the pellet was weighed. The QIamp DNA Mini Kit (Qiagen) was used (as per manufacturer's instructions) to extract genomic DNA.

Briefly, buffer ATL (lysis buffer) was added to the pellet to a total solution volume of 180µl (1 mg pellet = 1 µl buffer ATL). Then, 20 µl proteinase K was added, and the solution was vortexed for 10s until thoroughly mixed. This was to digest proteins and

remove contamination during DNA preparation. Samples were incubated at 56˚C for 1 h on a heat block (Dri-block heater DB-2D, Fisher Scientific); at 20-min intervals the samples were vortexed for 5 s to disperse the sample) to ensure bacteria were completely lysed. To ensure the genomic DNA was RNA-free, 4 µl RNase A (QIAGEN; 100 mg/mL) was added and mixed by pulse-vortexing (gently lift and lower sample on vortex mixer) for 15 s. Then, 200 µl buffer AL (lysis buffer) was added to the sample which was incubated at 70˚C for 10 min using a heat block. Following this, 200 µl ethanol (100%) was added to each sample and mixed thoroughly to ensure a homogeneous solution, by vortexing for 10 s. The sample was loaded into a QIAmp mini spin column and was centrifuged at 6297 xg for 1 min to remove filtrate. Buffer AW1 (wash buffer) was loaded onto the spin column and centrifuged at 6297 xg for 1 min to denature and remove proteins from sample. Buffer AW2 (wash buffer) was subsequently added according to manufacturer's instructions and centrifuged accordingly to remove impurities. The sample was eluted in 40 µl nuclease-free H_2O The sample yield was measured using a NanoDrop (ThermoScientific, Loughborough, UK). Sample yields were between 3.4 to 108.5 ng/mL and had a purity ratio (A260/A280) of 0.86 to 2.8 with a purity ratio of 2.0 being optimum.

2.3 Culture of avian and human intestinal epithelial cells

2.3.1 Routine cell culture

Throughout this project two cell types were cultured and used; avian intestinal epithelial cells (MM-CHiC clone: 8E11; Tentamedix GmbH; formerly Micromol, Germany) and human colon adenocarcinoma epithelial cells (CaCo-2; ECACC, Cat number 86010202).

The 8E11 cell line was maintained in Gibco DMEM/F12 Glutamax™ Supplemented with 8.8% FBS, 0.9% Pen-Strep (10,000 U/mL), 0.9% L-Glut (220 mM) and 0.9% Gibco Amphotericin B (250 µg/mL). The CaCo-2 cell line was maintained in Gibco DMEM, high glucose + pyruvate, supplemented with 8.8% FBS, 0.9% Pen-Strep (10,000 U/mL), 0.9% L-Glut (220 mM) and 0.9% Gibco MEM Non-essential Amino Acid Solution (100x). Both cell lines were grown in Cellstar® tissue culture treated flasks between 25 cm^2 to 175 cm^2 (dependent on quantity of cells required). Culture conditions for both cell types were 37˚C in a controlled 5% CO_2 humidified atmosphere.

Cell lines were sub-cultured at approximately 90% confluence (observed using a light microscope). Typically, 8E11 cells exhibited faster growth to confluency than CaCo-2

cell lines, as a result, 8E11 cells were seeded at a lower density to reach confluency within the desired time period. Spent media was removed and the cell monolayer was washed gently three times with 15 mL Gibco PBS (for cell culture). After the third wash, PBS was discarded and 15 mL Gibco TrypLE express enzyme added to disrupt the epithelial cell monolayer. TrypLE express was incubated in contact with cells for 5 min at 37˚C and monolayer detachment was monitored. When cells had successfully detached from the flask, 10 mL of cell-specific culture media was added to neutralise the trypsin digestion. The cells and neutralised TrypLE solution were removed from cell culture flask and transferred to a 50 mL centrifuge tube. This was centrifuged at 52.83 xg for 5 min (room temperature). The supernatant was carefully removed and discarded, and the remaining pellet was resuspended in 10 ml of fresh cell-specific media. The cell suspension was diluted into a new cell culture flask with fresh media (dilution varied depending on time requirement for confluent cells).

2.3.2 Treatment of epithelial cells with *Campylobacter*

Campylobacter strains were grown for 48 h at 37˚C or 42˚C under microaerobic conditions in a MAC cabinet. Then a single colony was inoculated into 5 mL of brucella broth containing CGS and incubated overnight at 42˚C under microaerobic conditions in a MAC cabinet. To ensure that the same number of bacterial cells was used in each experiment the overnight liquid culture was standardised (section 2.2.2.1) by measuring the OD 600 nm and standardising to 0.05 (approximately 4.9 x 10^6 cell/mL) using a mini spectrophotometer. CaCo-2 and 8E11 epithelial cell cultures were seeded in a 24-well tissue culture plate at approximately 1.05 x 10^5 and 1.65 x 10^5 cells per cm^2, respectively and incubated for 48 h or until confluent (approximately 3 days). The spent media from confluent epithelial monolayers was discarded and cells were washed three times with PBS (GIBCO) before 1 mL of fresh antibiotic free media (Table 2.3) was added and cells were infected with 30 µl of standardised bacterial suspension into duplicate wells (approximately 1.4 x 10^6 cfu per well). Infected monolayers were incubated for 4 h (Gentamicin Protection Assay) or 6 h (RNA isolation) at 37˚C in 5% CO_2 to allow for bacterial invasion of the epithelial cells. Uninfected cells treated with 30 µl brucella broth +CGS (3% v/v) or 5 µl lipopolysaccharide (500 ng/mL) served as negative and positive controls, respectively.

2.3.3 Treatment of epithelial cells with caprylate, butyrate, and chromium propionate

CaCo-2 and 8E11 epithelial cell cultures (from section 2.3.2) were seeded in a 24-well tissue culture plate at approximately 1.05 x 10^5 and 1.65 x 10^5 cells per cm^2 respectively and incubated until 90% confluent (usually 48 h) as measured with light microscopy. Feed additives stock solutions were prepared (Table 2.5). In brief, caprylic acid and butyric acid were diluted to 10% working concentrations using sterile water and standardised to a pH ~7.0 using HEPES buffer (Sigma) and sodium hydroxide respectively. The pH was measured using a HI-202 Edge Hybrid Multiparameter pH, EC, DO meter with an accuracy of ±0.01 pH (HANNA instruments; Bedfordshire, UK). Chromium propionate (10%) was diluted to a 0.1% working concentration using sterile water. Once confluent, epithelial cells were washed three times with PBS. Feed additives were diluted in antibiotic free media to target concentrations (Table 2.7) and 2 mL of this solution was added to each well of confluent cells. Treated cells were then incubated for 24 h at 37˚C in 5% CO_2.

2.3.4 Epithelial cell viability: AlamarBlue Assay

To assess cell viability the AlamarBlue Assay was used. The AlamarBlue Reagent (ThermoFisher Scientific) was used according to the manufacturer's guidelines.

In brief, 200 µl of 8E11 or CaCo-2 cells were seeded (10.5x10^4 8E11 cells or 6.67x10^4 CaCo-2 cells) into each well of a 96-well plate and incubated in 5% CO_2 at 37˚C until confluent. Spent media was removed from wells and 100 µl of fresh antibiotic free media treated with feed additives (Table 2.5) was added and incubated for a further 24 h at 37˚C in 5% CO_2, control wells were also included (media only). After 24 h, the spent media was removed and 50 µl fresh antibiotic free media (Table 2.5) was added to all wells. In addition, a toxicity control was included (49 µl antibiotic free media + 1 µl 10% Triton™ X-100 (Sigma-Aldrich)). Plates were incubated for 6 h in 5% CO_2 at 37˚C. Then, 10 µl cell viability reagent was added directly to each well and incubated for 4 h in 5% CO_2 at 37˚C. Absorbance levels were measured at a wavelength of 570 nm using a BMG plate reader. Results were presented as either the mean fluorescence (570 nm) of triplicate wells or the percentage viability (%) of cells, which was calculated using the following equation:

$$\text{Percentage viability (\%)} = \left(\frac{Average\ fluorescence\ treated\ cells}{Average\ fluorescence\ untreated\ cells}\right) \times 100$$

2.3.5 Gentamicin Protection Assay (GPA)

To assess epithelial cell invasion by *Campylobacter* a gentamicin protection assay was used. *Campylobacter* strains were cultured from bead stocks onto CBA plates and grown micro aerobically for 48 h at 37˚C or 42˚C. A single colony was sub-cultured in brucella broth containing CGS (section 2.3.2). Briefly, epithelial cell monolayers (passage 35 to 75), in antibiotic free assay medium or treatment medium were grown to confluency in a 24 well plate (as described in section 2.3.2 and 2.3.3 respectively). Confluent monolayers were washed three times with PBS and 1 mL fresh antibiotic free cell culture media was added to each well. Then, 1.4 x 10^6 CFU/mL of standardised bacterial suspension was added to duplicate wells and cells were co-incubated with bacterial suspension for 4 h at 37˚C in 5% CO_2. The remaining bacterial liquid culture was serially diluted in fresh brucella broth + CGS and plated out onto CBA plates for enumeration of *Campylobacter* (CFU/mL). At the end of the 4 h incubation period, the cell monolayer incubated with bacteria was washed gently, three times with PBS before 2 mL antibiotic free cell culture media and 4 µl gentamicin (125 µg/mL) was added to each well (to kill the extracellular bacteria) and incubated at 37˚C in 5% CO_2 for 1.5 h. Cells were then washed three times with PBS, and 2 mL 0.1% Triton X-100 in PBS (Table 2.3) was added to each well to lyse the cells and release the intracellular contents. After 10 min, the cell lysates were serially diluted in fresh brucella broth +CGS and plated onto CBA plates to enumerate invaded bacteria.

2.3.6 RNA isolation from epithelial cells

Total RNA was isolated from infected and/or treated 8E11 avian intestinal epithelial cells, (sections 2.3.2 and 2.3.3), grown in a 24 well tissue culture plate, using a RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. Total RNA (µg/mL) was quantified using a NanoDrop (ThermoScientific, Loughborough, UK). RNA yield from samples was between 75 to 637 µg/mL, with a purity ratio (A260/A280) of between 1.1 to 2.13, with a purity ratio of 2.0 being optimum.

2.3.7 cDNA synthesis using RNA from epithelial cells

Total RNA extracted from lysed cell monolayers was converted to cDNA using the iScript cDNA synthesis kit (Biorad) according to manufacturer's instructions. RNA

concentration was determined using the NanoDrop (as described in section 2.3.6) and a total of 1 µg of RNA was converted in each reaction. The RNA concentration was calculated using the following equation:

$$\text{Volume of } 1\mu\text{g RNA} = 1000/(\text{RNA concentration})$$

Briefly, reagents were combined in a single well of a qPCR tube strip, including 5x iScript Reaction Mix, iScript Reverse Transcriptase, nuclease free water and RNA template according to the number of treatments (Table 2.4).

The reaction tubes were covered with cover strips and centrifuged briefly to ensure the sample was at the bottom of the test wells. The test strips were placed in an AriaMx Real time PCR machine (Agilent) and incubated using optimal conditions (Table 2.9). Samples were removed from the AriaMx Real time PCR machine and stored at -20˚C.

Table 2.9 cDNA synthesis conditions

Step	Condition
Priming	25˚C for 5 min
Reverse transcription	46˚C for 20 min
Reverse transcriptase inactivation	95˚C for 1 min

2.3.8 Quantitative PCR of chicken epithelial cDNA (probe-based method)

Prior to the reaction, primers, and probes (Table 2.2) were reconstituted in nuclease-free water to a concentration of 10 pmol/µl and were vortexed thoroughly to mix. Quantitative PCR using the probe-based method was used to amplify the genes of interest (CXCLi1, CXCLi2 and TGFβ) and the housekeeping gene 28S ribosomal RNA. The 28S housekeeping gene was further diluted to 1/1000 before addition to the reaction mixture. Each PCR volume totalled 25 µl (Table 2.7).

Reactions were performed in duplicate wells of the AriaMx Real time PCR machine (Agilent, Cheshire, UK) to provide technical replicates per experiments. The primers and probes used within the study (Table 2.2) had specific annealing temperatures (Table 2.4), and the general working conditions used for the qPCR are summarised below (Table 2.10).

Table 2.10 qPCR conditions

Step	Condition	Number cycles

1: Activation	10 min at 95˚C	1
2: Denaturation	30s at 95˚C	40
3: Annealing	1 min at temperature defined by primers/probe being used (see Table 2.8)	

qPCR reactions were performed in an AriaMx real time PCR machine (Agilent). The Ct values were determined by measuring fluorescence of the probe specific to the gene of interest (Table 2.2), this was selected during PCR setup. Data from the AriaMx real time PCR machine was analysed using the Agilent AriaMx software (Agilent Aria 1.8). Data was loaded, and a graphical Table was produced of the Ct values from the selected PCR run. Threshold fluorescence was adjusted to 50 for all samples to ensure consistency.

Changes in gene transcription were assessed using the gene expression ratio and were calculated using the Pfaffl Method (Pfaffl, 2001) and AriaMx Software (Agilent). The Pfaffl method calculates relative gene expression while taking differences in primer efficiency into consideration (Bradburn, 2020); the Pfaffl equation is as follows:

$$\text{Gene Expression Ratio} = \frac{(primer\ efficiency\ GOI)^{\Delta Ct\ GOI}}{(primer\ efficiency\ HKG)^{\Delta Ct\ HKG}}$$

The Ct values of duplicate technical replicates was calculated. Using the control mean the change in Ct value (ΔCt) was calculated. Converted primer efficiencies were confirmed (see section 2.1; Table 2.3).

2.3.9 Genomic Analysis

Nineteen *Campylobacter* isolates were cultured (as described in section 2.2.2.1) and DNA was successfully extracted (as described in section 2.2.6). Genomic DNA was fully sequenced by Dr Matthew Hitchings at The Swansea Genome Centre.

Genomes were analysed using PATRIC (https://www.patricbrc.org/) and Galaxy. Within Galaxy, genomes were scanned for virulence genes using the virulence factor database (VFDB; Table 2.11) and antibiotic resistance genes using the Resfinder database, NDARO, and comprehensive antibiotic resistance database (CARD; Table 2.12).

Table 2.11 Virulence genes scanned for within the virulence factor database.

Gene	Gene Name	Reference
flaA	Flagellin A	

flaB	Flagellin B	(D. John, 2018)
flaC	Flagellin C	(D. John, 2018)
cadF	Outer membrane fibronectin-binding protein	(D. John, 2018)
cdtA	Cytolethal distending toxin A	(D. John, 2018)
cdtB	Cytolethal distending toxin B	(D. John, 2018)
cdtC	Cytolethal distending toxin C	(D. John, 2018)
cheA	Histidine autokinase	(Du et al., 2018)
cheV	Coupling scaffold protein	(Du et al., 2018)
cheY	Chemotaxis response regulator protein	(Du et al., 2018)
ciaB	*Campylobacter* invasion antigen B	(D. John, 2018)
ciaC	*Campylobacter* invasion antigen C	(D. John, 2018)
flhA	key component of flagellar export apparatus	(Carrillo et al., 2004)
flhB	key component of flagellar export apparatus	(Carrillo et al., 2004)
fliA	Flagellar biosynthesis RNA polymerase sigma factor	(D. John, 2018)
fliP	Component of flagellar export apparatus (T3SS)	(D. J. Bolton, 2015)
fliQ	Component of flagellar export apparatus (T3SS)	(D. J. Bolton, 2015)
fliR	Component of flagellar export apparatus (T3SS)	(D. J. Bolton, 2015)
jlpA	Surface exposed lipoprotein	(D. John, 2018)
pebA	Bi-functional adhesion/ABC transporter aspartate/ glutamate-binding protein	(D. John, 2018)
porA	Major outer membrane protein	(D. John, 2018)
flgR	Sigma-54 associated transcriptional activator	(D. John, 2018)
flgS	Signal transduction histidine kinase	(D. John, 2018)

Table 2.12 Antibiotic resistance genes screened for within the Resfinder database, national database of antibiotic resistant organismsand comprehensive antibiotic resistance database.

Gene	Antibiotic Class	Reference
blaOXA-184	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019; Evans & Amyes, 2014; Mouftah et al., 2021)
blaOXA-193	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019; Evans & Amyes, 2014; Mouftah et al., 2021)
blaOXA-452	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019; Evans & Amyes, 2014; Mouftah et al., 2021)
blaOXA-453	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019; Evans & Amyes, 2014; Mouftah et al., 2021)
blaOXA-605	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019; Evans & Amyes, 2014)
tet(O)	Tetracycline	(Alcock et al., 2019; Connell, 2003)
tet(O/32/O)	Tetracycline	(Alcock et al., 2019; Connell, 2003)
OXA-184	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019)
OXA-450	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019)
OXA-452	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019)
OXA-453	Penam, Carbapenem, Cephalosporin	(Alcock et al., 2019)
cmeA	Macrolide antibiotic, fusidane antibiotic, cephalosporin, fluoroquinolone antibiotic	(Alcock et al., 2019)
cmeB	Macrolide antibiotic, fusidane antibiotic, cephalosporin, fluoroquinolone antibiotic	(Alcock et al., 2019)
cmeC	Macrolide antibiotic, fusidane antibiotic, cephalosporin, fluoroquinolone antibiotic	(Alcock et al., 2019)
cmeR	Macrolide antibiotic, fusidane antibiotic, cephalosporin, fluoroquinolone antibiotic	(Alcock et al., 2019)

2.4 Analysis Software and Statistics

2.4.1 Software and Statistical Analysis

GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) was used to construct graphs and execute statistical analysis of data throughout this study.

Data from bacterial growth curves were presented as line graphs. The mean of three biological replicates +/- SEM was plotted. Differences between isolates/groups were assessed using two-way ANOVA, including a Tukey's Multiple Comparisons post-hoc test. A $p < 0.05$ was considered statistically significant.

Data from *Campylobacter* growth challenged with feed additives was presented as bar charts in a panel of six plots. Five plots included individual strains and the sixth plot combined a plot of all strains. Data was presented as the mean ± the SEM of a minimum of three biological replicates. Tests for normality included Anderson-Darling (A2*), D'Agostino-Pearson (K2), Shapiro-Wilk (W) and Kolmogorov-Smirnov (distance). Therefore, differences between isolates/groups were assessed using a Kruskal-Wallis test, including a Dunn's Multiple Comparisons post-hoc test. Data are presented as mean ± SEM. Statistical significance was ascribed when $p < 0.05$.

Campylobacter invasion data from cells pre-treated with feed additives were presented as bar charts in a panel of six plots. Five plots included individual strains and the sixth plot combined a plot of all strains. Data was presented as the mean +/- the SEM of a minimum of three biological replicates. Tests for normality included Anderson-Darling (A2*), D'Agostino-Pearson (K2), Shapiro-Wilk (W) and Kolmogorov-Smirnov (distance). Therefore, differences between isolates/groups were assessed using a Kruskal-Wallis test (non-parametric) or two-way ANOVA test (parametric), including a Dunn's or Dunnett's Multiple Comparisons post-hoc test (respectively). Data are presented as mean ± SEM. Statistical significance was ascribed when $p < 0.05$.

Viability assay data was presented as dot plots which presented three biological replicates. Each biological replicate was a mean of the triplicate experimental replicates. The mean of the three biological replicates was also plotted ± SEM. Tests for normality included Anderson-Darling (A2*), D'Agostino-Pearson (K2), Shapiro-Wilk (W) and Kolmogorov-Smirnov (distance). A Bartlett's test was used to test for differences in standard deviation. Therefore, differences between isolates/groups were assessed using one-way ANOVA (parametric) or Kruskal-Wallis test (non-parametric) including a Dunn's or Dunnett's multiple comparisons post-hoc test (respectively). If distribution was normal, but standard deviations significantly differed, a Brown-Forsythe and Welch's ANOVA test was used with a Dunnett's T3 multiple comparisons post-hoc test. Data are presented as mean ± SEM. Statistical significance was ascribed when $p < 0.05$.

Cytokine expression was presented as plots which presented the mean of a minimum of two biological replicates ± SEM. Tests for normality included Anderson-Darling (A2*), D'Agostino-Pearson (K2), Shapiro-Wilk (W) and Kolmogorov-Smirnov (distance). Therefore, differences between isolates/groups were assessed using a Kruskal-Wallis test

(non-parametric) or two-way ANOVA test (parametric), including a Dunn's or Dunnett's Multiple Comparisons post-hoc test (respectively). Data are presented as mean ± SEM. Statistical significance was ascribed when $p < 0.05$.

Chapter 3: Selection of standard *Campylobacter* strains for *in vitro* feed additive testing

3.1 Introduction

There is substantial evidence that the population structure of *Campylobacter* in intensive broiler production is complex and are extremely diverse in both genotype and phenotype (Colles & Maiden, 2012; D. John, 2018; Vidal et al., 2016). There are two major Multi-Locus Sequencing Type (MLST) clonal complexes (CC) (45 and 21) which exhibit distinctive infection rates, unique *in vivo* behaviours, and consistently colonise the chicken gastrointestinal (GI) tract (John et al., 2017).

In addition to genotypic variation, research has highlighted the importance of isolate source as a contributor to pathogenicity. For instance, cytotoxicity of strains isolated from poultry and human sources have been found to be 26.7% and 38.7%, respectively (Wysok et al., 2020). Research into *Campylobacter* heterogeneity has led to the identification of strains with a specifically invasive phenotype leading to successful extraintestinal spread within broiler chickens (Suzanne Humphrey et al., 2015). Despite the well-documented diversity between *Campylobacter* strains that can cause invasive disease and those that remain localised within the chicken GI tract, there is a poor understanding of the specific differences between strains and the underlying cause for these phenotypic differences (John et al., 2017).

Most studies, to date, have investigated few isolates and strains which poorly reflect the diversity of the genus. To apply scientific findings to *Campylobacter* as a genus, especially for the development of *Campylobacter* targeted treatments (e.g., feed additives), it is of the upmost importance that research is conducted on a range of isolates that represent this diverse bacterial genus.

3.1.1 *Campylobacter* genetic diversity

Sequence types (ST) are determined by genotyping *Campylobacter* samples using MLST of seven housekeeping genes, and this system is commonly used to categorise *Campylobacter* strains (Rawson et al., 2022). Despite the growth cycle of a broiler flock being short, there is sufficient time for multiple *Campylobacter* STs to colonise simultaneously (Lydekaitienė & Kudirkienė, 2020; Rawson et al., 2022). *Campylobacter* with a ST that match central genotype can be further categorised into a clonal complex

(CC) (Jolley et al., 2018). For *C. jejuni* specifically there are 11,884 distinct STs spanning across 45 CCs (Šoprek et al., 2022).

Housekeeping genes from *C. jejuni* strains show little sequence diversity, with a small pool of alleles, with high rates of recombination between isolates (Suerbaum et al., 2001). Despite the limited number of mechanisms by which *Campylobacter* may acquire genetic diversity, research has indicated that there is a large degree of intraspecific genotypic diversity (Dorrell et al., 2001)

Important core genes are shared by *Campylobacter* isolates regardless of source, e.g., *cadF* (virulence gene that works within contact regions to facilitate adherence to fibronectin), however, there are also accessory genes which are only functionally important for poultry isolates e.g., *ciaB* (caecal colonisation virulence gene) (Reddy & Zishiri, 2018). Thus, each strain of *Campylobacter* may utilise different mechanisms to spread throughout the host GI tract and this is down to genetic diversity, source diversity and an interaction of these two factors (Jeon et al., 2010).

3.1.2 *Campylobacter* diversity in growth and survival

The genotypic and phenotypic heterogeneity among *Campylobacter* results in differences in the ability of individual strains to grow and survive (Dzianach et al., 2022). The best example of this is variation in the ability to colonise and persist in the chicken's GI tract or in other hosts (El-Shibiny et al., 2007). For instance, *Campylobacter* isolated from the environment (mammalian origin) or poultry products are likely to have grown at either 37˚C or 42 ˚C respectively due to the internal body temperatures of the respective hosts (Duffy & Dykes, 2006; El-Shibiny et al., 2007). Mechanistic understanding of this was provided by Duffy and Dykes (2006), who demonstrated that genes involved in the stress response are differentially regulated at 37˚C and 42˚C, affecting survival on beef, chicken and in water using four genetically distinct strains and the study concluded that recovery of *Campylobacter* from food is influenced by different temperatures in a strain specific manner. In addition to this Khanna, Bhavsar and Kapadnis (2006) found that growth and chemotaxis of *C. jejuni* was greater at 37˚C than at 42˚C, indicating that mammalian core temperature is favourable for *Campylobacter* virulence.

Survival times for *Campylobacter* in water vary between 2-4 weeks but have also been reported up to 4 months (Chan et al., 2001; Rollins & Colwell, 1986). This variation may reflect strain diversity or differences in experimental conditions (e.g., water type,

incubation conditions) (Cools et al., 2003). Avian isolates have demonstrated prolonged survival in water *in vitro*, compared to clinical and water isolates, emphasising the role of drinking water as a campylobacteriosis transmission route for strains of specific origin (Cools et al., 2003).

Campylobacter strains rarely exist independently of other strains, both in the environment and within the poultry GI tract. It has been demonstrated using two distinct *Campylobacter* strains (*C. jejuni* OR1 and *C. coli* OR12), that the exponential phase of growth is similar during co-culture, however strain sensitivity to excess numbers of other strains or products, was observed at higher ratios during the stationary phase (El-Shibiny et al., 2007).

Genome sequence analysis and monitoring the respiratory activity of cells that are metabolically active revealed that some strains (e.g., *C. jejuni* NCTC 11168) are capable of catabolising fucose due to a novel L-fucose pathway present within a 9kb genomic island (absent in *C. jejuni* 81-176) (Gundogdu et al., 2007; Hofreuter, 2014; Line et al., 2010; Wagley et al., 2014). Growth temperature has been shown to affect the oxidation and utilisation of growth substrates at 42˚C, and Line *et al.* (2010) showed that the genome sequenced strain *C. jejuni* NCTC 11168 was able to better oxidise nearly 190 substrates as a potential source of carbon.

Campylobacter cells require O_2 for growth but are highly sensitive to normal atmospheric oxygen tensions (Kaakoush et al., 2007). Despite microaerophilic requirements, *Campylobacter* can survive in conditions of atmospheric oxygen tension, e.g., on chicken meat for prolonged periods (Hilbert et al., 2010) – this is termed oxygen tolerance and is an important factor in *Campylobacter* virulence and pathogenesis. Studies have been conducted which demonstrate that some *Campylobacter* isolates are obligate microaerophiles with varying degrees of oxygen tolerance that could be attributed to the presence of eighteen genes identified by Kaakoush *et al.* (2007) (Table 3.1). There are several other mechanistic assumptions by which *Campylobacter* isolates can tolerate varying oxygen tensions such as biofilm formation or interaction with other microorganisms such as *Pseudomonas* species (Hilbert et al., 2010).

Table 3.1 Eighteen genes identified that encode proteins involved in oxygen tolerance in *C. jejuni* strains NCTC11168 and RM1221 (Kaakoush *et al*., 2007).

Gene	Function
cj0264c	Dimethylsulfoxide reductase
cj0203	Putative transmembrane transport protein downregulated at low oxygen tension
cjo239c	NifU protein homologue downregulated at low oxygen tension
cj0240c	NifS protein homologue downregulated at low oxygen tension
cj0298c	Oxobutanoate hydroxy methyltransferase downregulated at low oxygen tension
cj0414	Putative oxidoreductase subunit downregulated at low oxygen tension
cj0415	Putative oxidoreductase subunit downregulated at low oxygen tension
cj0425	Putative periplasmic protein downregulated at low oxygen tension
cj0628	Putative lipoprotein downregulated at low oxygen tension
cj0629	Possible lipoprotein downregulated at low oxygen tension
cj0779	Thiol peroxidase downregulated at low oxygen tension
cj0780	Periplasmic nitrate reductase
cj1183c	Putative fatty-acyl-phospholipid synthase downregulated at low oxygen tension
cj0864	Putative periplasmic protein upregulated at low oxygen tension
cj0874c	Cytochrome C upregulated at low oxygen tension
cj0876c	Putative periplasmic protein upregulated at low oxygen tension
cj1357c	Putative periplasmic cytochrome C upregulated at low oxygen tension
cj1358c	Putative periplasmic cytochrome C upregulated at low oxygen tension

3.1.3 Diversity in *Campylobacter* pathogenicity

3.1.3.1 Differences in the presence and absence of *Campylobacter* invasive genes

There are specific genes that are crucial for facilitating and enabling invasion of *Campylobacter* into epithelial cell lines and intracellular survival (Table 3.2), and it is well documented that the presence of these genes varies between strains. To accurately represent the invasive spectrum of *Campylobacter*, and apply scientific findings appropriately, research must be conducted on strains isolated from different, but relevant sources (environmental, veterinary, or clinical) and tested on both human and avian cell lines (D. A. John et al., 2017).

Table 3.2 Summary of genes essential for bacterial internalisation by host epithelial cells.

Gene	Function	Reference
flaA	Adherence to and invasion of epithelial cells	(D. A. John et al., 2017; Zheng et al., 2006b)
ciaA	Invasion of epithelial cells	(D. A. John et al., 2017)
ciaI	Intracellular survival	(D. A. John et al., 2017)
iamA	Invasion of epithelial cells and intracellular survival	(Frazão et al., 2017)
ciaB	Invasion of epithelial cells and intracellular survival	(Frazão et al., 2017)
pldA	Invasion of epithelial cells and intracellular survival	(Frazão et al., 2017)
Various genes within pVir plasmid	Invasion of epithelial cells	(Zheng et al., 2006b)
cadF	Adherence to and invasion of epithelial cells	(Zheng et al., 2006b)

3.1.3.2 Diversity of *in vitro Campylobacter* induced immune responses

Campylobacter demonstrates phenotypic diversity through differences in adhesion and invasion to epithelial cell lines, toxin production, serum resistance (Backert et al., 2013; Wassennaar et al., 1993; Zheng et al., 2006b). While *Campylobacter* is pathogenic *in vivo* to both human and avian hosts (Black et al., 1988; Griekspoor et al., 2015; Knudsen et al., 2006; L. K. Williams et al., 2013), it has been shown that there is significant variation in *Campylobacter* isolates to interact *in vitro* with cultured epithelial cells (Backert et al., 2013). The pathogenesis of *Campylobacter* has been studied extensively *in vitro* by focusing on human intestinal cell responses (such as HT-29, T84 and CaCo-2) to *Campylobacter* infection. This process involves *Campylobacter* internalisation and activation of downstream signalling pathways such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK) and p38 MAPK (D. A. John et al., 2017; Larson et al., 2013; MacCallum, Haddock, et al., 2005; Zheng et al., 2006b). The activation of these pathways leads to production of interleukin 8 (IL-8), an inflammatory chemokine, and IL-10, an anti-inflammatory cytokine (D. A. John et al., 2017).

Backert *et al.* (2013) found that *C. jejuni* CG8486 is approximately 1000-fold less invasive than *C. jejuni* 81-176 in INT-407 cells, but in contrast no differences in invasion levels were detected in CaCo-2 cells. It has been observed that *Campylobacter* isolates from patients with severe GI symptoms invade cultured epithelial cells *in vitro* more than isolates with mild symptoms (Fauchere et al., 1986). In addition to evidence that there is a difference in efficiency and invasion capabilities between strains into cultured cell lines of certain host or tissue origins, there has been investigation into strain source as a

contributing factor to pathogenic diversity into specific cell lines (Backert et al., 2013; Zheng et al., 2006b).

3.1.4 Phenotypic diversity should be addressed when product testing

Despite the extensive research into human cell line interaction with *Campylobacter* there are limited studies that have studied interactions with avian cells (D. A. John et al., 2017). A recent study conducted by John *et al.* (2017) investigated the difference in cytokine responses of avian 8E11 and human HT-29 cells to challenge with 100 *Campylobacter* isolates from a variety of sources with varying STs; they found that the induction of inflammatory cytokines varied widely (up to 100,000-fold) between infected vs uninfected cell lines but were unable to identify differences in response between isolate source or sequence type. Interestingly, the reference strains, *C. jejuni* M1 and *C. jejuni* NCTC 11168, produced responses that equated to the mean for the whole study population, indicating that these strains were appropriate to represent the diversity of this study population (D. A. John et al., 2017).

3.1.5 Aims

A collection of *Campylobacter* strains (BBSRC LifeEnd BB/M009610/1) isolated from the liver, ileum or caeca of free-range broiler chickens were used to study species diversity in *in vitro* growth and epithelial cell invasion. The strains had all been previously characterised at the genomic level (Sheppard *et al.*, 2011; Sheppard *et al.*, 2013). The present study used genomic data to identify the presence and absence of virulence and antibiotic resistance genes to further determine the genetic diversity within the *Campylobacter* strain collection. The data collected in this chapter was used to select a standard set of strains that reflected the diversity and invasive spectrum of *Campylobacter*. The specific chapter aims were to:

- Investigate genetic diversity within 19 *Campylobacter* isolates focussing on virulence and antimicrobial resistance genes.
- Investigate the effect of temperature and source on the growth response of 21 *Campylobacter* strains.
- Investigate the effect of source on the invasive capabilities of 23 *Campylobacter* strains isolated from different anatomical areas of the chicken.
- Select a subset of *Campylobacter* strains that show consistent *in vitro* responses for testing *in vitro* effects of feed additives in future chapters.

3.2 Materials and Methods

3.2.1 *Campylobacter* isolates

This study used a total of 27 isolates (Table 2.8) of *C. jejuni* and *C. coli* isolated from naturally infected broiler chickens throughout different stages of the study. Strains were isolated from the caeca (C) (n=9), ileum (G) (n=9), or liver (L) (n=9) (BBSRC LifeEnd BB/M009610/). Two reference strains were used throughout this study (*C. jejuni* M1 and *C. jejuni* NCTC 11168; Table 2.8). Isolates were cultured as described in section 2.2.2.1. Not all strains were used in each assay because ofstock contamination.

3.2.2 Epithelial cell culture

Avian intestinal epithelial cells (MM-CHiC clone: 8E11; Tentamedix GmbH; formerly Micromol, Germany) and human colon adenocarcinoma epithelial cells (CaCo-2; ECACC, Cat number 86010202) were cultured as described in section 2.3.1.

3.2.3 *Campylobacter* DNA Isolation and Genomic Analysis

Nineteen of the *Campylobacter* isolates were cultured as described in section 2.2.2.1 and DNA successfully extracted (section 2.2.6). Genomic DNA was sequenced at The Swansea Genome Centre. Full genomic sequences were analysed using Galaxy and heat maps generated using Morpheus.

Genomes were analysed for the presence and absence of genes encoding *Campylobacter* virulence factors identified in the virulence factor database (VFDB) (Table 2.11). Genomes were also analysed for the presence and absence of antibiotic resistance genes using the Resfinder database (Table 2.12), NDARO (Table 2.12), and comprehensive antibiotic resistance database (CARD) (Table 2.12).

3.2.4 *Campylobacter* growth assay

The growth assay was performed as described in section 2.2.3.1.

3.2.5 Invasion of epithelial cell lines by *Campylobacter*

Epithelial cells were cultured in a 24-well plate as described in section 2.3.2 and 2.3.3, respectively. The gentamicin protection assay was performed as described in section 2.3.5.

The number of bacterial cells recovered from epithelial cells was compared to the original bacterial inoculum and used to calculate percentage invasion.

3.3 Results

3.3.1 Genomic Evaluation of *Campylobacter* Isolates for Virulence Factors and Antibiotic Resistance

To compare the presence and absence of virulence genes between isolates, the virulence factor database (VFDB) was used (Figure 3.1). *C. jejuni* NCTC 11168 contained all twenty-three genes screened for by the VFDB. Two out of 19 isolates contained both *flaA* and *flaB* (*C. jejuni* NCTC 11168 and *C. coli* C11), whilst *flaC* was present in all 19 isolates. Thirteen genes including *cadF* were present in all isolates (Figure 3.1). All three *cdt* genes were present in 12 isolates; L7 was the only isolate that contained only two out of three *cdt* genes (Figure 3.1).

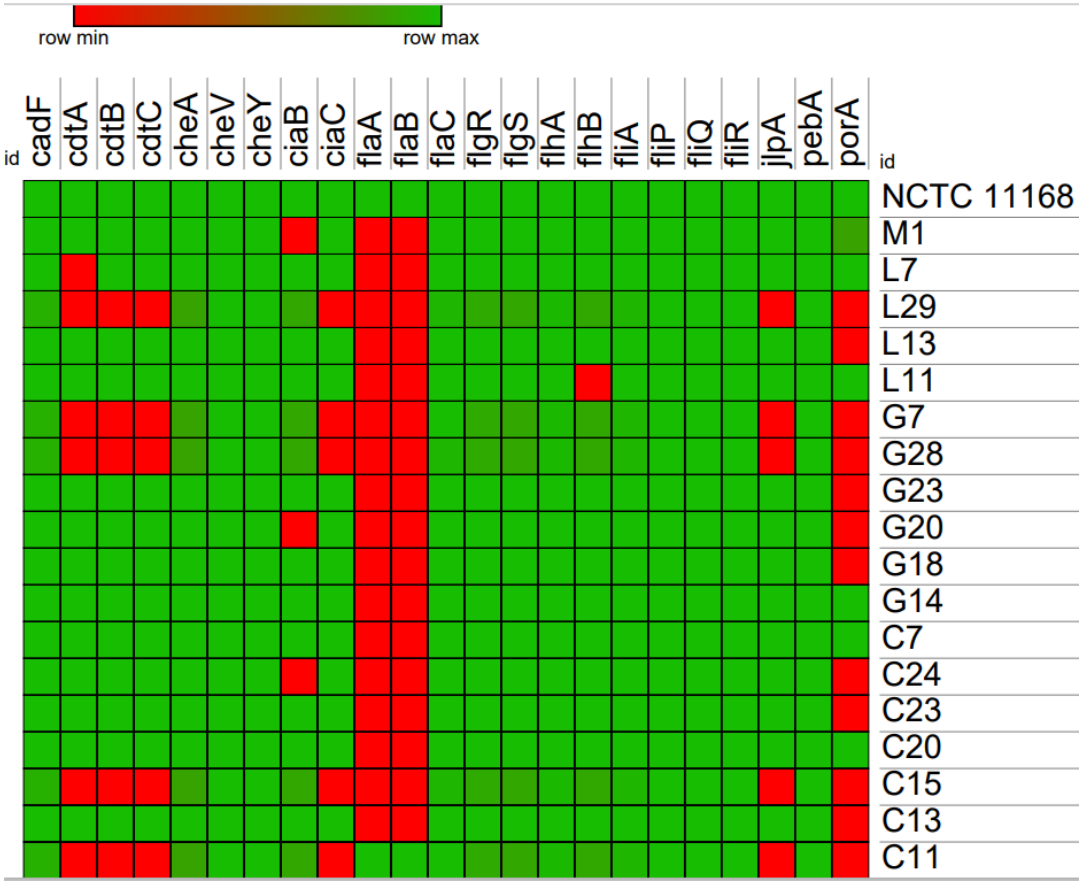

Figure 3.1 Presence and absence of genes encoding virulence factors in 19 *Campylobacter* isolates.

Genomes of 19 *Campylobacter* isolates were compared to the VFDB for presence and absence of 23 genes known to encode *Campylobacter* related virulence factors. Green squares indicate presence of the gene, red squares indicate gene absence.

Three databases were utilised to compare antibiotic resistance across the 19 *Campylobacter* isolates (Figure 3.2; Table 2.8). *C. jejuni* M1 was the only isolate to

contain the blaOXA-184 gene and this was consistent across all three databases. Presence/absence of bla-OXA genes was consistent across all three databases (Figure 3.2).

The Resfinder database (Figure 3.2 (A)) produced results for tet(O) presence/absence that did not agree with results produced from NDARO (Figure 3.2 (B)) and CARD (Figure 3.2 (C)). Both the Resfinder and CARD databases screened isolates for blaOXA-452 and blaOXA-453 (OXA-452, OXA-453) genes (Figure 3.2 (A and C)), which confirmed presence of the blaOXA-452 gene in a singular isolate (C20) and blaOXA-453 in two isolates (G7 and C11).

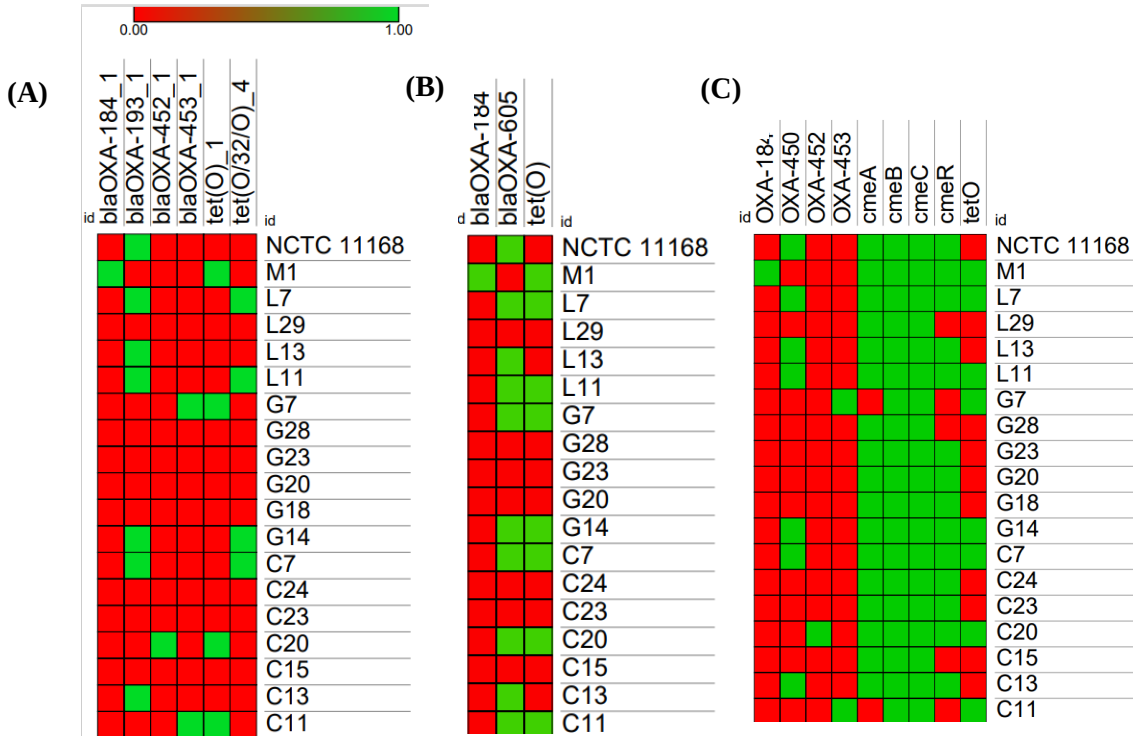

Figure 3.2 Presence and absence of genes encoding antibiotic resistance in 19 *Campylobacter* isolates.

Genomes of 19 *Campylobacter* isolates were compared to (A) the Resfinder database for presence and absence of 6 genes known to confer antibiotic resistance, (B) NDARO for presence and absence of 3 genes known to confer antibiotic resistance, and (C) the CARD for the presence and absence of 9 genes known to confer antibiotic resistance. Green squares indicate presence of the gene, red squares indicate gene absence.

3.3.2 *Campylobacter* Growth

Campylobacter isolates from caeca grown (Figure 3.3) at 42˚C had significantly higher growth rates compared to the same isolates grown at 37˚C (n=7) (Table 3.1). However,

the same was not observed for ileal and liver isolates (Figure 3.4 and Figure 3.5, respectively), where temperature did not affect growth rate ($p>0.05$).

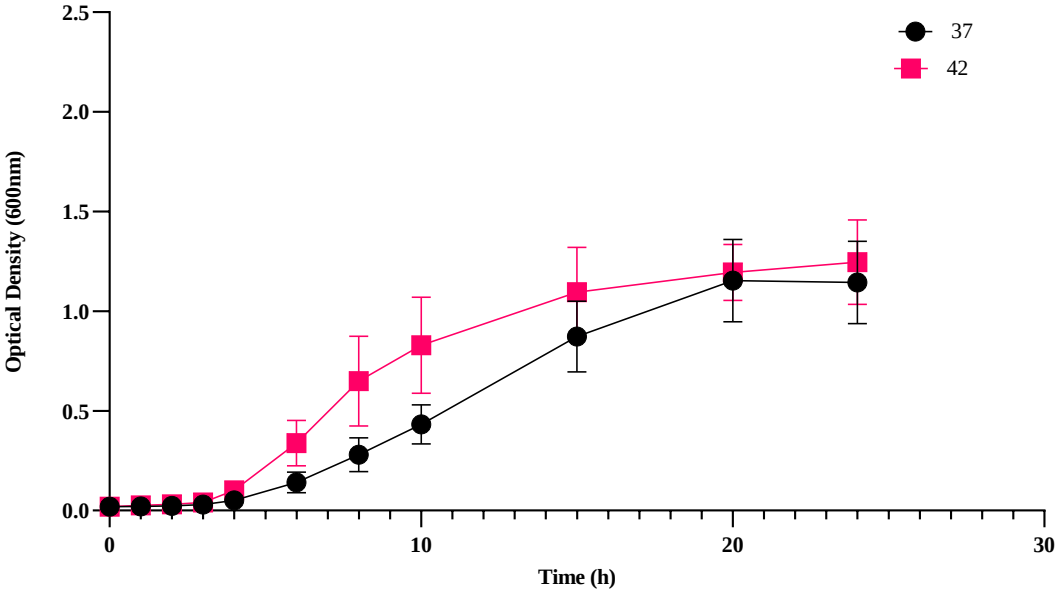

Figure 3.3 Growth of *Campylobacter* caecal isolates over 24 h at different temperatures.

Optical density (600 nm) of *Campylobacter* isolates of caecal origin (isolates n = 7) incubated at 37˚C and 42˚C, over 24 h was measured. Mean optical density plotted ± SEM (biological replicate n = 3-5). A two-way ANOVA revealed temperature as a significant cause of growth variation ($p = 0.0234$).

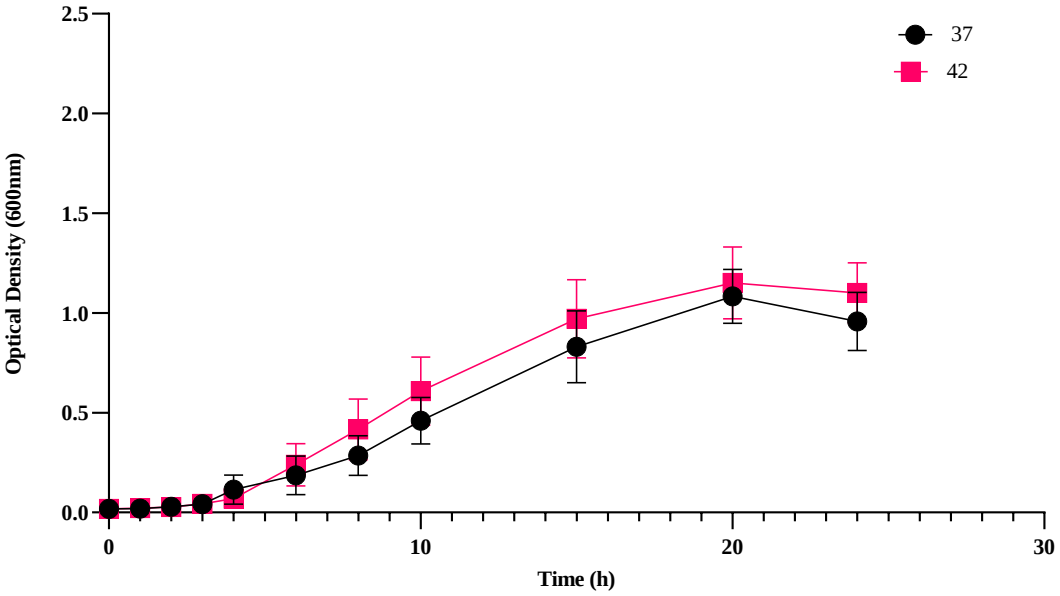

Figure 3.4 Growth of *Campylobacter* ileal isolates over 24h at different temperatures.

Optical density (600 nm) of *Campylobacter* isolates of ileal origin (isolates n=8) incubated at 37˚C and 42˚C, over 24 h was measured. Mean optical density plotted ± SEM (biological replicates n = 3-5). A two-way ANOVA revealed temperature had no significant effect on growth of ileal isolates (p = 0.218).

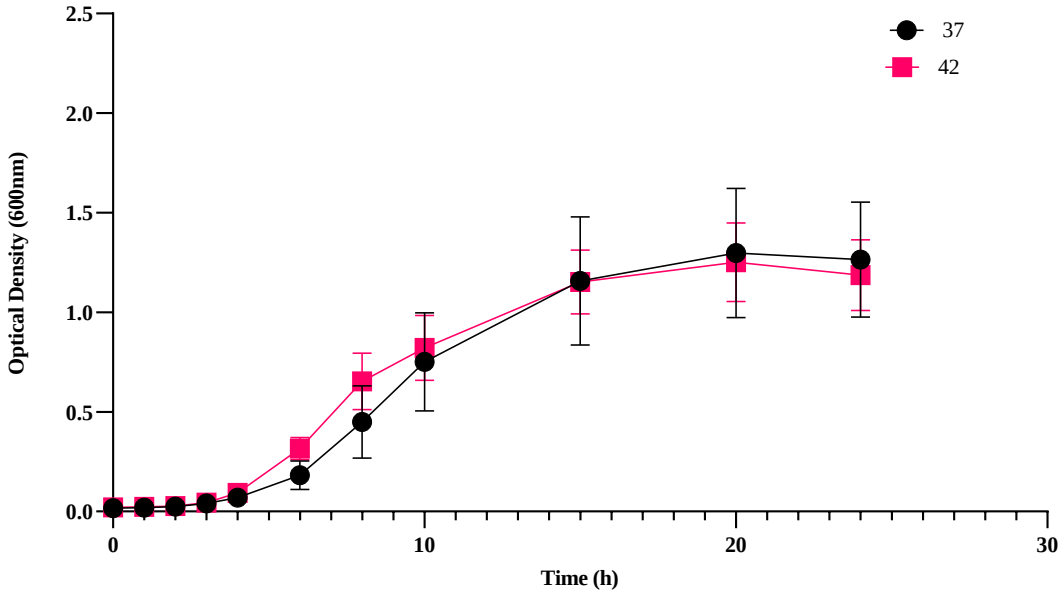

Figure 3.5 Growth of *Campylobacter* liver isolates over 24h at different temperatures.

Optical density (600 nm) of *Campylobacter* isolates of liver origin (isolate n = 6) incubated at 37˚C and 42˚C, over 24 h was measured. Mean optical density plotted ± SEM (biological replicate n = 3-5). A two-way ANOVA revealed temperature had no significant effect on growth of liver isolates (p = 0.674).

3.3.3 *Campylobacter* Invasion

Twenty-one *Campylobacter* isolates from different sections of the chicken gut and liver were tested *in vitro* for their invasive capability into 8E11 avian (Figure 3.6) and CaCo-2 human (Figure 3.7) epithelial cell lines. Two reference strains (*C. jejuni* M1 and *C. jejuni* NCTC 11168) were also used. A gentamicin protection assay (GPA) was used to measure the percentage of bacterial cells internalised by epithelial cells from a standardised inoculum (section 2.2.2.1 and 2.3.5). The GPA methodology does differ in the literature making comparison between studies difficult, and therefore the accuracy of the assay has been questioned and evaluated (Friis et al., 2010) One out of the seven caecal isolates (C20) had a mean invasion of over 1% of the original inoculum into CaCo-2 cells (Figure 3.7), all seven isolates achieved <1% invasion into 8E11 cells. No statistically significant difference was found between individual caecal isolates.

Out of the eight ileal isolates, one isolate achieved, on average, >1% invasion into CaCo-2 cells (G28) (Figure 3.7); similarly, to the caecal isolates, all ileal isolates achieved <1% invasion into 8E11 cells (Figure 3.7). A Dunn's Multiple comparisons statistical test compared invasion percentage of individual isolates and revealed a significant difference ($p < 0.05$) in invasion between two of the ileal isolates (G25 & G28; Figure 3.7), however this was only seen in the CaCo-2 cell line.

Of the six liver isolates, all achieved <1% invasion into 8E11 cells (Figure 3.8); only four of the isolates were successfully measured using the GPA into CaCo-2 cells, of which one isolate achieved >1% invasion (L29). No statistically significant difference was found between individual liver isolates.

The mean invasion ability of all isolates was compared and there was no statistically significant difference found between isolates from various sources of the chicken gut or liver.

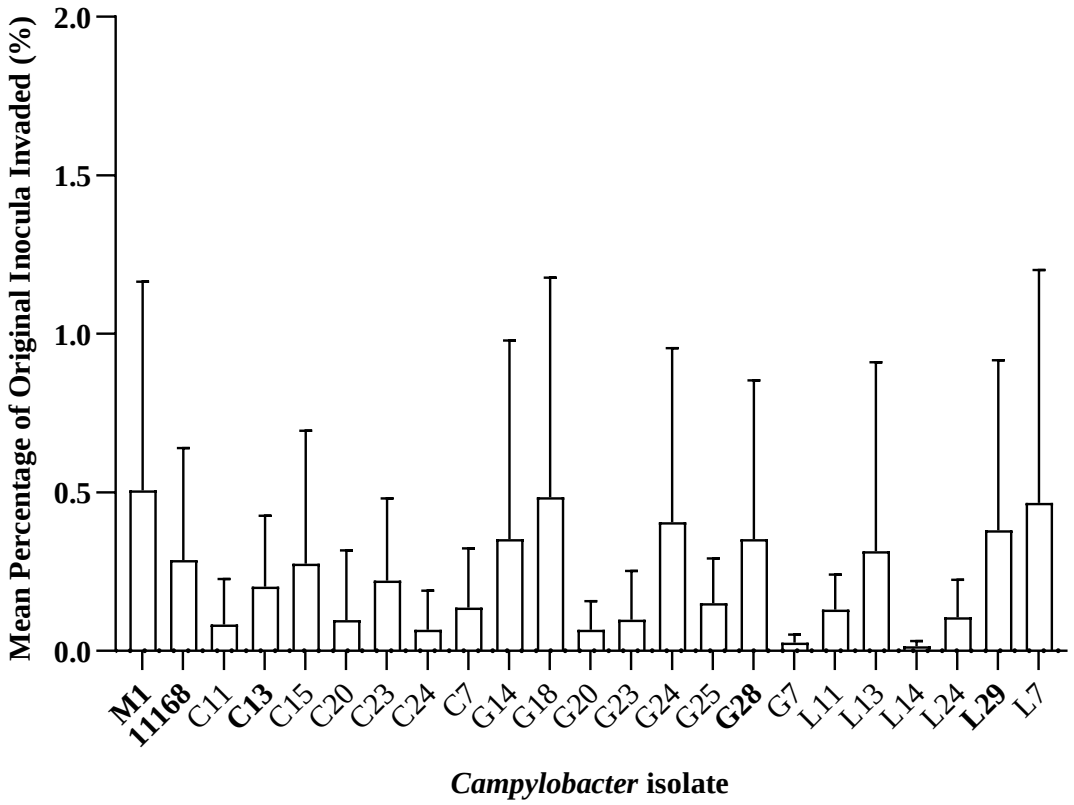

Figure 3.6 Invasion (%) of *Campylobacter* isolates into 8E11 cells

Epithelial cells were grown to confluence and infected with *Campylobacter* strains for 4 h. Reference strains were *C. jejuni* M1 and *C. jejuni* 11168 and they were compared to caecal (C), ileal (G), and liver (L) isolates. Data is presented as the mean of biological replicates (n= 3) ±

SEM. Isolates in bold were carried forward for experimental analysis with feed additives and probiotics. A Kruskal-Wallis test and Dunn's multiple comparisons post-hoc test was applied to the data and revealed no significant differences of isolate invasion into epithelial cells.

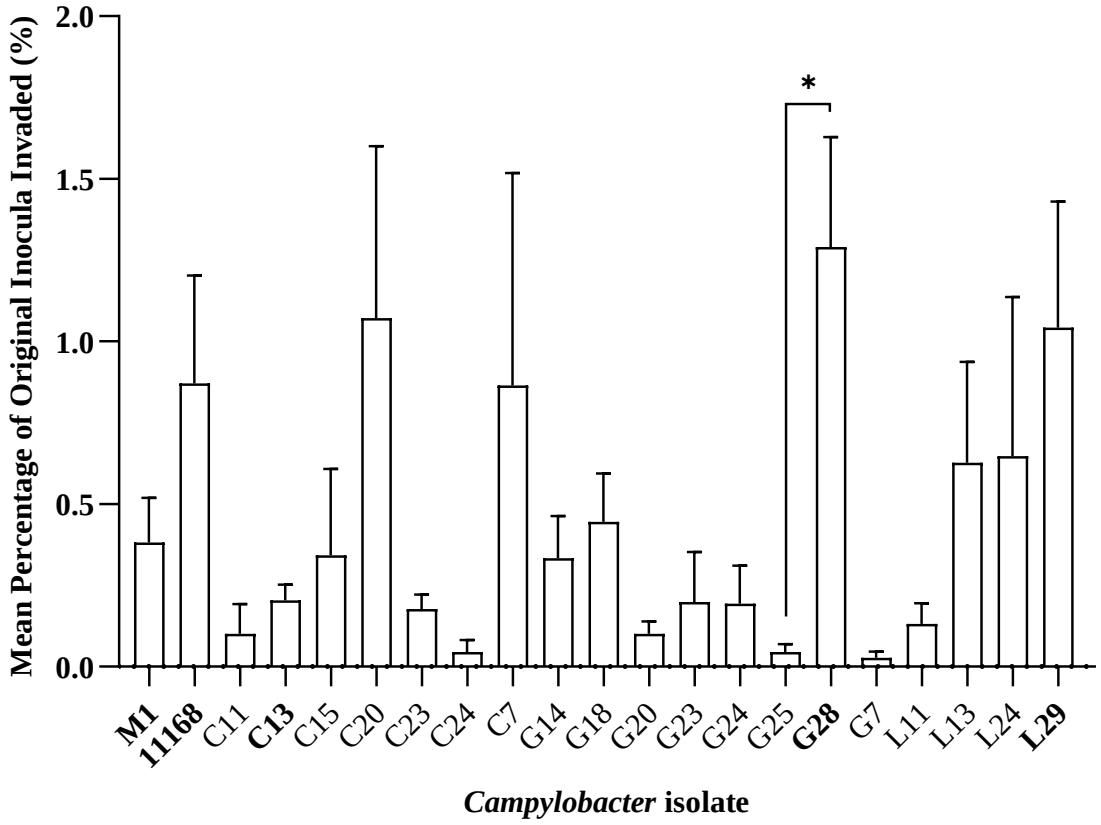

Figure 3.7 Invasion (%) of *Campylobacter* isolates into CaCo-2 cells

Epithelial cells were grown to confluence and infected with *Campylobacter* strains for 4 h. Reference strains were *C. jejuni* M1 and *C. jejuni* 11168 and they were compared to caecal (C), ileal (G), and liver (L) isolates. Data is presented as the mean of biological replicates (n= 3) ± SEM. Isolates in bold were carried forward for experimental analysis with feed additives and probiotics. A Kruskal-Wallis test and Dunn's multiple comparisons post-hoc test was applied to the data and revealed a significant variation between isolate invasion into epithelial cells ($p = 0.0012$); invasion of isolate G25 and G28 were significantly different (* $p < 0.05$).

Isolates were grouped and a mean invasion capability was calculated for liver, caecal and ileal isolates (Figure 3.8). The highest recorded individual invasion measurement was from a caecal isolate into CaCo-2 cells (C7 - 4.762%), but the highest mean invasion was measured when liver isolates invaded CaCo-2 cells (Figure 3.8).

Caecal and liver isolates (Figure 3.8) both expressed higher invasive capabilities into CaCo-2 cells than 8E11, this was statistically significant ($p = 0.021$, $p = 0.013$,

respectively). There was no statistically significant difference between ileal isolate invasion into 8E11 or CaCo-2 cells (Figure 3.8).

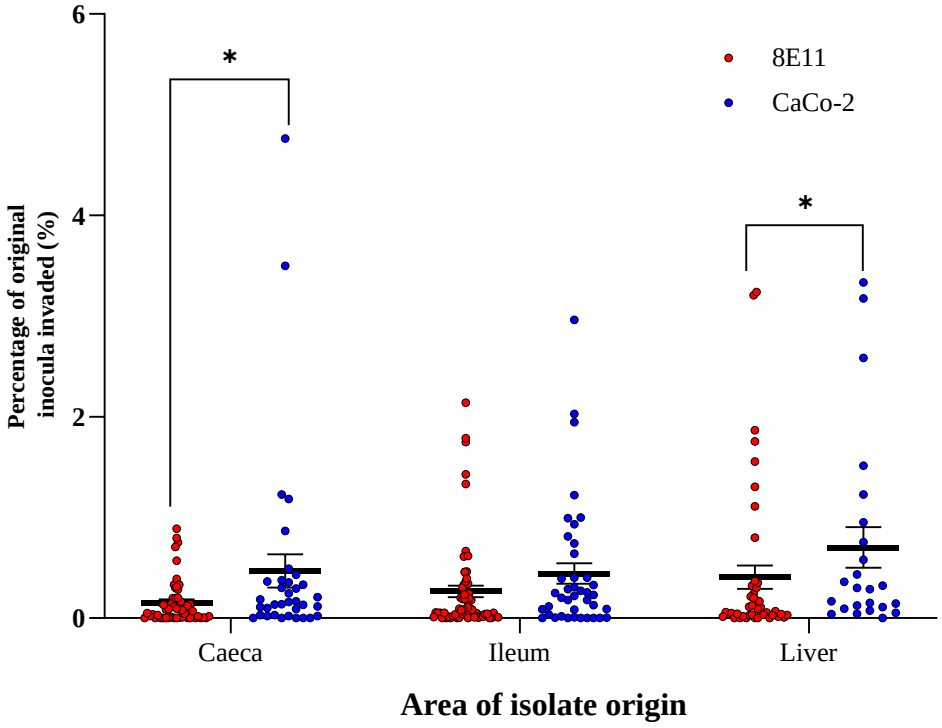

Figure 3.8 Invasion (%) of *Campylobacter* isolates into CaCo-2 and 8E11 cells

Epithelial cells were grown to confluence and infected with *Campylobacter* strains for 4 h. Reference strains were *C. jejuni* M1 and *C. jejuni* 11168 and they were compared to caecal (C), ileal (G), and liver (L) isolates. Data is presented as the mean of all isolates from a given source ± SEM (variation of isolates within each group is also plotted). A Mann-Whitney test was applied to the data and revealed caecal and liver isolates had a significantly higher invasion percentage into CaCo-2 cells (* $p < 0.05$).

3.3.4 Comparison of *Campylobacter* invasion to published dataset

To select strains for use in product testing later in the thesis, the invasion data generated from the GPA in this study was plotted against data generated in a previous study that utilised the same assay for invasion analysis (John, 2018; Figure 3.9 and 3.10). A simple linear regression was produced and the r^2 calculated for invasion into 8E11 cells was 0.0005 ($p = 0.7708$) (Figure 3.8) and 0.0245 for invasion into CaCo-2 ($p = 0.1162$) (Figure 3.10). These results indicated that the *Campylobacter* strains did not invade consistently between experiments.

In addition to the reference strains (*C. jejuni* M1 and *C. jejuni* NCTC 11168), three experimental strans, including C13 (low invader), and G28 and L29 (high invaders) were selected to carry forward for the remainder of the *in vitro* experimentation (Table 3.6).

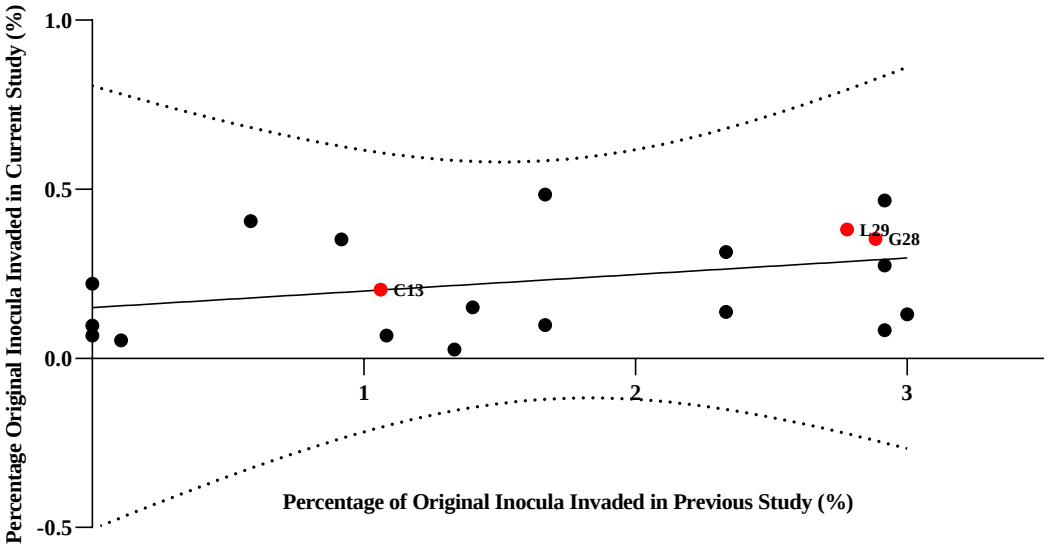

Figure 3.9 Comparison of invasion (%) of *Campylobacter* between previous study (John, 2018) and current study into 8E11 cells.

A simple linear regression was conducted on invasion data of *Campylobacter* isolates into CaCo-2 cells from the current study and a previous study using the same isolates; mean of biological replicates (n = 3 to 15) is plotted. Data points in red indicate isolates selected and carried forward for experimental analysis with feed additives and probiotics.

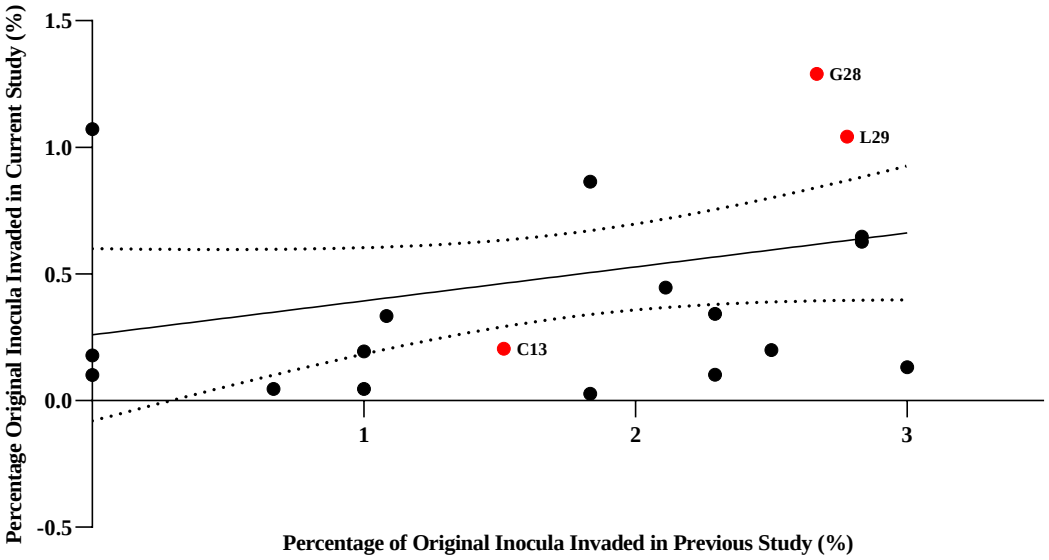

Figure 3.10 Comparison of invasion (%) of *Campylobacter* between previous study (John, 2018) and current study into CaCo-2 cells.

A simple linear regression was conducted on invasion data of *Campylobacter* isolates into CaCo-2 cells from the current study and previous study using the same isolates; mean of biological replicates (n = 3 to 15) is plotted. Data points in red indicate isolates selected and carried forward for experimental analysis with feed additives and probiotics.

Table 3.3 Summary of isolates carried forward for feed additive testing based on invasion data compared between current study and previous study (John, 2018).

	Invasion (%) into 8E11 cells (±SEM)		Invasion (%) into CaCo-2 cells (±SEM)	
Isolate	**Previous study**	**Current study**	**Previous study**	**Current study**
C13	1.061	0.203 ±0.074	1.515	0.205 ±0.047
G28	2.883	0.353 ±0.144	2.667	1.29 ±0.338
L29	2.778	0.381 ±0.162	2.778	1.042 ±0.388

3.4 Discussion

A total of 29 *Campylobacter* isolates from various sources within the avian GI tract and with different sequence types were analysed (27 isolates from naturally infected broiler chickens and two reference strains (*C. jejuni* M1 and *C. jejuni* NCTC 11168; Table 3.2)). The results in the present study emphasise the diverse behaviour of *Campylobacter* isolates in a sterile and controlled laboratory environment. In addition, a diverse phenotypic variation was observed for isolates from the same source, including growth rate and invasion potential into epithelial cell lines of different origins (Bourke, 2002; van Putten et al., 2009).

Cools *et al.* (2003) reported that isolates from various sources (avian, human, environmental) have different optimum atmospheric requirements for *in vitro* culture. Here, the ability of isolates of avian and human origin (Table 3.2) to grow at both 37˚C and 42˚C with temperature resulting in no significant difference in population density at 24h was demonstrated.

The isolates analysed in the present study were evaluated by John (2018), however, the protocol used in the current study was updated due to isolates exhibiting increased resistance to the previously used gentamicin concentration which was demonstrated by optimisation assays and gentamicin washes. In the current study preliminary GPAs were conducted with each assay increasing the concentration of gentamicin for the extracellular killing of non-invaded bacterial cells. It was found that 125 µg/mL was optimum for ensuring no survival of extracellular bacteria compared to 100 µg/mL in the original protocol, however this increase in gentamicin concentration used may have led to intracellular killing of bacteria, resulting in a lower percentage invasion being reported. The invasion results we report here are dissimilar to the previous study (despite use of the same epithelial cell lines). The isolates invaded at approximately 10-fold lower than previously reported, further confirming the unpredictable behaviour of *Campylobacter* strains and the influence of a laboratory environment on resistance to antibiotics.

The main objective of this chapter was to select three strains for further testing (Chapter 4 and Chapter 5) that most accurately represented *Campylobacter* populations present within broiler production and produced consistent results during *in vitro* testing.

Nineteen of the twenty-nine isolates were genome sequenced and subjected to genomic analysis. The *cadF* gene was present in 100% of isolates screened, including the two reference stains of human origin. There is a growing number of studies that have reported expression of CadF in 100% of strains analysed (human and avian origin) (Bang et al., 2004; Krause-Gruszczynska et al., 2007). However other studies have reported a variation in this statistic, for example, detection of *cadF* presence in avian isolates was reported as 76% (37 out of 49 isolates) by Chansiripornchai and Sasipreeyajan (2009). The variation in detection of *cadF* between studies could be a result of geographical differences or due to differences associated with isolate source (Chansiripornchai and Sasipreeyajan, 2009).

Within the isolates assessed in the current study, 10.5% of isolates contained the *flaA* gene which is an extraordinarily low prevalence compared to results in the literature that report 100% presence (Andrzejewska et al., 2011; Bang et al., 2004). Motility is considered an essential function for survival within, and colonisation of both avian and human GI tracts (D. J. Bolton, 2015). *FlaA* and *flaB* are the two primary genes that encode the flagellar filament and the expression of both (in a defined ratio) is believed to be a prerequisite for successful motility (Lertsethtakarn et al., 2011; van Vliet & Ketley, 2001; Wassennaar et al., 1993). In this study, the low incidence of *flaA* in genomes compared to previous reports could be due to isolate source.

Coincidentally, *flaB* was present in the same two isolates out of 19 screened indicating the synchronous existence of both FlaA and FlaB proteins to encode the flagellar filament. However, studies suggest that motility of *Campylobacter* is possible without the expression of *flaB* (Wassennaar et al., 1993). Lower prevalence of *flaB* among *Campylobacter* populations compared to *flaA* has been reported and this variation is attributed to isolate source (Krutkiewicz & Klimuszko, 2010).

Out of the 19 isolates sequenced, the Cdt cluster was present in 12 and a partial cluster present in a singular isolate. Cdt is a multi-subunit toxin produced by both *C. jejuni* and *C. coli* and is an important determinant of virulence (Asakura et al., 2008). The presence of *cdtA, cdtB,* and *cdtC* is required for functional Cdt activity – absence of one or more of these genes produces low or no Cdt activity (Asakura et al., 2008; Bang et al., 2004).

Ripabelli *et al.* (2010) looked at the variation in Cdt gene expression across 65 *Campylobacter* isolates from human, animal and food sources and reported a higher prevalence of the Cdt cluster (96.9%) than the current study. Discrepancies between the two studies could be attributed to differences in isolate source, and number of isolates analysed. The current study only analyses two strains of human origin and the remainder from chickens, however, isolates from food, animals and clinical isolates from human disease were investigated by Ripabelli *et al.* (2010), suggesting increased toxicity of clinical isolates.

In the current study, genes encoding tetracycline resistance (tet(O) and tet(O/32/O)) were present in 21 to 37% of isolates analysed, but this varied between the databases used. Antibiotics and antibiotic growth promoters (AGPs) are banned in poultry farming due to EU legislation, however, in human treatment of campylobacteriosis macrolides and fluoroquinolones are routinely used (Kurincic et al., 2005; Wagenaar et al., 2006). Resistance to tetracycline varies based on geography and source, for example, resistance in *C. coli* isolates from pigs varied between 1 to 83% across six European countries. The same analysis was conducted on *C. jejuni* isolates from chickens across the same six countries and resistance varied between 1 to 67% (Wagenaar et al., 2006). Isolate source, country of origin, and the database used for analysis appear to be crucial factors in confirming antibiotic resistance amongst isolates.

The genomes of 19 *Campylobacter* isolates were processed through three independent anti-microbial resistance (AMR) databases (Resfinder, CARD, and the NCBI database). Discrepancies between database outputs for the isolates screened could be due to several reasons. Indeed, variable results between Resfinder and CARD (as seen in this study) have been previously reported (Mahfouz et al., 2020). A review by Papp and Solymosi (2022) rigorously compared the three AMR databases used in this study (and other AMR databases) and matched the number of sequences with the associated count of unique genes stored within them. NDARO and Resfinder had 13 and 9 duplicate sequences for specific genes (respectively) whereas CARD was the only database where the number of unique genes and sequences was equal. The AMR databases used in this study are all accessible and regularly updated, however their architecture and content vary, reflected in the differing outputs. An appropriate database must therefore be chosen by each researcher that suits the research question needs (Papp & Solymosi, 2022).

For this specific study, there was a large focus on detection of genes encoding important virulence factors that contribute to *Campylobacter* pathogenesis. In the present study only *C. jejuni* M1 was considered a high invader in 8E11 cells (above 0.5% invasion) despite *C. jejuni* 11168 and isolate C13 testing positive for the more virulence associated genes; in CaCo-2 cells a total of seven isolates (*C. jejuni* NCTC 11168, and isolates C20, C7, G28, L13, L24 and L29) exhibited invasion levels of above 0.5%. From the observed results a link between virulence genes and invasion could not be established. Investigating AMR between isolates in this study is not crucial for determining the invasivity of specific isolates, however it would be preferable to utilise a database (such as CARD) where there is a single sequence matched to each unique gene, especially when using a small number of unique isolates.

The results from this study found invasion between individual isolates to be diverse, however caecal and liver isolates were significantly more invasive toward avian (8E11) cells than human (CaCo-2) cells. Strain specific difference in invasivity was only observed between two isolates (G25 and G28) into CaCo-2 cells; John (2018) reported similar findings where some isolates were unable to invade either cell line, and that invasion potential into cells is highly variable and is dependent on strain type. When grouped based on anatomical area of isolation (liver, ileum, or caeca) there was no significant difference between the isolates for invasion into 8E11 or CaCo-2 cells. John (2018) found that under the same conditions, liver isolates invaded cell lines at a much higher rate compared to caecal or ileal isolates. There are numerous other studies that support these findings which suggest that a higher level of invasion is required for isolates to reach the liver from the gastrointestinal tract (Van Deun, Pasmans, Ducatelle, et al., 2008; L. K. Williams et al., 2013). Ileal isolates showed no difference in invasive capabilities regardless of cell line, however caecal and liver isolates invaded CaCo-2 cells at a significantly higher rate than 8E11 cells (D. A. John et al., 2017). It is well documented that the choice of cell line can have an influence on the invasion potential of isolates (D. John, 2018). CaCo-2 cells are commonly used for both *in vitro* and *in vivo* experimentation with *Campylobacter* (Hänel et al., 2004; D. A. John et al., 2017; C M Szymanski et al., 1995). The avian 8E11 cell line is a novel cell line with few studies to date that have utilised this cell line with *Campylobacter* to investigate pathogenesis (D. John, 2018; D. A. John et al., 2017).

Five isolates were selected including two reference isolates chosen due to frequent use in published research and consistent phenotypic behaviours both *in vitro* and *in vivo* (*C. jejuni* M1, *C. jejuni* NCTC 11168). Isolates C13, G28 and L29 were selected because they all showed similar presence/absence for virulence genes, the only motile strain selected was the caecal isolate due to testing positive for *flaA* and *flaB.* The selected isolates were further chosen based on their consistency over multiple studies. The invasion recorded in the current study was on a whole lower than that recorded previously, however the invasion of the C13, G28 and L29 into 8E11 cells was the most consistent between the two studies. The invasion of isolates into CaCo-2 cells, however, was not similar for 12 of the isolates; therefore, it was decided to choose isolates that showed consistent results for invasion into 8E11 cells only as this study focusses on preventing extra intestinal spread in the avian GI tract.

A single isolate was chosen from the caeca, ileum, and liver due to the well documented variability in strains based on anatomical source (AbuOun et al., 2005; D. John, 2018). However, all isolates used in this study (aside from reference strains which are of human origin) were of avian origin from free range chickens of the same species.

3.5 Conclusion

To conclude, the presence/absence of virulence factor associated genes is highly variable between isolates regardless of source and there is variability between genomic databases which could influence the genomic outputs and therefore should be considered before use. Incubation temperature during the growth phase of *Campylobacter* isolates *in vitro* has no significant effect on the final population density at 24 h (Table 3.4); *Campylobacter* isolates of avian origin are equally capable of growth at human and avian internal temperatures demonstrating the adaptations this species has made to survive variable environments and aid pathogenesis. Invasive capability *in vitro* is highly variable between isolates and dependent on the cell line used, suggesting there is no simply defined level of invasive potential that can be assigned to all strains; isolate source and cell line are influential on bacterial invasion. Three isolates (*C. coli* C13, *C. coli* G28, *C. coli* L29) and two reference strains (*C. jejuni* M1 and *C. jejuni* NCTC 11168) were selected for further testing with feed additives in the chapters 4 and 5.

Table 3.4 Summary of main results from Chapter 3

Experiment	**Result**
Genomic Variation – Virulence Genes	*C. jejuni* NCTC 11168 was the only isolate to contain all virulence factors *C. jeuni* NCTC 11168 and *C. coli* C11 were the only isolates to contain *flaA* and *flaB* genes
Genomic variation – Antibiotic resistance genes	*C. jejuni* M1 was the only isolate to contain the blaOXA-184 gene
Campylobacter growth	Caecal isolates had significantly higher growth rates at 42˚C compared to the same isolates grown at 37 ˚C There was no variation in growth of isolates of ileal or liver origin dependent on temperature
Campylobacter invasion	Ileal isolate G28 had a significantly higher invasion into CaCo-2 cells compared to ileal isolate G25 On average, liver and caecal isolates had a higher invasion into CaCo-2 cells than 8E11 cells

Chapter 4: Direct effects of feed additives on *in vitro* growth and motility of *Campylobacter*

4.1 Introduction

Newly hatched chicks are highly susceptible to colonisation by pathogenic microorganisms due to an immature and sterile gastrointestinal (GI) tract (Panda et al., 2009). For over 50 years, antimicrobials and antibiotics have been used to suppress and eliminate harmful enteropathogens (Panda et al., 2009). In addition to treating sick animals and preventing disease, antibiotics have been administered to production animals to enhance growth since the 1940s when it was discovered that their interaction with the intestinal microbiota induced a growth promoting effect (Castanon, 2007; Vazquez, 2016). Due to increasing antibiotic resistance associated with zoonotic pathogens there have been modifications to the legislation for the use of antibiotic growth promotors (AGPs) within the United States (US) and the European Union (EU) (Panda et al., 2009; Vazquez, 2016). Within the US, AGPs have not been withdrawn completely from animal production, however the Food and Drug Administration (FDA) has issued guidelines for the animal production industry to voluntarily withdraw AGPs of medical importance (Teillant & Laxminarayan, 2015; Vazquez, 2016). Despite the FDA recommendations it was estimated that 14.6 million kg of antibiotics were sold for use in production animals across the US in 2012 (Teillant & Laxminarayan, 2015). Remarkably, this exceeded the number of antibiotics sold for human use in the US by over 4-times (Vazquez, 2016). In the EU, however, there has been a total ban on AGPs since January 2006, due to the increasing antibiotic resistance and the residual effects on human consumers of treated animal products (Castanon, 2007; Panda et al., 2009).

The restrictions put in place for AGP use in poultry production have stimulated research into alternative compounds and substances that can be used (Ahsan et al., 2016; Panda et al., 2009; Redondo et al., 2014; Sugiharto, 2016). Broiler chickens have a short lifespan (5 to 7 weeks). Vaccination is not recommended against enteropathogens such as *Salmonella* due to the immature immune system of young broilers resulting in poor antibody responses (de Zoete et al., 2007; Mot et al., 2014). In addition, a successful vaccination against *Campylobacter* infection in chickens has not yet been developed (Bennett et al., 2018; F. Van Immerseel et al., 2005). Therefore, there has been extensive research into the potential for isolated nutrients, dietary supplements, herbal compounds,

and genetically modified foods to be used as alternatives to AGPs (Ahsan et al., 2016). An economically viable and practical alternative should result in a 2-log reduction in *Campylobacter* populations on poultry carcass contaminations, which could bring a 30-fold reduction in human campylobacteriosis cases (F. Solis de los Santos et al., 2009; Grant et al., 2018; Jayaraman et al., 2017; Singh & Kim, 2021).

Organic acids are a widely used group of compounds suitable for use as feed additives due to their significant antimicrobial activities (Van Immerseel *et al.*, 2004; Filip Van Immerseel *et al.*, 2004; Hermans *et al.*, 2010; Ahsan *et al.*, 2016). They are known to selectively stimulate the growth and activity of beneficial gut microbes, improving host gut health (Ahsan et al., 2016). SCFA are naturally occurring compounds found in the GI tract of poultry as the result of microbial metabolism or carbohydrate fermentation (Ahsan et al., 2016). Medium chain fatty acids (MCFAs) are naturally found in some mammalian milk and coconut oil and have been found to modify virulence factor expression, e.g., decrease in *hilA* expression, thus decreasing invasiveness of *Salmonella in vitro* (F. Solis de los Santos et al., 2009). Organic acid efficacy as a feed additive to control microbes is variable, and heavily influenced by concentration, acid form and degree of dissociation when it arrives in the intestine and is taken up by bacterial cells (Leeson et al., 2005). When studied *in vitro,* MCFA and monoglycerides of MCFA were bactericidal against *Campylobacter,* however *in vivo* research remains inconsistent (Marta Isabel Gracia et al., 2016).

Pro- and prebiotics could also provide the host with improved gut health and protection from enteropathogens (Abdelqader & Al-Fataftah, 2016). The ingestion of pro- and prebiotic species can enhance microbial fermentation in the small intestine, leading to increased production of SCFA (Abdelqader & Al-Fataftah, 2016; Deepa et al., 2018). There is *in vitro* evidence to suggest that pro- and prebiotics also possess direct antimicrobial qualities, specifically against *C. jejuni*, however this has not been replicated *in vivo* (Marta Isabel Gracia et al., 2016).

4.1.1 Butyric Acid

Butyric acid may also be referred to as butanoic acid, 1-propanecarboxylic acid, or propanecarboxylic acid (Deepa et al., 2018). It has a molecular weight of 88.12 g/mol and a pKa of 4.82 (weak acid) and is corrosive and volatile in nature. In the feed

manufacturing process it is most commonly available as sodium butyrate as this combination is easier to handle (Ahsan et al., 2016; Deepa et al., 2018).

Upon hatching, the gut microbiome of the chick is undeveloped and levels of SCFA in the distal small intestine and ceca gradually increase and plateau by day 15 post hatch (Leeson et al., 2005). There is little information available on the metabolism of butyrate by poultry, however it is known to be a major modulator of epithelial cell activity, stimulant of villi growth and modulator of intestinal microflora (Abdelqader & Al-Fataftah, 2016; Ahsan et al., 2016; Leeson et al., 2005). Free butyrate is quickly absorbed in the upper digestive tract; however sodium butyrate reaches the small intestine where it is converted into butyric acid and then absorbed by enterocytes (Ahsan et al., 2016; Leeson et al., 2005). It improves intestinal health through various mechanisms, although the efficacy of sodium butyrate is dependent on the pKa value of the butyric acid and pH of the section of the digestive tract in which it is absorbed (Ahsan et al., 2016). This was confirmed by Leeson *et al.* (2005) who reported a correlation between pathogen control and the levels of undissociated butyrate in the caeca of birds. Similarly, Deepa *et al.* (2018) reported that the increases in villi height, and the villi height:crypt depth ratio varied between different forms of butyric acid treatment.

Supplementation of poultry feed with butyric acid has produced performance and antimicrobial results like those achieved with oxytetracycline supplementation (Deepa et al., 2018). When undissociated SCFA are taken up by bacterial cells, this reduces the cytoplasmic pH and modifies purine bases, denatures enzymes, leading to cell death. Butyric acid is the most potent SCFA against acid-tolerant enteropathogens such as *E. coli* and *Salmonella* (Ahsan et al., 2016; Panda et al., 2009).

To investigate the *in vivo* bactericidal capabilities of butyric acid against enteropathogens, multiple studies (de los Santos *et al.*, 2008; Van Deun, Pasmans, Van Immerseel, *et al.*, 2008; de los Santos *et al.*, 2009; Xiao *et al.*, 2017) have been conducted to determine the range of concentrations that might achieve desired results without negatively impacting bird productivity. Panda *et al.*, 2009 found that addition of butyric acid to poultry diets at 0.4% per weight of feed was equally as effective as the control AGP in reducing *E. coli* counts from the crop, while also achieving optimum weight gain and feed conversion ratios (FCR) throughout the study. Butyric acid feed supplementation at 0.4% (w/w) in the form of butyrate glycerides has been considered to maintain intestinal villi structure

and is beneficial to poultry in comparison to the negative side effects associated with antibiotic use (Leeson et al., 2005). Cox *et al.* (1994) showed that *Salmonella* colonisation of the poultry intestine could be effectively reduced by butyric acid. In 2005, Van Immerseel *et al.* compared the ability of coated (sodium salt of n-butyric acid in microencapsulated form) and uncoated (powder form) butyric acid in reducing *Salmonella* within the caeca and internal organs and found that 0.063% of the coated form could significantly reduce the shedding of *Salmonella* with coated feed more efficient at decreasing caecal colonisation by slaughter age. Coated butyric acid was shown to be more a more effective bactericidal agent against *C. jejuni* when compared to propionic acid, acetic acid, and L-lactate, with a mechanism that directly induces bacterial cell death, and indirectly by favouring the production of bacteria that compete for nutrition and space within the GI tract e.g., *Lactobacillus salivarius* (Ahsan et al., 2016; Zhao & Doyle, 2006).

4.1.2 Caprylic Acid

Caprylic acid is an 8-carbon MCFA that is a natural component of coconut oil, mammalian breast and bovine milk (de los Santos *et al.*, 2008; de los Santos *et al.*, 2009). It is generally recognised as safe (GRAS) by the US FDA and represents a practical and economical alternative to AGPs that could be implemented immediately by poultry farmers (de los Santos *et al.*, 2008; de los Santos *et al.*, 2009)..

The benefits of caprylic acid include maintaining gastrointestinal health, homeostasis, and microbial control (de los Santos *et al.*, 2008). It has been speculated that caprylic acid may exhibit a similar mechanism of action to SCFA (such as butyric acid), by lowering the pH of the GI tract, being directly bactericidal and reducing the expression of virulence factors required for intestinal colonisation (Harrison et al., 2013; D. Hermans et al., 2012).

In contrast to butyric acid, there has been more research conducted on the direct effects of caprylic acid (and other MCFA) on *Campylobacter* control within poultry. Hermans *et al.* (2010) conducted both *in vitro* and *in vivo* experiments to determine the applicability of MCFA (including caprylic and capric acid) to achieve a 2-log reduction in caecal numbers of *Campylobacter* and found that the minimum inhibitory concentration (MIC) of MCFA were 10-fold lower compared with the MIC of butyric acid. In addition, there was a significant concentration dependent bactericidal activity of caprylic acid toward *Campylobacter* (D. Hermans et al., 2010). *In vivo*, 1% caprylic acid (coated or uncoated)

had no effect on caecal *Campylobacter* numbers, however, the authors did note that formulation differences, *Campylobacter* strain differences, or the genetic backgrounds of the birds used within the trial were confounding factors (D. Hermans et al., 2010).

In contrast to Hermans *et al.* (2010), Solis de los Santos *et al.* (2008; 2009) found that caprylic acid administered at 0.7% reduced caecal *Campylobacter* counts pre-slaughter. The reduction in *Campylobacter* populations in infected chicks by 0.7% and 1.4% caprylic acid was 3- to 4- log higher than the recommended reduction to significantly impact the number of human campylobacteriosis cases each year (Solis de los Santos *et al.*, 2008). The ability of caprylic acid to reduce caecal numbers of *Campylobacter* in pre-infected birds makes it an appealing option for poultry farmers.

4.1.3 Chromium Propionate

Chromium (Cr) is a trace element that is essential within the body for the metabolism of carbohydrates, protein, and fats, however it has great potential for toxicity depending on its different forms (Hayat et al., 2020; R. U. Khan et al., 2014; Rajalekshmi et al., 2014). It is found in the environment in various oxidation states, trivalent chromium (Cr^{3+}) is the most stable and bioavailable, in contrast, hexavalent chromium (Cr^{6+}) is toxic, and inorganic (Hayat et al., 2020; R. U. Khan et al., 2014). Cr is transported in the body by chromomodulin, where it activates many enzymes required for the synthesis of nucleic acids and proteins (Arif, Hussain, et al., 2019; Hayat et al., 2020).

In poultry, Cr plays an important role in glucose homeostasis, as it is present in insulin sensitive tissues and potentiates the action of insulin (Rajalekshmi et al., 2014; Spears et al., 2019). Studies (Hayat et al., 2020; R. U. Khan et al., 2014) suggest that Cr can improve the immune status of heat stressed broilers, as it stimulates the production of corticosterone which interferes with leukocyte function and upregulates IFN-gamma expression. Chromium propionate is an organic source of Cr that is more efficiently absorbed compared to other chromium sources. At present it is the only US FDA Centre for Veterinary Medicine approved Cr source that can be used to supplement broiler diets, with up to 0.2 mg chromium propionate/kg feed (0.00002%) being permitted (Hayat et al., 2020; Rajalekshmi et al., 2014). Cr^{3+} dose must be monitored as overdose of the mineral could lead to hepatotoxic, nephrotoxic, oxidative and DNA damaging effects (Hayat et al., 2020). Spears *et al.* (2019) investigated the safety of chromium propionate where doses of Cr were 2x and 10x the US FDA approved dose. Cr at 0.00004% did not

affect the residual Cr concentration found in the broiler breast muscle or skin, however 0.0002% Cr supplementation did results in a significant increase of Cr in the liver. The European Food Safety Authority (EFSA) thoroughly investigated the use of Cr in broiler diets and concluded that 0.00004% chromium propionate (KemTRACE™) could be used safely as a zootechnical supplement for the fattening process (Bampidis et al., 2021) and did not pose a health threat to animals or human consumers. There have been additional studies that have investigated Cr supplementation up to 0.00032% for birds under normal environmental conditions and have resulted in improved antibody responses to vaccinations and improved lymphocyte proliferation, although such studies provided limited results regarding bacterial diseases (Lee et al., 2003; Rajalekshmi et al., 2014; Uyanik et al., 2002).

Most Cr studies (R. U. Khan et al., 2014; Piray & Foroutanifar, 2022; Rajalekshmi et al., 2014; Uyanik et al., 2002) investigate impact on broiler performance (feed conversion, product quality etc.) and immune system however the results between studies have shown to be highly variable (Hayat et al., 2020). Generally, Cr dosage and breast meat yield increase linearly, a similar trend is seen between dose and FCR (Arif, Hussain, et al., 2019; Rajalekshmi et al., 2014). Supplementation of 0.000015% Cr3+ significantly improved FCR and increased jejunal wall thickness and intestinal crypt depth (Hayat et al., 2020). This study also suggested that Cr3+ could have a regulatory effect on cytokines (Hayat et al., 2020). To date there is no conclusive evidence that Cr is directly bactericidal, however Leeson *et al.* (2005) reported a correlation between the presence of undissociated chromium propionate levels and pathogen control.

4.1.4 *Bacillus* spp.

For over 100 years, the benefits of consuming probiotic microorganisms have been recognised by the scientific community (Cutting, 2011; Vazquez, 2016). They improve diversity of the gut microbiome, promote the growth of beneficial bacteria to the host that produce SCFA (e.g., butyric acid), and inhibit pathogen colonisation (Kabir, 2009; Vazquez, 2016). Frequently administered probiotic bacterial genera within broiler production systems are *Lactobacillus* spp., *Bifidobacterium*, *Bacillus* spp. and *Saccharomyces* (Kabir, 2009). There are challenges with producing probiotics such as *Lactobacillus* and *Bifidobacterium* on a large scale, due to their microaerophilic and/or anaerobic requirements, slow growing nature, and requirement for storage at low

temperatures; thus, their production is complex with relatively high costs (Vazquez, 2016).

Bacillus is a genus of Gram-positive, rod-shaped bacteria used as a probiotic for 50 years (Cutting, 2011; Vazquez, 2016). It can produce spores under a stress response and these spore forming species are able to survive and multiply within the intestinal tract of animals (Vazquez, 2016). *Bacillus subtilis* is well studied at the genetic and phenotype level and has been shown to be bactericidal against *Helicobacter pylori in vitro* due to the production of the antibiotic amicoumacin (Cutting, 2011; Kobayashi et al., 2003; Pinchuk et al., 2001). Competitive exclusion of *Campylobacter* by *B. subtilis* has also been investigated (Balta et al., 2022). Thomrongsuwannakij, Chuanchuen and Chansiripornchai (2016) conducted an *in vivo* investigation into competitive exclusion and reported that *B. subtilis* was not capable of competing with *C. jejuni* within the GI tract, due to the complex pathogenesis mechanisms employed by *Campylobacter* to survive environmental stressors.

4.1.5 Aims

This study investigated the direct effects of feed additives on the growth and motility of a subset of *Campylobacter* strains identified in Chapter 3. These *Campylobacter* strains were previously selected for their consistent *in vitro* behaviours over numerous cultures. Two organic acids (caprylate and butyrate), one mineral compound (chromium propionate), and two strains of a probiotic genera (*Bacillus*) were tested based on their known bactericidal effects, contribution to gut health, and interaction with other enteric pathogens. The specific chapter aims were to:

- Determine the direct bactericidal effect of caprylate, butyrate, and chromium propionate on *Campylobacter* growth *in vitro.*
- Determine the direct bactericidal effect of media conditioned with *Bacillus* species on *Campylobacter* growth *in vitro.*
- Investigate the direct effect of chromium propionate on *Campylobacter* motility *in vitro.*

4.2 Materials and Methods

4.2.1 *Campylobacter* isolates

Three *Campylobacter* isolates (C13, G28, L29) (Table 3.3) were specifically selected from an original collection of 21 (Table 2.8). Two reference strains were used throughout this study (*C. jejuni* M1 and *C. jejuni* NCTC 11168; Table 2.8). Isolates were cultured as described in section 2.2.2.1.

4.2.2 *Campylobacter* growth assay challenged with Butyrate

Growth assays were conducted as outlined in section 2.2.4. The final concentrations of Butyric Acid in solution were 0.2% (22.6 mM), 0.6% (67.8 mM), 1.0% (113 mM) and 1.4% (158.2 mM) (v/v) with a pH of 7.0 (±0.2) achieved by adding sodium hydroxide and monitoring pH with a pH probe.

4.2.3 *Campylobacter* growth assay challenged with Caprylate

Growth assays were conducted as outlined in section 2.2.4. The final concentrations of Caprylate in solution were 0.25% (17.25 mM), 0.75% (51.76 mM), 1.25% (86.25 mM) and 1.75% (120.75 mM) (v/v) with a pH of 7.0 (±0.2) achieved by adding HEPES buffer.

4.2.4 *Campylobacter* growth assay challenged with Chromium Propionate

Growth assays were conducted as outlined in section 2.2.4. The final concentrations of Chromium Propionate in solution were 0.00002%, 0.00006%, 0.0001% and 0.00014% (v/v)

4.2.5 Preparation of *Bacillus* spp. conditioned media

Bacillus subtilis PB6 and *Bacillus licheniformis* were grown in antibiotic free 8E11 or CaCo-2 media as described in section 2.2.2.2. The 0.1% (w/v) suspension of *Bacillus* spp. in media was incubated for 4 or 24 h, under aerobic or anaerobic conditions and at 42°C (Table 4.1). After incubation, the tube was agitated for 10 s using an IKA Vortex genie 3 (Oxford, England). The optical density (600 nm) of 1mL of the solution was measured and recorded (Table 4.2). Some of the suspensions had to be further diluted with fresh antibiotic free media to achieve a reading within the limit of detection.

Growth assays with *Bacillus subtilis* PB6 and *Bacillus licheniformis* conditioned media were conducted as outlined in section 2.2.4.

4.2.6 *Campylobacter* motility assay

A bacterial motility assay was conducted as described in section 2.2.5. In addition, a second bacterial motility assay (as per section 2.2.5) was conducted with agar containing 0.00002%, 0.00006%, 0.0001% and 0.00014% chromium propionate. To achieve this, 1 mL 2X brucella liquid medium (Table 2.1) was combined with 1 mL 2X chromium propionate working concentrations (Table 2.5). This was then supplemented with 0.3% agar and protocol followed as described in section 2.2.5.

4.3 Results

4.3.1 Growth of *Campylobacter* directly challenged with feed additives

4.3.1.1 Growth of *Campylobacter* over 24 hours directly challenged with Butyrate

The five *Campylobacter* isolates selected (Table 3.3) from the original collection (Table 2.8) were grown at 42˚C in a microaerobic atmosphere (5% O_2; 10% CO_2;85 % N_2) in brucella broth and a range of concentrations from 0.2 to 1.4% of butyrate for a period of 24 h. The percentage change in optical density over 24 h was calculated (Figure 4.1) and compared to *Campylobacter* grown in untreated brucella broth. Results of a two-way ANOVA indicated that the concentration of butyrate treatment significantly affected the growth of *Campylobacter in vitro* ($p < 0.0001$). At the strain level (Figure 4.1 A-E) butyrate treatment had a significant effect on the growth of *Campylobacter* G28 and *Campylobacter* L29 at concentrations between 0.6 - 1.4%. ($p < 0.05$). The effect of butyrate treatment on all strains combined (Figure 4.1 F) resulted in significant reduction in growth of strains treated with 1.0% and 1.4% butyrate ($p < 0.05$) compared to untreated control.

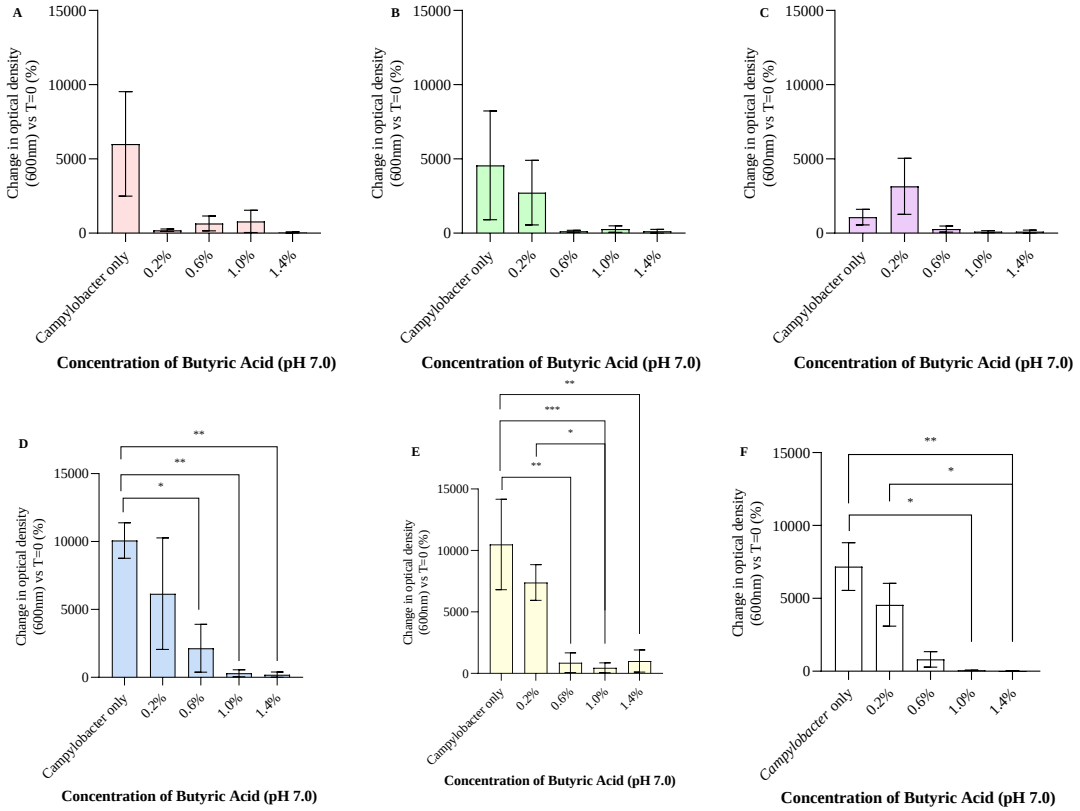

Figure 4.1 Growth of *Campylobacter* from avian sources challenged with butyrate

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in butyric acid (~pH 7.0) at 42˚C ± SEM (biological replicate n=4). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E). The mean of all five strains was also calculated (F). A two-way ANOVA with Dunnett multiple comparisons was conducted on data presented in graphs A-E (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); a Kruskal-Wallis with multiple comparisons was conducted on data present in graph F (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.1.2 Growth of *Campylobacter* over 24 hours directly challenged with Caprylate

Five *Campylobacter* isolates were grown at 42˚C in a microaerobic atmosphere (5% O_2; 10% CO_2; 85% N_2) in brucella broth and caprylate at varying concentrations (0.25-1.75%) over 24 h. the percentage change in optical density over 24 h was calculated (Figure 4.2). No significant difference was detected in the growth of *Campylobacter* challenged with caprylate at the strain level (Figure 4.2 A-E) or on average when strains were combined (Figure 4.2 F) compared to untreated control.

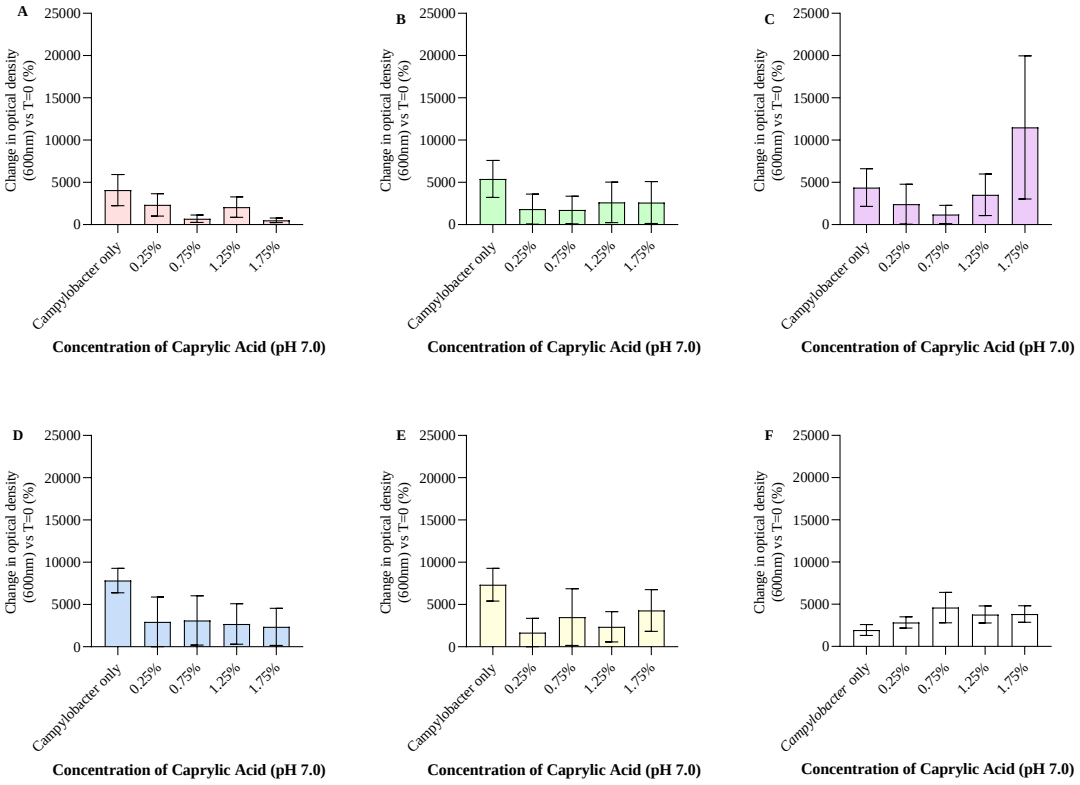

Figure 4.2 Growth of *Campylobacter* from avian sources challenged with caprylate

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in caprylic acid (~pH 7.0) at 42˚C ± SEM (biological replicate n=4). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), the mean of all five strains was also calculated (F). A two-way ANOVA with Dunnett multiple comparisons was conducted on data presented in graphs A-E; a Kruskal-Wallis with multiple comparisons was conducted on data present in graph F.

4.3.1.3 Growth of *Campylobacter* over 24 hours directly challenged with Chromium Propionate

Five *Campylobacter* isolates were grown at 42˚C in a microaerobic atmosphere (5% O_2; 10% CO_2; 85% N_2) in brucella broth and chromium propionate at varying concentrations (0.00002-0.00014%) over 24 h; the percentage change in optical density over 24 h was calculated (Figure 4.3). No significant difference in the growth of *Campylobacter* was detected when challenged with chromium propionate at the strain level (Figure 4.3 A-E) or on average when strains were combined (Figure 4.3 F) compared to untreated control.

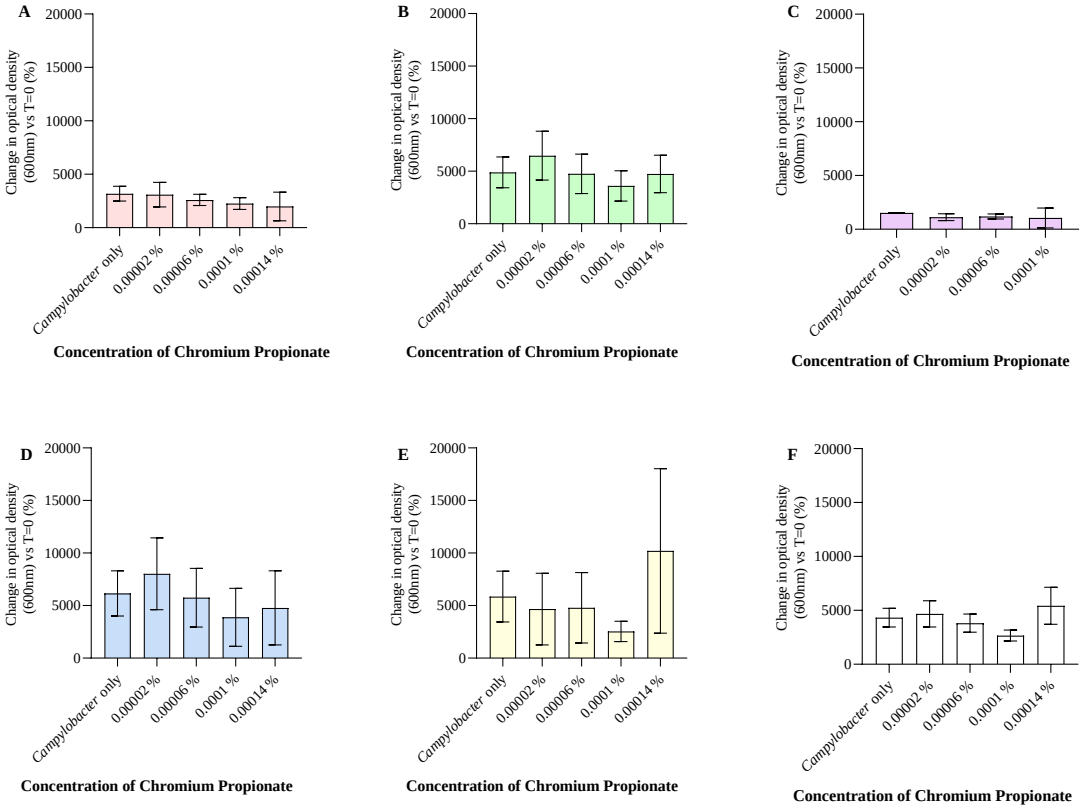

Figure 4.3 Growth of *Campylobacter* from avian sources challenged with chromium propionate

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in chromium propionate at 42˚C ± SEM (biological replicate n=3). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), the mean of all five strains was also calculated (F). A two-way ANOVA with Dunnett multiple comparisons was conducted on data presented in graphs A-E; a Kruskal-Wallis with multiple comparisons was conducted on data present in graph F.

4.3.2 Growth of *Campylobacter* directly challenged with *Bacillus* conditioned cell culture media

Conditioned media used in this experiment showed indications of undesired bacterial growth. Incubation of filtered conditioned media not challenged with *Campylobacter* inoculum resulted in bacterial growth and there was inconsistent growth between experiments.

4.3.2.1 Growth of *Campylobacter* over 24 hours directly challenged with *Bacillus subtilis* PB6 conditioned growth media

Five *Campylobacter* isolates were grown at 42˚C in a microaerobic atmosphere (5% O_2; 10% CO_2; 85% N_2) in 8E11 and CaCo-2 cell culture media conditioned with *B. subtilis* PB6 (Table 4.1) over 24 h. The percentage change in optical density over 24 h was calculated (Figure 4.4 and Figure 4.5). At the strain level, there was no significant difference in growth of individual strains when challenged with different formulations of *B. subtilis* PB6 conditioned 8E11 or CaCo-2 media (Figure 4.4 and Figure 4.5 respectively). The abbreviations used in Figure 4.4 – 4.7 are summarised in Table 4.1.

Table 4.1 Descriptions of abbreviations used in Figure 4.4-4.7

Abbreviation	Description
M 4 1/100	Conditioned media cultured with probiotic for 4 h under microaerobic conditions, diluted 1/100
M 4 1/1000	Conditioned media cultured with probiotic for 4 h under microaerobic conditions, diluted 1/1000
M 24 1/100	Conditioned media cultured with probiotic for 24 h under microaerobic conditions, diluted 1/100
M 24 1/1000	Conditioned media cultured with probiotic for 24 h under microaerobic conditions, diluted 1/1000
A 4 1/100	Conditioned media cultured with probiotic for 4 h under aerobic conditions, diluted 1/100
A 4 1/1000	Conditioned media cultured with probiotic for 4 h under aerobic conditions, diluted 1/1000
A 24 1/100	Conditioned media cultured with probiotic for 24 h under aerobic conditions, diluted 1/100
A 24 1/1000	Conditioned media cultured with probiotic for 24 h under aerobic conditions, diluted 1/1000

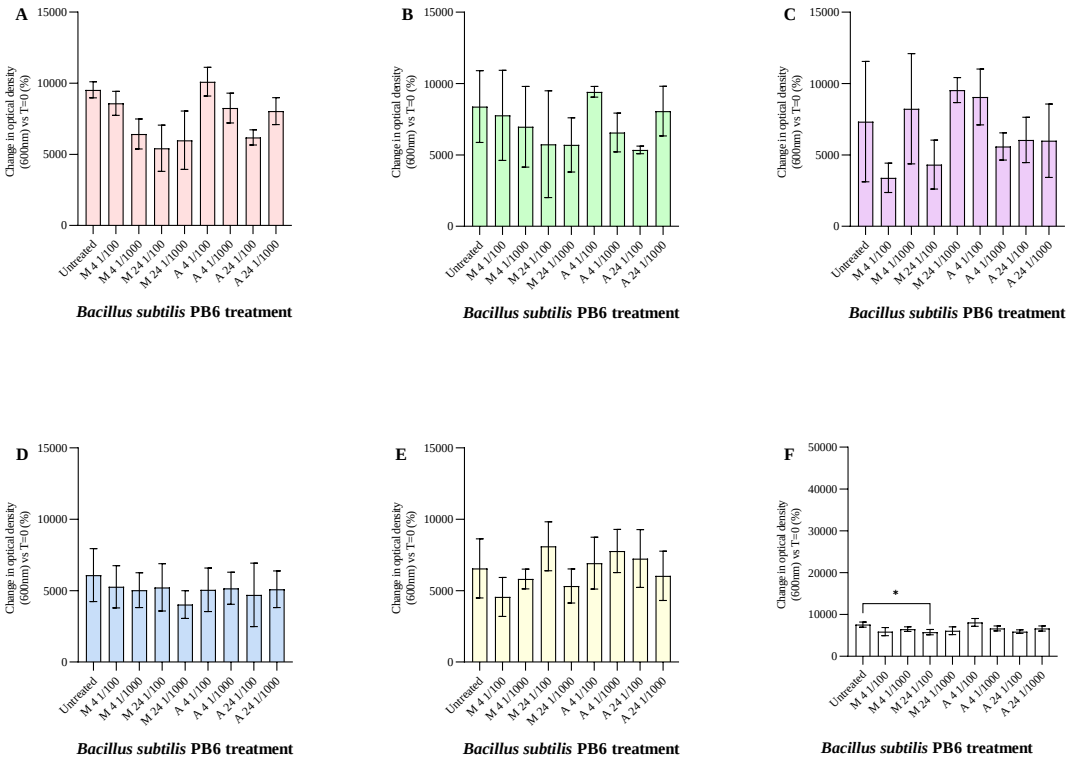

Figure 4.4 Growth of *Campylobacter* from avian sources challenged with *Bacillus subtilis* PB6 in 8E11 conditioned media

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in *Bacillus subtilis* PB6 conditioned 8E11 media at 42˚C ± SEM (biological replicate n=3). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), a mean response from all five strains combined was also plotted (F). A two-way ANOVA with Dunnett multiple comparisons was conducted.

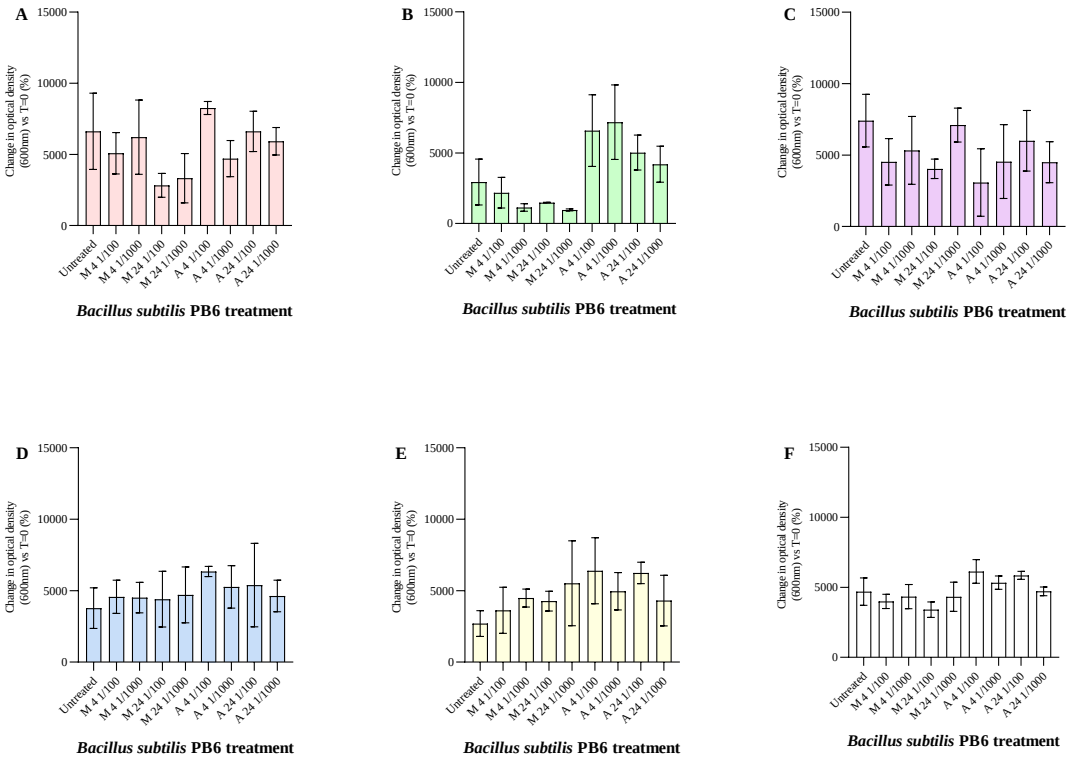

Figure 4.5 Growth of *Campylobacter* from avian sources challenged with *Bacillus subtilis* PB6 in CaCo-2 conditioned media

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in *Bacillus subtilis* PB6 conditioned CaCo-2 media at 42˚C ± SEM (biological replicate n=3). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), a mean response from all five strains combined was also plotted (F). A two-way ANOVA with Dunnett multiple comparisons was conducted.

4.3.2.2 Growth of *Campylobacter* over 24 hours directly challenged with *Bacillus licheniformis* conditioned growth media

Five *Campylobacter* isolates were grown at 42˚C in a microaerobic atmosphere (5% O_2; 10% CO_2; 85% N_2) in 8E11 and CaCo-2 cell culture media conditioned with *B. licheniformis* (Table 4.1) over 24 h. The percentage change in optical density over 24 h was calculated (Figure 4.6 and Figure 4.7). At the strain level there was a significant increase ($p < 0.05$) in the growth of *C. jejuni* M1 grown in unconditioned media and grown in treated *B. licheniformis* media in a microaerobic environment for 24 h and diluted 1/100 (Figure 4.6A). A significant reduction in growth of *Campylobacter* on average was observed when grown in microaerobically treated *B. licheniformis* media grown for 24 h and diluted 1/100 ($p < 0.05$) (Figure 4.6F). In addition, at the strain level

there was no significant difference in growth of individual strains when challenged with different formulations of *B. licheniformis* conditioned CaCo-2 media (Figure 4.7).

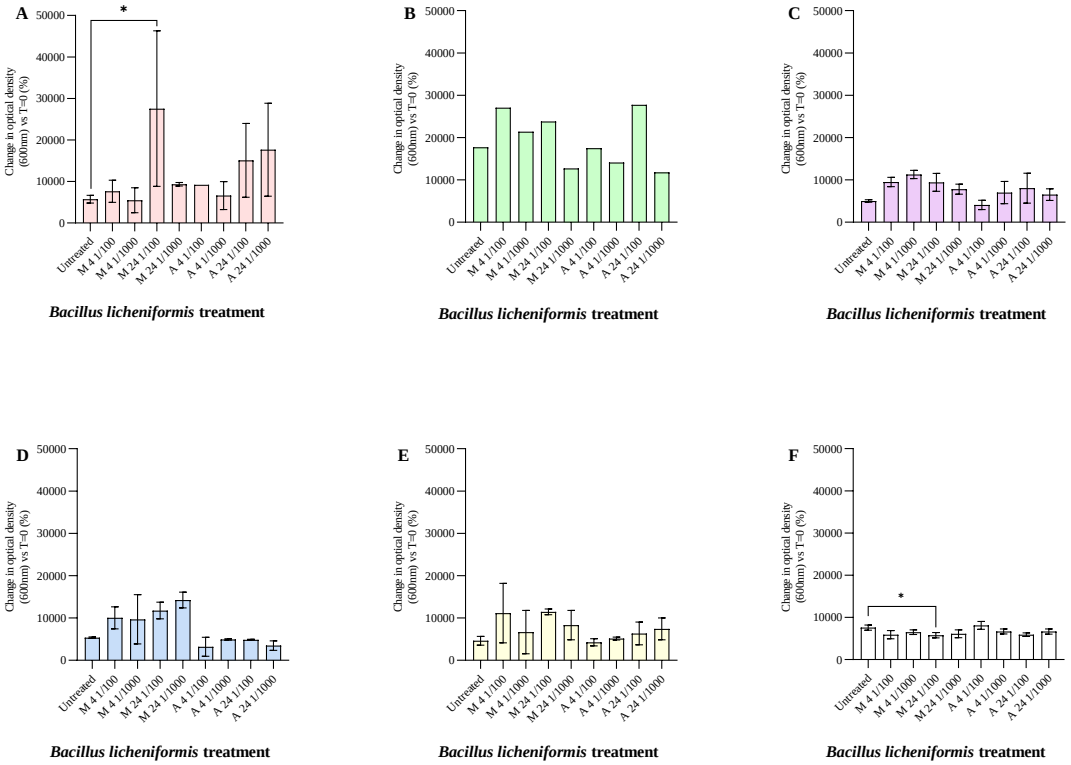

Figure 4.6 Growth of *Campylobacter* from avian sources challenged with *Bacillus licheniformis* conditioned 8E11 media

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in *Bacillus licheniformis* conditioned 8E11 media at 42˚C ± SEM (biological replicate n=3). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), a mean response from all five strains combined was also plotted (F). A two-way ANOVA with Dunnett multiple comparisons was conducted (* $p < 0.05$). An SEM could not be calculated for plot B due to only one biological replicate being conducted.

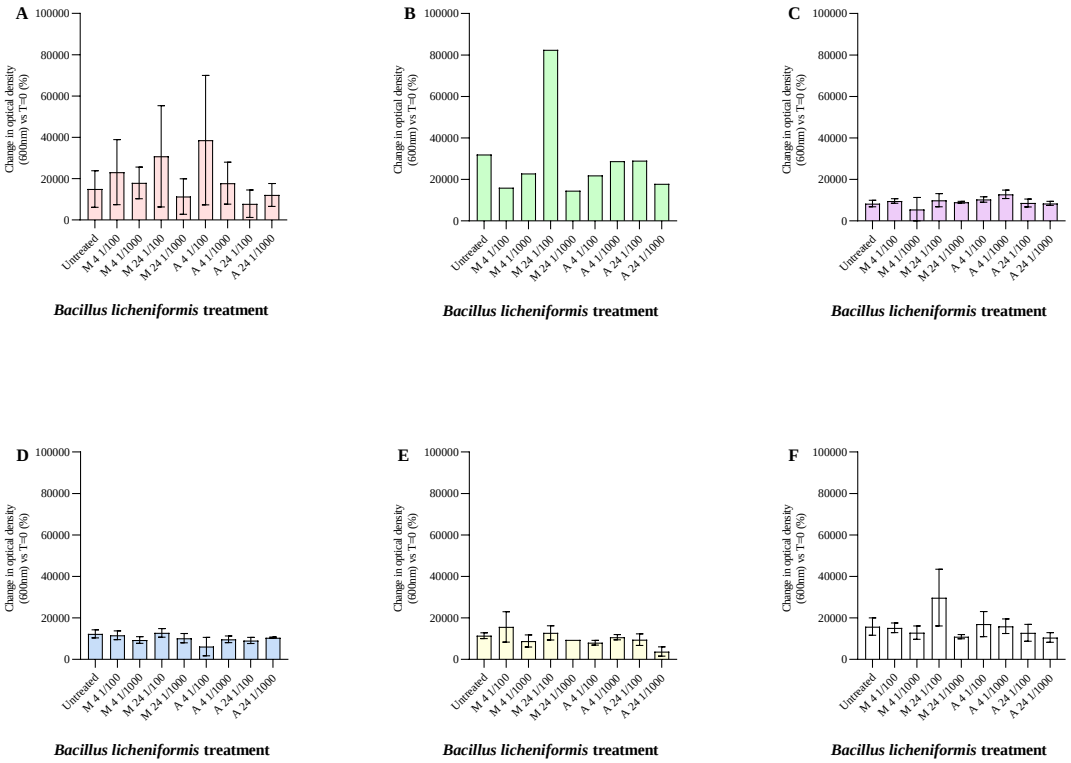

Figure 4.7 Growth of *Campylobacter* from avian sources challenged with *Bacillus licheniformis* conditioned CaCo-2 media

The change in optical density (600 nm) was calculated over 24 h for *Campylobacter* isolates grown in *Bacillus licheniformis* conditioned CaCo-2 media at 42˚C ± SEM (biological replicate n=3). Reference strains included *C. jejuni* M1 (A) and *C. jejuni* NCTC 11168 (B) and were compared to *Campylobacter* C13 (C), *Campylobacter* G28 (D) and *Campylobacter* L29 (E), a mean response from all five strains combined was also plotted (F). A two-way ANOVA with Dunnett multiple comparisons was conducted. An SEM could not be calculated for plot B due to only one biological replicate being conducted.

4.3.3 Motility of *Campylobacter*

4.3.3.1 Motility of *Campylobacter* strains in brucella agar

The motility of *Campylobacter* was assessed in brucella agar with and without *Campylobacter* growth supplement (Oxoid;Figure 4.8; Figure 4.9). At the strain level, supplementation with the growth supplement had no significant effect on growth; however, in brucella 0.3% agar, motility of *Campylobacter* C13 and *Campylobacter* L29 was significantly increased compared to reference strain *Campylobacter* M1 ($p < 0.05$) (Figure 4.8).

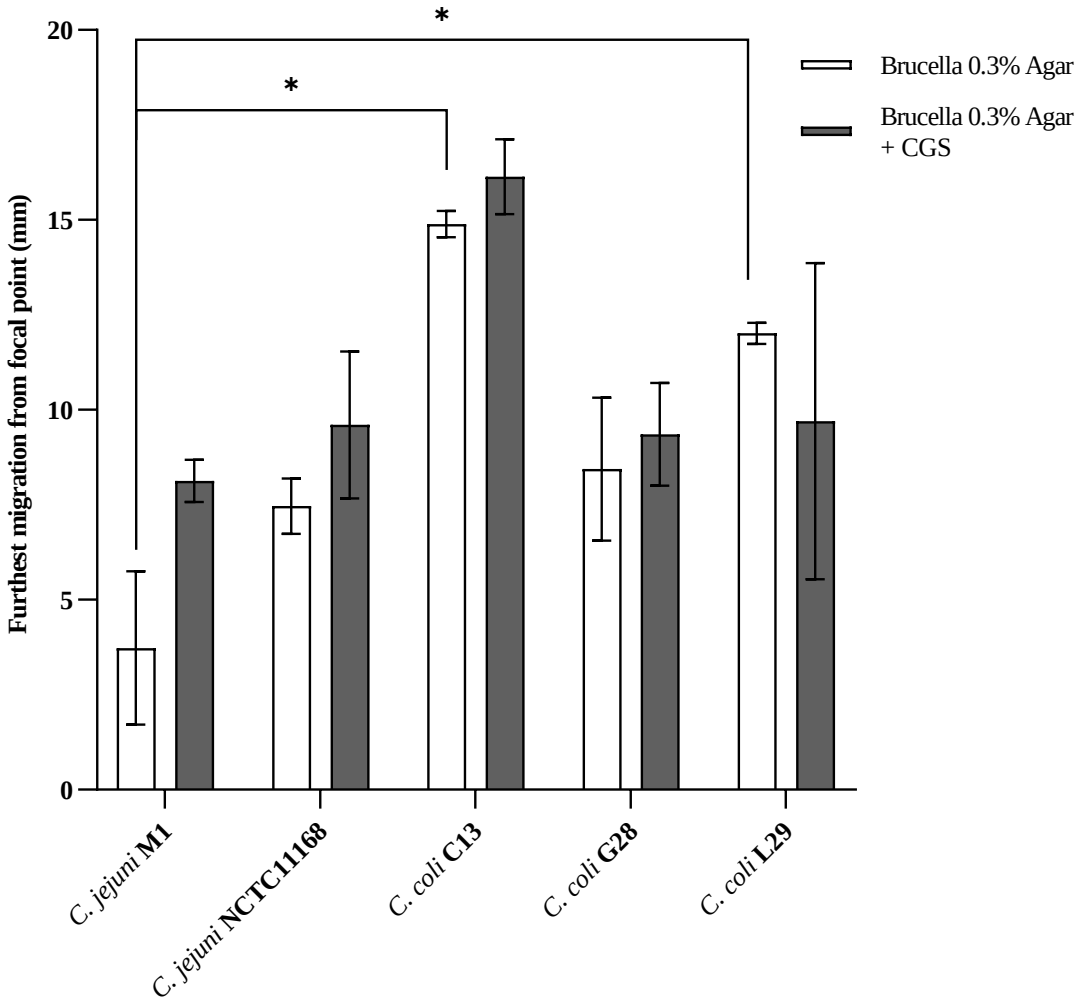

Figure 4.8 Motility of *Campylobacter* isolates from avian sources in brucella 0.3% agar with and without *Campylobacter* growth supplement (CGS) (Oxoid)

The motility of *Campylobacter* isolates of 24 h was measured from a central point in brucella agar with or without the addition of a growth supplement (experimental replicate n=3 ± SEM). Reference strains included *C. jejuni* M1 and *C. jejuni* NCTC 11168 and were compared to *Campylobacter* C13, *Campylobacter* G28 and *Campylobacter* L29. A two-way ANOVA with Sidak multiple comparisons was conducted (* $p < 0.05$).

When motility of all strains was combined a paired t-test confirmed that addition of *Campylobacter* growth supplement had no significant effect on the motility of *Campylobacter* (Figure 4.9).

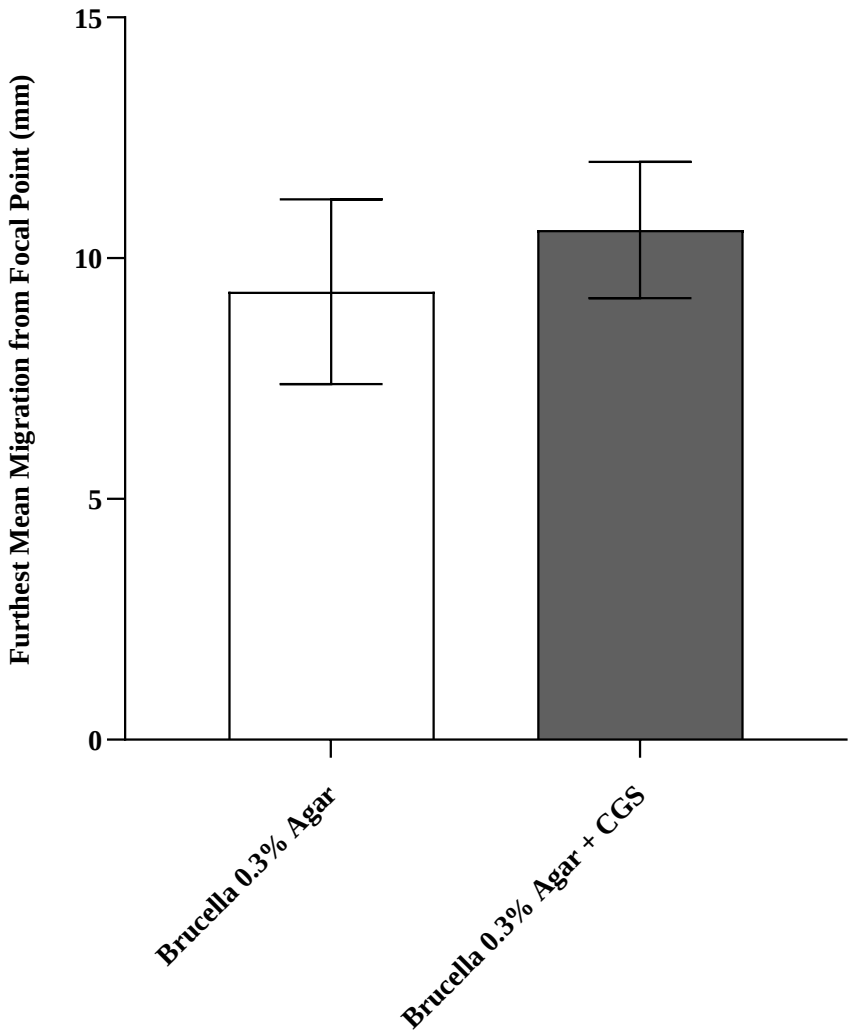

Figure 4.9 Mean motility of *Campylobacter* from avian sources in brucella 0.3% agar with and without *Campylobacter* growth supplement (CGS) (Oxoid)

The motility of *Campylobacter* isolates of 24 h was measured from a central point in brucella agar with or without the addition of a growth supplement (isolate replicate n=5 ± SEM). Reference strains included *C. jejuni* M1 and *C. jejuni* NCTC 11168 and were compared to *Campylobacter* C13, *Campylobacter* G28 and *Campylobacter* L29. This graph shows the mean of the five strains. A paired T-test was conducted on data presented.

4.3.3.2 Motility of *Campylobacter* strains in brucella agar (without CGS) with Chromium Propionate

The motility of *Campylobacter* isolates in the presence of increasing concentrations of chromium propionate (0.00006% and 0.00014%) (Figure 4.10; Figure 4.11). At the strain level the motility of *C. jejuni* M1 and *Campylobacter* G28 were significantly increased in the presence of 0.0006% chromium propionate ($p < 0.05$), 0.00014% chromium propionate significantly increased the motility of *Campylobacter* C13 ($p < 0.01$), and

motility of *Campylobacter* L29 was increased at both concentrations ($p < 0.0001$) (Figure 4.10). For *C. jejuni* NCTC 11168, C13 and L29 a concentration dependant increase in motility is seen, although only significant for C13 and L29.

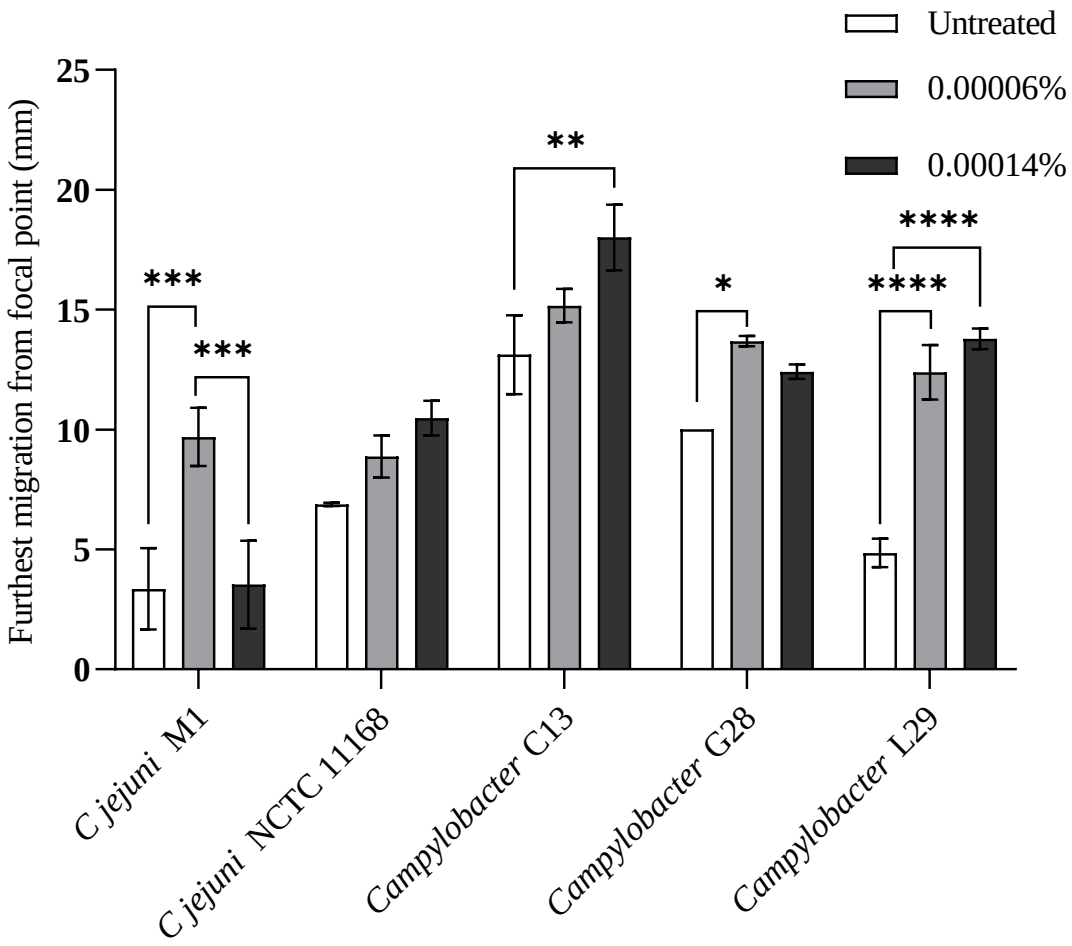

Figure 4.10 Motility of *Campylobacter* isolates from avian sources with chromium propionate treated brucella 0.3% agar

The mean motility of *Campylobacter* isolates of 24 h was measured from a central point in brucella agar with or without the addition of chromium propionate (n=3 experimental replicates ± SEM). Reference strains included *C. jejuni* M1 and *C. jejuni* NCTC 11168 and were compared to *Campylobacter* C13, *Campylobacter* G28 and *Campylobacter* L29. A two-way ANOVA with Dunnett multiple comparisons was conducted on data presented (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

A two-way ANOVA confirmed that the mean motility of *Campylobacter* was significantly increased in the presence of 0.00006% and 0.00014% chromium propionate ($p < 0.0001$) (Figure 4.11).

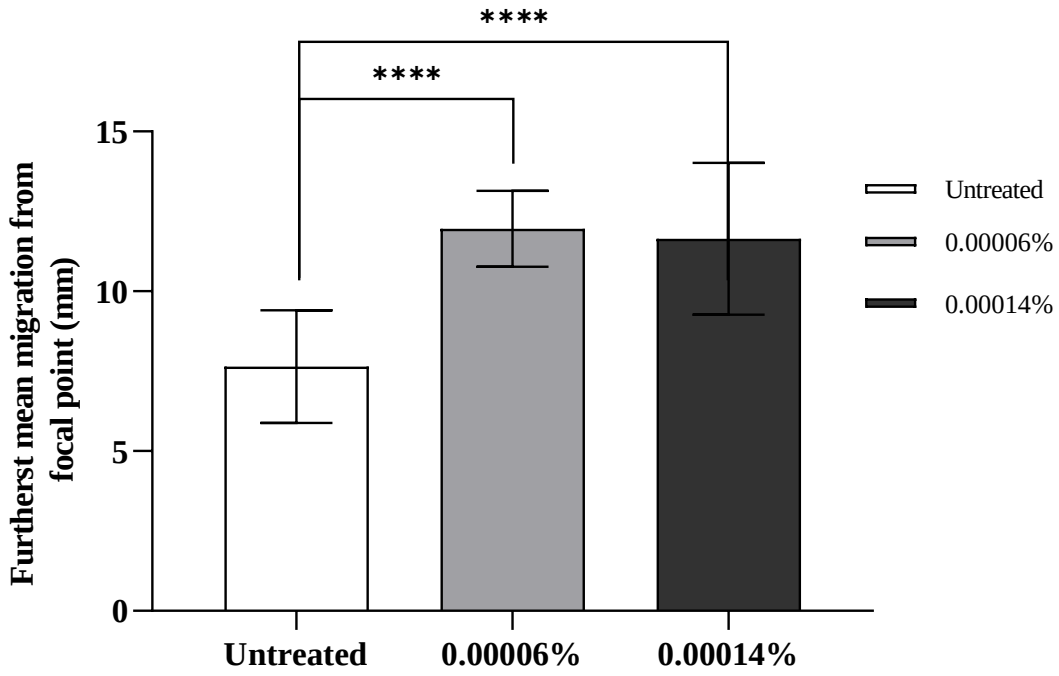

Figure 4.11 Mean motility of *Campylobacter* from avian sources with chromium propionate treated brucella 0.3% agar.

The motility of *Campylobacter* isolates of 24 h was measured from a central point in brucella agar with or without the addition of chromium propionate (n=3 experimental replicates ± SEM). Reference strains included *C. jejuni* M1 and *C. jejuni* NCTC 11168 and were compared to *Campylobacter* C13, *Campylobacter* G28 and *Campylobacter* L29. A two-way ANOVA with Dunnett multiple comparisons was conducted on data presented (**** $p < 0.0001$).

4.4 Discussion

Five different *Campylobacter* strains were used to determine the effect of feed additives on growth; two reference strains of human origin and three strains isolated from chicken origin. Three experimental isolates were selected to represent and demonstrate the diversity of strain behaviour documented in experimental research and the variability in responses from different strains under the same conditions. Results from individual strains were combined to provide an insight into the general effect of the feed additives on *Campylobacter* independent of strain variation (Sahin, Morishita and Zhang, 2002).

Despite the aim of this study being to identify a compound that can reduce *Campylobacter* load within the chicken, we used strains of both human and avian origin (Sahin, Morishita and Zhang, 2002). The three isolates were selected because of *in vitro* testing in chapter 3 (C13, G28, L29) were all *C. coli* strains. *C. coli* does not contribute to human disease to the extent of *C. jejuni*, however the non-reference *C. jejuni* strains from the original collection did not show high stability and consistency across assays and studies. This

could be considered a major limitation to this current work as the results cannot be fully generalised to strains that are known to cause human disease. However, our two reference strains (*C. jejuni* M1 and *C. jejuni* NCTC 11168) are both known to cause human disease and *C. jejuni* M1 has also be isolated from poultry. In future work, or replications of the work in this study it would be advisable to select *C. jejuni* isolates in addition to *C. coli* to be able to conclude more definitively if the effects of feed additives can be generalised to clinically relevant strains.

This study gave insight into the potential phenotypic and genotypic differences between *C. jejuni* and *C. coli* strains. For example, two of the *C. coli* strains (C13 and L29) were significantly more motile than the control strain *C. jejuni* M1. This could be a contributing factor to a lack of human disease caused by *C. coli*. The bacterium may move faster through the human GI tract and therefore not efficiently adhere to and invade human intestinal epithelial cells, on the contrary, increased motility may facilitate adhesion too and movement across the intestinal epithelial barrier

In addition to using strains of different origin regarding human or avian, three strains isolated from different areas of the chicken GI tract were used, largely due to the observations of varying colonisation ability in chickens with dominant isolates able to displace others, potentially owing to a better growth rate under replicate conditions and therefore surviving longer within the GI tract and able to spread to extended parts of the GI tract (Jacobs-Reitsma *et al.*, 1995; Korolik, 1998; Sahin, Morishita and Zhang, 2002). Even though there are reports of *Campylobacter* isolates of different serotypes and genotypes colonising chicken flocks during the same production cycle, a single chicken being infected by more than one strain is extremely rare which further supports the "dominant strain" theory (Jacobs-Reitsma *et al.*, 1995; Stern *et al.*, 1997; Korolik, 1998; Sahin, Morishita and Zhang, 2002). The results from the current study further support the notion that there is significant difference in strain behaviour and reaction to challenge with feed additives. The greatest variation in response to butyrate was where four out of five isolates showed a decrease growth rate at 0.2% butyrate, however *Campylobacter* isolate C13 spiked in growth at the 0.2% concentration, however C13 proceeded to follow the decrease in growth trend observed across other strains. When an "overall observation" approach was adopted by combining the effect of butyrate on all five *Campylobacter* isolates a clear growth reduction was shown as a result of butyrate application at the 1.0% and 1.4% concentrations.

Chromium propionate has largely been investigated as a supplement that could have positive effects on poultry growth performance and carcass characteristics (Hayat et al., 2020; R. U. Khan et al., 2014; Rajalekshmi et al., 2014) with no evidence of bactericidal properties. Therefore, it was unsurprising to observe no bactericidal effect of this compound in the current study.

The acids supplied by Kemin Animal Nutrition and Health Ltd. were standardised to pH 7.0 (± 0.2) prior to *in vitro* experimentation with *Campylobacter* isolates and epithelial cell lines (Chapter 5). *In vitro* studies with MCFA, specifically caprylic acid, have reported a pH dependent bactericidal effect of these compounds toward *Campylobacter* (D. Hermans et al., 2010) The lack of bactericidal effect seen in the current study could be explained by the standardisation of all concentrations of caprylic acid to pH 7.0. The proposed mechanism of action (MOA) of MCFA (and SCFA) is internalisation of the associated acid, after which it dissociates and a subsequent increase of anions leads to intracellular acidification and bacterial cell death, by increasing the pH of caprylic acid to 7.0, caprylate might have been ineffective in this process (D. Hermans et al., 2010). However, the SCFA, butyric acid, was also standardised to the same pH, and a significant bactericidal effect was still observed; it should be noted that due to addition of buffer to reduce the pH, the effect of butyrate was being measured, not the effect of butyric acid. It could be speculated that SCFA have a second MOA that also results in bacterial cell death and is not reliant on an acidic pH.

It must be recognised that the pH of caprylic acid was altered with HEPES buffer, and butyric acid was altered with sodium hydroxide. A study with sodium hydroxide revealed a bactericidal effect of this chemical on *Campylobacter* populations when tested at 0.05 or 0.1 N within 1 minute of application (Zhao & Doyle, 2006), the bactericidal effect still observed by butyrate at a pH 7.0 could therefore be attributed to the effect of the sodium hydroxide buffer. Future research should include investigation into the bactericidal effect of sodium hydroxide against *Campylobacter*, independent of butyric acid, and the effect of caprylic acid pH on its bactericidal properties.

Bacilli-based probiotics are considered to have highly antagonistic activity against bacterial pathogens (Cutting, 2011; Sorokulova et al., 1997). Upon administration and digestion they produce substances (such as SCFA) that have been shown to increase organism resistance to pathogens (Kabir, 2009; Sorokulova et al., 1997; Vazquez, 2016).

Two species of *Bacillus (B. subtilis and B. licheniformis)* were used in the current study to determine if there was a characteristic difference in the antimicrobial properties of these commonly used probiotic species. Previously, an investigation into the production of extracellular antimicrobial substances by these species of *Bacillus* revealed that the antimicrobial properties of the strains differed under varying conditions (Korenblum et al., 2005). These observations influenced the decision to prepare conditioned media under varying conditions (incubation time, temperature, base media type). Korenblum *et al.* (2005) demonstrated that during a 4-day growth phase, the antimicrobial activity of *B licheniformis* and *B subtilis* was inversely parallel – at day 1 the antimicrobial activity of *B subtilis* was at its greatest, however, the peak antimicrobial activity of *B licheniformis* was observed at day 3-4; both strains, however, showed similar trends in spore formation in culture.

Two strains of *Bacillus* were also used to determine if there was a difference in *Campylobacter* response to different species of the same genus, due to difference in molecules secreted at each stage of growth. A preliminary growth curve of the two *Bacillus* species in 8E11 and CaCo-2 cell media was conducted over 24 hours prior to conditioned media preparation (Appendix 5 and Appendix 6).The growth of *B licheniformis* was higher than *B subtilis* in both cell culture media, and growth of *B subtilis* PB6 was higher in 8E11 media compared to CaCo-2 over a 24h period. Prospective future work aimed to apply the *Bacillus* conditioned media to both avian and human cell lines (8E11 and CaCo-2 respectively) which additionally influenced the decision to prepare a variety of conditioned media using the two complete cell media solutions.

A major drawback to the conditioned media experiment was the contamination of the conditioned media with *Bacillus* spores after filtration. The technique used to filter the conditioned media was not effective and resulted in media with unwanted bacterial growth. Due to this, it cannot be concluded that the results observed in this study were entirely accurate and would explain the high degree of error observed in the results. Despite this obvious drawback in the current study, the study has highlighted the potential for products of *Bacillus* fermentation within the chicken GI tract as inhibitors of *Campylobacter*, although further investigation into the specific molecules secreted by different *Bacillus* species is required.

It was shown in Chapter 3 (section 3.3.1) that *C. jejuni* NCTC 11168 was the only selected strain to test positive for the *flaA* and *flaB* gene, indicating that it might be expected to be the most motile of the five strains tested in the present study due to the presence of both genes a prerequisite for a fully active flagellar filament (P Guerry et al., 1991). However, it was found that the caecal isolate (C13) was the most motile strain of *Campylobacter* tested in untreated agar. There are several other flagella related genes (class II genes) that were not tested for in chapter 3 (Lertsethtakarn et al., 2011), which could explain the high level of motility exhibited by the caecal isolate.

The results obtained from the motility analysis further supports the observations of strain diversity and the large degree of variation exhibited from strains of different sources. The degree of motility exhibited by the avian isolates may correspond to the region of isolation and the ability of the strains to move through the GI tract. The caecum is the farthest point in the avian GI tract from which isolates were extracted, increased motility of these strains may enable movement through the intestinal mucosa and residency within this area.

The effect of CrProp due to its organic stable form was also investigated, providing a readily available source of Cr3+ which is efficiently absorbed in comparison to other Cr sources. The isolates (as predicted) did not respond to CrProp in a consistent manner with no clear pattern observable based on source (at species or anatomical level). What was found was overall both concentrations of CrProp trialled *in vitro* (0.00006% and 0.00014%) significantly increased *Campylobacter* motility (Figure 4.11). Biosorption of Cr3+ has been observed in bacterial cells and happens more readily than absorption of trivalent chromium in the non-ion form; however, microbial interactions are more often with Cr6+ and result in reduction to Cr3+ (Gutiérrez-Corona et al., 2016). There is very little research on trivalent Cr due to its inefficient biosorption by cells, and therefore most research is focussed on hexavalent Cr and its effects on biological processes (Gutiérrez-Corona et al., 2016; Zhen et al., 2016). The reduction of hexavalent Cr to trivalent Cr within bacterial cells suggests that some of the observations made in previous studies could be attributed to the bioaccumulation of Cr3+ within the cytoplasm. Effects of Cr6+ on motility have been observed in several studies that primarily use spermatozoan cells from different mammalian species, with a reduction in motility observed and this has been linked to Cr6+ facilitated down-regulation of protein tyrosine phosphorylation within the sperm cell (Marouani et al., 2012; Pokhrel et al., 2020; Zhen et al., 2016). However, increased motility has been observed in two colorectal cancer cell lines (DLD-1 and

HT29) exposed to Cr6+ due to activation of focal adhesion kinase (FAK) – a regulator of cell motility (D Chen, 2017). FAK plays an important role in *Campylobacter* pathogenesis and is recognised as an integral virulence factor that enables efficient invasion of epithelial cells (Boehm et al., 2011). It could be that Cr has a negative effect on phosphorylation of the FlaA and FlaB proteins but enhance FAK activity. Four of the five strains tested lack the FlaA and FlaB proteins that could potentially be negatively affected by Cr exposure, however all five strains are assumed to be FAK dependent for invasion, this study presents a novel observation of increased flagella-independent motility in *Campylobacter* isolates facilitated by Cr3+ activation of the FAK pathway.

When the motility assay was trialled with agar treated with caprylate or butyrate, we found that the *Campylobacter* growth limiting effects of these feed additives prevented visible migration (section 4.3.1.1 and 4.3.1.2).

Motility has historically been considered a virulence factor that enhances the pathogenesis of *Campylobacters* (D. J. Bolton, 2015). In the current study, chromium propionate enhances motility of *Campylobacter* at the species level, despite seeing a variation in results at isolate level. Motility at an optimum level may serve as an efficient mechanism to enhance *Campylobacter* virulence and pathogenicity regarding movement to edible tissues within poultry; however, it could be argued that increasing motility significantly may reduce virulence of strains, providing them with the motility to move through the intestinal mucosa at a faster rate and thereby decreasing the extraintestinal spread across the intestinal epithelium and reducing *Campylobacter* load in edible tissues.

4.5 Conclusion

This work demonstrated that the direct addition of sodium butyrate at pH 7.0 limits growth of *Campylobacter in vitro*. The effect of caprylic acid on growth may have been suppressed by the pH adjustment as suggested by a previous study (D. Hermans et al., 2010).

A novel observation of increased motility in *Campylobacter* isolates with exposure to Cr was demonstrated (Table 4.1). Future research should be conducted to identify the specific biological interactions which occur to result in these observations. Based on previous studies it is proposed that increased motility observed is independent of flagella proteins and is facilitated by Cr activation of FAK, a known facilitator of motility and

specifically required for *Campylobacter* invasion into epithelial cells (Boehm et al., 2011; D Chen, 2017).

Table 4.1 Summary of main results from Chapter 4

Experiment	**Result**
Isolate growth with conditioned media	*Bacillus subtilis* PB6 and *Bacillus licheniformis* 24h conditioned 8E11 media diluted 1/100 resulted in a significant reduction in *Campylobacter* growth
Campylobacter motility	*C. coli* C13 and *C. coli* L29 had significantly greater motility than *C. jejuni* M1 Chromium propionate significantly improved the motility of *Campylobacter* isolates at 0.0006% and 0.00014%

Chapter 5: Indirect effects of feed additives on *Campylobacter* invasion into epithelial cells and cytokine production *in vitro*

5.1 Introduction

Pathogenesis of *Campylobacter* and its interactions with intestinal epithelial cells has been extensively researched in recent years (Backert et al., 2013; Dhar & McAuley, 2019; Hameed, 2019; Konkel et al., 2020; Novik et al., 2010). Damage to the avian intestinal epithelium during pathogenesis, facilitates the extraintestinal spread of *Campylobacter* to edible tissues, in turn increasing contamination of poultry products at retail sale for human consumption. Here the focus was on the effects of feed additives on epithelial interactions following *Campylobacter* infection, namely toxicity, invasion, and induction of inflammatory cytokine production. These responses play a significant role in *Campylobacter* extraintestinal spread from the chicken gut.

To date, studies have revealed variation in invasion, cytokine production and toxicity(W. Awad et al., 2017; M. L. Chen et al., 2006; MacCallum, Haddock, et al., 2005). However this has been attributed to the diverse behaviour exhibited by various *Campylobacter* phenotypes and/or the various interactions that occur with host cells of different origins (van Putten et al., 2009). A large amount of *in vitro* research has been focussed on human cell line interactions with *Campylobacter* and comparatively little focussed on avian intestinal cell systems, despite the main contributor to acquiring human campylobacteriosis being through consumption of poultry products (D. A. John et al., 2017). It is vital that interactions between *Campylobacter* and cells of the avian GI tract are more extensively studied to determine the mechanisms employed by the pathogen to aid spread to edible tissues.

5.1.1 *Campylobacter* interaction with host epithelial cells

When colonising the avian and human gastrointestinal tract there are numerous interactions between enteric bacterial pathogens and host cells, resulting in enhanced survival within the host and/or damage to host cells (Backert et al., 2013; Elmi et al., 2016; Ó Cróinín & Backert, 2012). *Campylobacter* is an anomaly compared to other Gram-negative pathogens, as there is no specific and readily identifiable secretion system that delivers virulence factors into host cells. It can be assumed, however, that intimate

contact with host cells is required for virulence factor secretion mechanisms to succeed (Elmi et al., 2016).

The three main types of *Campylobacter* interaction with epithelial cells are (i) adhesion, (ii) invasion, and (iii) cytotoxicity.

5.1.1.1 *Campylobacter* adherence to host epithelial cells

The ability of *Campylobacter* to adhere to the epithelial cell surface is considered a prerequisite for successful colonisation and contributes to the increased concentration of bacterial products in the local area, making it an important step in bacterial pathogenesis (Jain et al., 2008; Rubinchik et al., 2012). There is a reported correlation between level of adherence to some cell types (e.g., HeLa) and the severity of clinical symptoms that manifest in human patients (Rubinchik et al., 2012). Successful *Campylobacter* adherence to host cells does not require mediation by fimbria or pili, instead there are several other virulence mechanisms that have been identified enabling *Campylobacter* to interact with host cell surface adhesins (Rubinchik et al., 2012; van Putten et al., 2009).

Campylobacter express two microbial surface components recognising adhesive matrix molecule(s) (MSCRAMMa) and various glycans on its cell surface (Lipooligosaccharide (LOS) and glycoproteins) that can interact with lectin-like host cell receptors (Rubinchik et al., 2012; Talukdar et al., 2020). CadF is an outer membrane protein of *Campylobacter* that belongs to the outer membrane protein A- porin proteins (Omp-A) family. CadF promotes adherence to host cells by binding to fibronectin (Fn) via a four amino acid motif (Phe-Arg-Lue-Ser) (Rubinchik et al., 2012; Talukdar et al., 2020; van Putten et al., 2009). CadF may also be referred to as a Fibronectin (Fn)-binding protein (FNBP), and is a member of the MSCRAMMs family (Talukdar et al., 2020). Fn is a glycoprotein expressed in the extracellular matrix of many types of differentiated host cells, it is also present in blood and connective tissue (Rubinchik et al., 2012; Ruoslahti, 1981). The functions of Fn include mediating cellular interactions with the extracellular matrix, facilitating cell adhesion, differentiation, growth, and migration (Pankov & Yamada, 2002). In addition to CadF, *Campylobacter* also possess FlpA which binds specifically to Fn-binding site on human and chicken cell lines that has been localised to a span of nine amino acids (Trp-Arg-Pro-His-Pro-Asp-Phe-Arg-Val) (Rubinchik et al., 2012; Talukdar et al., 2020).

Campylobacter can also adhere to host epithelial cells via another binding protein, PEB1 (van Putten et al., 2009). PEB1 is a conserved aspartate/glutamate binding protein and is considered a major cell adherence molecule of both *C. jejuni* and *C. coli* (Pei & Blaser, 1993; van Putten et al., 2009). The PEB1 binding protein belongs to the family of cluster three binding proteins of bacterial ATP transporters, it mediates adhesion to host cells by binding L-glutamate and L-aspartate (van Putten et al., 2009).

5.1.1.2 *Campylobacter* invasion of host epithelial cells

Both *in vitro* and *in vivo* studies have reported *Campylobacter* bacterial cells residing within the cytoplasm of host epithelial cells, either in membrane bound vesicles or not associated with organelles, indicating the ability of *C. coli* and *C. jejuni* to invade epithelial cells (Backert et al., 2013; Wooldridge & Ketley, 1997). There are several components to the invasive process; flagellum mediated motility, actin polymerisation, host cell microtubules, and the expression of *Campylobacter* invasion antigens (Cia) (Elmi et al., 2016; D. A. John et al., 2017; van Putten et al., 2009; Wooldridge & Ketley, 1997). When *Campylobacter* makes contact with the host cell surfaces this initiates membrane ruffling and formation of invaginations which facilitate the uptake of *Campylobacter* cells polar tip first (Backert et al., 2013; van Putten et al., 2009).

A key factor identified in all *Campylobacter* uptake studies using epithelial cell lines is the presence of functional flagella (van Putten et al., 2009). John *et al.* (2017) reported the specific importance of FlaA (*flaA*) in invasion of epithelial cells, when the isolate in question lacks flagellum or has FlaB dominant flagellum (short flagellum) reduced invasion capabilities are reported (van Putten et al., 2009; Wooldridge & Ketley, 1997). Functional flagella (FlaA dominant) also act as export apparatus in secretion of CiaC and FlaC (non-flagellar proteins) that also aid the invasion process (Elmi et al., 2016; D. A. John et al., 2017).

When contact is made with the host cell surface this initiates local depolymerisation of cortical actin filaments and formation of membrane projections that are host cell microtubule-based (D. A. John et al., 2017; van Putten et al., 2009). Actin depolymerisation consists of a series of signalling events that integrate with the actin filaments in the cytoskeleton of the plasma membrane and have been observed in several invasion studies (van Putten et al., 2009; Wooldridge & Ketley, 1997). When interacting with host cells, *Campylobacter* can disrupt both tight junctions and adherens junctions,

both of which are associated with the actin cytoskeleton (Elmi et al., 2016). CaCo-2 cells can partially differentiate resulting in microvilli production and tight junction formation. The cells can also express apical surface enzymes that are characteristic of intestinal enterocytes (Wooldridge & Ketley, 1997). Further evidence of microtubule dependent invasion has been shown when microtubule depolymerisation agents such as nocodazole, reduce (and in some cases completely block) *C. jejuni* invasion (Biswas et al., 2003; van Putten et al., 2009). Invasion of avian epithelial cells is microtubule-, microfilament- and caveolin-dependent as highlighted by John *et al.* (2017).

Outer membrane vesicles (OMVs) interact with host cells as a way of delivering proteins and other moieties into host cells. These vesicles are enriched with phospholipids, lipooligosaccharides, outer membrane and periplasmic proteins (Elmi et al., 2016). OMVs are crucial for pathogenicity of Gram-negative bacteria. They are important in host colonisation, virulence factor deliverance and modulation of the host response (Elmi et al., 2016). The production and secretion of Cia proteins (*Campylobacter* invasion antigens) is triggered (as are many invasion mechanisms) by direct contact with host cells and some of these have been associated with OMV activity (Elmi et al., 2016; van Putten et al., 2009). The role of Cia proteins is still under investigation, however CiaB has been referred to as a strain-specific invasion antigen and is not required for successful invasion by all *Campylobacter* strains (van Putten et al., 2009).

5.1.1.3 *Campylobacter* induced cytokine production within host epithelial cells

A commonly recognised component of enteric infections is the induction of inflammatory cytokines and associated responses (Al-Banna et al., 2018; Hickey et al., 2000). The adherence and invasion of human intestinal epithelial cells induces an immune response which activates transcription factors (NF-kappaβ and AP-1), subsequently causing phosphorylation of the ERK pathway and P38 mitogen-activated protein kinases (van Putten et al., 2009). The activation of the MAP kinase, ERK and P38 pathways leads to production of proinflammatory cytokines IL-8 and IL-10 (Hickey et al., 2000). In addition, other proinflammatory cytokines of primary interest within the human innate immune system are IL-1α, IL-1β, IL-6, and TNF-α (D. A. John et al., 2017; C. K. Smith et al., 2005; van Putten et al., 2009).

Avian systems have fewer cytokines compared to human systems (C. K. Smith et al., 2005). Despite not possessing an IL-8 chemokine, two orthologs have been identified, CXCLi1 and CXCLi2, which are both induced by *Campylobacter* (D. A. John et al., 2017). The induction of IL-8 and CXCLi1/2 in humans and chickens (respectively), is important for the infiltration of neutrophils in the gut (D. A. John et al., 2017). In addition to IL-8 orthologs, important *Campylobacter*-associated cytokines in the avian response from non-epithelial cells (e.g., spleenocytes) are IL-1β and IFN-γ (W. A. Awad et al., 2018; Barjesteh et al., 2013).

John *et al.* (2017) investigated the potential link between cytokine production, cellular toxicity, and invasion, stating that whilst the three are not always linked, an association can be made between production of IL-8, CXCLi1, and CXCli2, and toxicity, suggesting similar induction mechanisms. Results from the study by John *et al.* (2017) supported the hypothesis that invasion of epithelial cells by *Campylobacter* is required for efficient cytokine production in avian systems.

5.1.2 *Campylobacter* induced damage to host epithelial cell monolayers

Maintenance of gut barrier integrity is crucial for limiting and preventing pathogenesis of *Campylobacter* (W. A. Awad et al., 2018). The function of the host epithelial cell monolayer is damaged by interactions with *Campylobacter*. Direct damage is caused by *Campylobacter* adhesion/invasion and toxin production, but indirect damage is caused by induction of an inflammatory response in the epithelial cells themselves (Al-Banna et al., 2018; Wooldridge & Ketley, 1997). When adhering to and invading intestinal epithelial cells *in vitro*, campylobacters generate toxins that impair cell function and thus the function of the epithelium as a primary barrier against extra-intestinal spread of pathogens (Man, 2011).

5.1.2.1 *Campylobacter* induced damage due to adhesion and invasion of epithelial cells

Campylobacter isolates with a greater ability to adhere to intestinal epithelial cells, typically have higher invading ability. In addition to this, adhesion is a prerequisite for the paracellular passage of *Campylobacter* between epithelial cells and into the blood stream (W. A. Awad et al., 2018). The adhesion, invasion and paracellular movement of *Campylobacter* is accompanied by cytopathic effects. Of these three virulence mechanisms, invasion induces the greatest degree of damage to the colonic mucosa which

leads to inflammation of the intestinal epithelium (Jain et al., 2008; van Vliet & Ketley, 2001; Wooldridge & Ketley, 1997).

There is evidence of species-specific cell responses to *Campylobacter* infection, indicating that not all epithelial cells will be damaged to the same extent when infected by *Campylobacter* isolates (van Putten et al., 2009). CaCo-2 cells challenged with *C. jejuni* for 6h showed upregulation of genes involved in cell growth, gene transcription, steroid biosynthesis, and inflammation, however this was not observed in CT-62 cells (murine intestinal epithelial cells) (van Putten et al., 2009).

The movement of *Campylobacter* between and into epithelial cells, compromises the epithelial barrier by damaging tight junctions (W. A. Awad et al., 2018). The integral proteins within the tight junction structure are disrupted, leading to both increased paracellular passage of the bacterium and increasing inflammation. HtrA is a serine protease that combines protease and chaperone functions and is in the bacterial periplasmic space. HtrA has been identified as a key contributor to virulence due to its interaction with occludin (key protein in tight junctions) (Harrer et al., 2019). Redistribution of tight junction proteins (specifically occludin) from an intercellular to intracellular location as a result of *Campylobacter* challenge has been observed in CaCo-2 and T84 epithelial cell monolayers *in vitro* (M. L. Chen et al., 2006; MacCallum, Hardy, et al., 2005). The reduction of tight junction integrity contributes to the loss of absorptive function seen in intestinal epithelium, as demonstrated by a loss in transepithelial electrical resistance (TEER), and collapse of epithelial fluid transport (MacCallum, Hardy, et al., 2005).

5.1.2.2 *Campylobacter* induced damage due to direct cytotoxicity

Permeability of the intestinal epithelium, whilst being directly affected by invasion (section 5.1.2.1), can also be mediated by bacterial toxins (W. A. Awad et al., 2018). CDT is the best characterised toxin in *Campylobacter* species (AbuOun et al., 2005; Jain et al., 2008). There are three subunits that make up CDT (CdtA, CdtB, and CdtC), and it belongs to the AB2-type toxins (Al-Edany et al., 2015; Elmi et al., 2016; Reddy & Zishiri, 2018). Subunits A and C facilitate the movement of CdtB into host cells. CdtB damages host cell DNA by interfering and ultimately causing arrest in G2/M phase of the eukaryotic cell cycle (Elmi et al., 2016; Hickey et al., 2000; van Putten et al., 2009). By disrupting the G2/M phase of the cell cycle, the renewal of the intestinal epithelium is compromised and

this primary barrier against extra-intestinal spread of bacteria is compromised (van Putten et al., 2009).

Once *Campylobacter* is within the host epithelial cell, there are numerous cytotoxins that are released (Hickey et al., 2000). The best characterised toxin associated with *Campylobacter* is a multi-subunit toxin previously mentioned, CDT (Purdy *et al.*, 2000; John *et al.*, 2017). Not all *Campylobacter* strains are positive for CDT, in particular *C. coli* strains lack CDT in their membranes (AbuOun et al., 2005; Hickey et al., 2000). The active subunit of CDT (CdtB) is commonly present in *C. jejuni* isolates. However *C. coli* strains do not naturally possess this toxin subunit (Jain et al., 2008). In *C. jejuni* isolates delivery of biologically active CDT into host cells has been facilitated by OMVs and this is an important part of *C. jejuni* pathogenesis (Elmi et al., 2016). C. *jejuni* NCTC 11168 OMVs are cytotoxic toward CaCo-2 intestinal epithelial cells *in vitro* (Elmi et al., 2016).

CDT, whilst primarily functioning as a cell targeted toxin, also induces IL-8 production in epithelial cells. This was supported by the induced IL-8 activity observed with *C. coli* strains with a shuttle plasmid containing the CDT operon inserted into the membrane, the same strains lacking the plasmid were unable to induce the same IL-8 response (Hickey et al., 2000). IL-8 production from epithelial cells may also be induced by the adhesin factor Jlp (C. K. Smith et al., 2005).

5.1.2.3 *Campylobacter* induced damage due to cytokine production

Cell signalling pathway analysis has led to the observation that the innate immune response induced by *Campylobacter* contributes to the inflammatory pathology seen in the avian and human gastrointestinal tract (W. A. Awad et al., 2018; D. A. John et al., 2017; van Putten et al., 2009). An over-exuberant immune response leads to the dysregulation of cytokine production, promoting tissue damage and the recruitment of neutrophils and monocytes. The damage to the intestinal epithelium that this causes allows for increased bacterial invasion both paracellularly and transcellularly (D. A. John et al., 2017; van Putten et al., 2009). During the progressive phase of human campylobacteriosis significantly higher concentrations of proinflammatory cytokines has been observed (Nyati & Nyati, 2013).

As mentioned above (section 5.1.2.2), the permeability of the intestinal epithelium is mediated by *Campylobacter*-associated toxins, this not only increases the ability of microbes and toxins to spread extra-intestinally but further damages host cells by aiding

immune response activation (W. A. Awad et al., 2018). “Leaky gut”, as this increased permeability is often referred to, increases the transcellular and paracellular passage of *Campylobacter* aiding the bacterial dissemination toward other organs (W. A. Awad et al., 2018).

The structure of tight junctions between the intestinal epithelial cells is damaged by elevated levels of TNF-α and IFN-γ, consequently compromising the integrity of this aspect of the intestinal barrier (W. A. Awad et al., 2018; Rees et al., 2008). TNF-α decreases the expression of two major tight junction proteins, occluding and ZO-1 (He et al., 2012). Some studies have alluded to the contribution of IL-6 in disruption of tight junction structure by elevating claudin-2 expression (Suzuki et al., 2011). TNF-α has been frequently associated with structural damage to tight junctions; *C. jejuni* 81116 in the presence of IFN-γ and TNF-α, for example, increased cellular damage and induced a redistribution of occluding within 24 hours (Dodson, 2010). Disruption of tight junction function due to claudin and occludin redistribution by *C. jejuni* and TNF- α has been further confirmed by multiple studies (Konkel et al., 2020; Lamb-Rosteski et al., 2008; Rees et al., 2008).

5.1.3 Anti-inflammatory strategies

Probiotics are a viable tool for *Campylobacter* reduction in chickens due to positive effects on animal performance, and gut microbiota leading to SCFA production which in turn, improves barrier integrity of epithelial cells (Balta et al., 2022). Probiotics work in several ways to alleviate *Campylobacter* pathogenesis and have been demonstrated both *in vivo* and *in vitro*. Firstly, probiotic interaction with host cells induces IFN-γ production, which reduces the severity of disease progression in the host, in addition enhanced levels of IFN-α have been reported as an immunomodulatory mechanism as a result of orally administered probiotics (Balta et al., 2022; Mazziotta et al., 2023). Secondly, competitive exclusion by probiotics depletes the availability of nutrients in the gastrointestinal tract and blocks *Campylobacter* interaction with host cell receptors, reducing the induction of pro-inflammatory cytokines that are stimulated during *Campylobacter* infection (Balta et al., 2022; M. Khan, 2019). Pre-treatment of cell lines with probiotics (e.g. *E. coli Nissle* 1917, Aviguard® formulation, and *Lactobacillus* spp. mixture) has been shown to alter the expression of pro-inflammatory cytokines (decrease in IL-6, IL-8, IL-18, MAPK, IFN-γ and TNF-α; increase in CXCLi2, IL-10) and stimulates production of short chain

fatty acids that act as antimicrobial molecules (Balta et al., 2022; Helmy et al., 2021; Taha-Abdelaziz et al., 2019).

Vinolo *et al.* (2011) reported the importance of SCFAs (acetate, propionate, and butyrate) as nutrients for gastrointestinal epithelial cells but also as potential modulators of gut immunity. SCFA are found naturally in the gastrointestinal tract of mammals and birds, produced by fermentation of polysaccharides, oligosaccharides and glycoprotein precursors (McNeil, 1984; Tralongo et al., 2014; Vinolo et al., 2011). Modulation of inflammatory mediators released by macrophages can be achieved by SCFA, specifically the suppression of known pro-inflammatory mediators involved in *Campylobacter* pathogenesis (Vinolo et al., 2011).

5.1.4 Anti-inflammatory activity of Butyrate

Butyrate is the most studied SCFA and enhances production of IL-10, a known anti-inflammatory cytokine (Vinolo et al., 2011). There have been several studies focussed on the mechanism of action of butyric acid, regarding anti-inflammatory properties (Pituch et al., 2013). It is well known that there is an anti-inflammatory effect of butyrate and its associated salts, specifically in the intestinal epithelium (Pituch et al., 2013). In human cells, sodium butyrate is the most potent inhibitor of histone deacetylase (HDAC), the resulting increase in histone and non-histone protein acetylation modulates gene expression of important immunomodulatory cytokines released by macrophages and monocytes (Vinolo et al., 2011; Waldecker et al., 2008). Studies have investigated the effect of butyric acid on several cell types found in the intestine and liver (monocytes, macrophages, murine BV2 cells, and Kupffer cells) and reported consistent decrease in TNF-α, nitric oxide, and IFN-γ, and consistent upregulation of IL-10 (Fukae et al., 2005; J.-S. Park et al., 2005; Perez et al., 1998; Pituch et al., 2013; Säemann et al., 2000).

Butyrate has been further shown to increase the TEER of CaCo-2 cell monolayers *in vitro* and consequently protect these cells from *C. jejuni* invasion and translocation (Tralongo et al., 2014). These results implied a concentration dependent decrease in the intestinal mucosa permeability. This could be due to strengthening the tight junctions and stabilising the over-exuberant inflammatory response by decreasing the expression of pro-inflammatory cytokines which has been demonstrated by a reduction in mRNA expression of these cytokines in intestinal biopsies (Tralongo et al., 2014).

5.1.5 Aims

This chapter investigated the indirect effects of feed additives on *Campylobacter* induced damage to epithelial cell monolayers *in vitro*, arising from invasion and inflammatory cytokine production. Supplementation of chicken feed with SCFA is a practical potential solution for the modulation of inflammation seen in *Campylobacter* pathogenesis, providing a practical and economically viable dose can be determined. Three feed additives were carried forward from Chapter 4, two organic acids (caprylic and butyric) and one mineral compound (chromium propionate). It was hypothesised that caprylate and butyrate would modulate the expression of cytokines produced by epithelial cell monolayers The specific chapter aims were to:

- Determine toxicity of feed additives to avian and human epithelial cells at defined concentrations.
- Determine if feed additives protect avian epithelial cells against *Campylobacter* invasion.
- Quantify inflammatory cytokines expressed by avian epithelial cells when exposed to *Campylobacter* isolates.
- Determine if pre-exposure of epithelial cells to feed additives affects cytokine induced by *Campylobacter* exposure.

5.2 Materials and Methods

5.2.1 *Campylobacter* isolates

Three *Campylobacter* isolates (C13, G28, L29) (Table 3.3) were specifically selected from an original collection of 21 (Table 2.8). Two reference strains were used throughout this study (*C. jejuni* M1 and *C. jejuni* NCTC 11168; Table 2.8). Isolates were cultured as described in section 2.2.2.1.

5.2.2 Culture of epithelial cell monolayers

Human and avian epithelial cell lines (CaCo-2 and 8E11, respectively) were used in this study and cultured as per routine procedure outlined in section 2.3.1. For the AlamarBlue assay, cell lines were cultured in a 96-well tissue culture plate until confluent. For the gentamicin protection assay (GPA) and cytokine quantification, cell lines were cultured in 24-well tissue culture plate and treatment of cell lines began when cells were approximately 90% confluent as estimated with light microscopy.

5.2.3 Epithelial cell viability: AlamarBlue Assay

To assess the toxicity of feed additives towards epithelial cell lines an AlamarBlue assay was conducted as outlined in section 2.3.4. An untreated control, positive control (Triton X-100), and a range of concentrations of feed additives were applied to cell lines to determine the spectrum of toxicity across a wide range of concentrations (Table 4.1).

Table 5.1 Concentrations of controls and feed additives used in the AlamarBlue cell viability assay.

Feed additive	Concentration(s) (% v/v)
Untreated	n/a
Triton X-100 (positive control)	$2x10^{-4}$
Butyrate (pH 7.0 ±0.2)	$2x10^{-7}$, $2x10^{-6}$, $2x10^{-5}$, $2x10^{-4}$, $2x10^{-3}$, 0.02, 0.2, 0.6, 1.0, 1.4
Caprylate (pH 7.0 ±0.2)	$2.5x10^{-7}$, $2.5x10^{-6}$, $2.5x10^{-5}$, $2.5x10^{-4}$, $2.5x10^{-3}$, 0.025, 0.25, 0.75, 1.25, 1.75
Chromium propionate	$2x10^{-12}$, $2x10^{-11}$, $2x10^{-10}$, $2x10^{-9}$, $2x10^{-8}$, $2x10^{-7}$, $2x10^{-6}$, $6x10^{-6}$, $1x10^{-5}$, $14x10^{-5}$,

5.2.4 *Campylobacter* invasion assay with epithelial cells pre-incubated with feed additives compounds

To quantify *Campylobacter* invasion into epithelial cell lines a GPA was conducted as outlined in section 2.3.5. Prior to performing the GPA, epithelial cell monolayers were incubated with feed additives for 24 h as outlined in section 2.3.3.

5.2.5 Quantification of cytokine mRNA expression from epithelial cell lines exposed to *Campylobacter*

Avian epithelial cells (8E11) were cultured to 90% confluency (section 2.3.1 and 4.2.1) and exposed to *Campylobacter* isolates for 6 h (section 2.3.2). RNA was extracted from epithelial cells as outlined in section 2.3.6, followed by conversion to cDNA (section 2.3.7). Quantitative PCR using the probe-based method (section 2.3.8) was used to quantify mRNA expression by 8E11 cells during exposure to *Campylobacter* isolates.

5.2.6 Quantification of cytokines from epithelial cell lines pre-treated with feed additives before exposure to *Campylobacter*

Prior to exposure to *Campylobacter* isolates for 6 h, avian cell monolayers were pre-treated with feed additives (Table 4.2) for 24 h. The RNA from treated and infected

monolayers was extracted and converted to cDNA for quantitative PCR to quantity cytokine expression (sections 2.3.7 and 2.3.8).

Table 5.2 Concentrations of feed additive used in the treatment of avian cell lines prior to exposure to *Campylobacter* isolates for measurement of inflammatory cytokines.

Feed additive	Concentration(s) (% v/v)
Butyrate (pH 7.0 ±0.2)	0.02, 0.2, 0.6, 1.0, 1.4
Caprylate (pH 7.0 ±0.2)	0.025, 0.25, 0.75, 1.25, 1.75
Chromium propionate	0.00002, 0.00004, 0.0001, 0.00014

5.3 Results

5.3.1 Epithelial cell viability

Viability of epithelial cells was presented as fluorescence at 570 nm using AlamarBlue (Figure 5.1-5.3). Standard cell culture media (untreated control) was considered optimum for 100% of cells in the monolayer to be considered viable. Reduction in fluorescence compared to the untreated control indicated a reduction in cell metabolism, whereas increased fluorescence compared to the untreated control indicated an increase in cell respiration due to the reduction of resazurin to resorufin – this could be a product of increased cell metabolism or proliferation. The Triton X-100 despite not being a positive control (as intended) did result in a reduction in fluorescence compared to untreated cell lines (Figure 5.1, 5.2 & 5.3).

5.3.1.1 Butyrate effect on epithelial cell viability

Butyrate had no significant effect on viability of 8E11 epithelial cells at any of the concentrations trialled during this experiment (Figure 5.1(A)). However, for CaCo-2 cells at the higher concentrations (0.6 to 1.4%) a significant increase in fluorescence was observed, with the 0.6% concentration having the greatest effect (Figure 5.1(B)). No concentrations of butyrate were significantly cytotoxic to the epithelial cells.

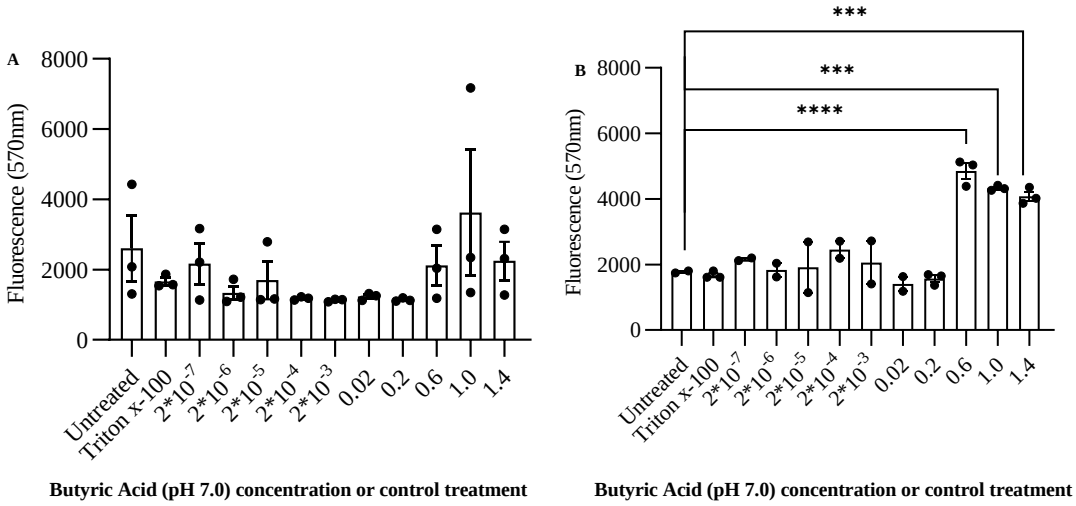

Figure 5.1 Viability of avian (8E11) and human (CaCo-2) cells after incubation with butyrate.

Fluorescence (570 nm) was measured from 8E11 (A) and CaCo-2 (B) cell lines incubated with Butyric acid (pH 7.0) (n=3 ± SEM). A Kruskal-Wallis ANOVA with multiple comparisons was used to determine significance of results in 8E11 cells (A) and an ordinary one-way ANOVA with multiple comparisons was used to determine significance of results in CaCo-2 cells (B). *** $p < 0.001$, **** $p < 0.0001$.

5.3.1.2 Caprylate effect on epithelial cell viability.

Caprylate had no significant effect on the viability of 8E11 epithelial cells at any of the concentrations trialled during this experiment (Figure 5.2(A)). In CaCo-2 cells a significant increase in fluorescence at the three lowest concentrations was observed (0.00000025 to 0.000025%) and 0.025 to 0.75% (Figure 5.2(B)). No concentrations of caprylate were significantly cytotoxic to the epithelial cells.

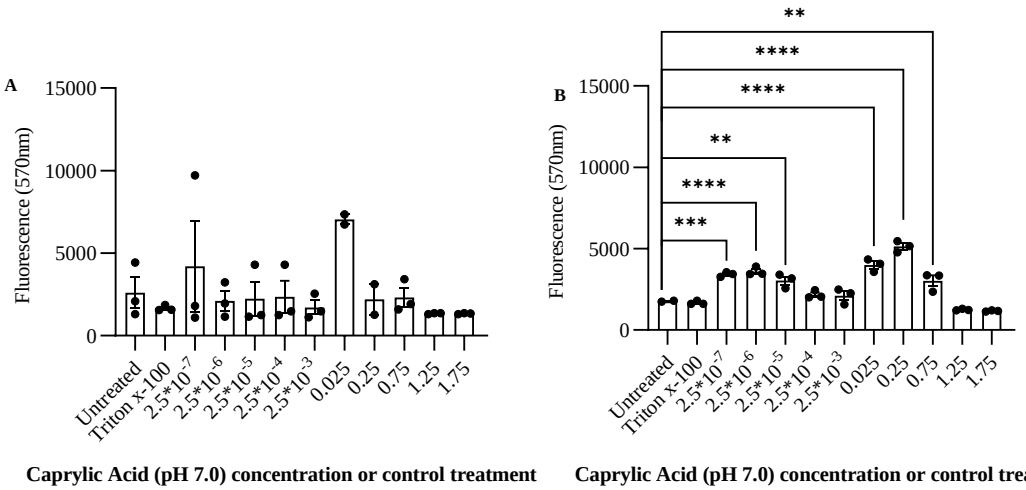

Figure 5.2 Viability of avian (8E11) and human (CaCo-2) cells after incubation with caprylate.

Fluorescence (570 nm) was measured from 8E11 (A) and CaCo-2 (B) cell lines incubated with caprylic acid (pH 7.0) (n=3 ± SEM). A Kruskal-Wallis ANOVA with multiple comparisons was used to determine significance of results in 8E11 cells (A) and an ordinary one-way ANOVA with multiple comparisons was used to determine significance of results in CaCo-2 cells (B). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.1.3 Chromium Propionate effect on epithelial cell viability

Chromium propionate significantly increased fluorescence of 8E11 epithelial cell monolayers at all concentrations above 0.00000002% (Figure 5.3(A)). A sharp increase in fluorescence was observed at 0.000000002%. Furthermore, at no concentration was chromium considered significantly toxic to 8E11 cells. Chromium had no significant effect on CaCo-2 cell monolayers (Figure 5.3(B)).

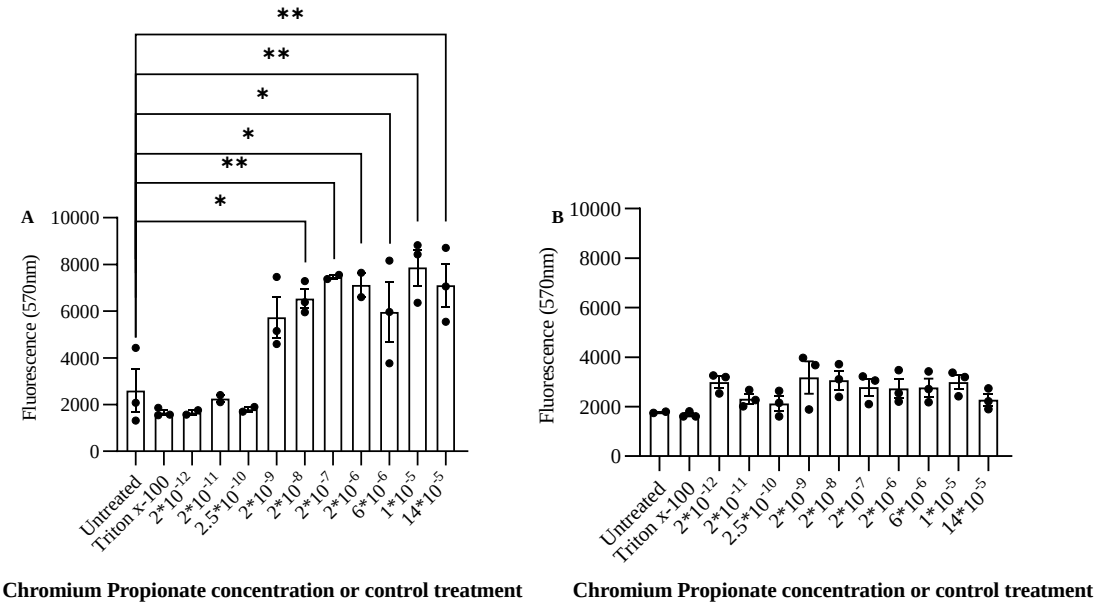

Figure 5.3 Viability of avian (8E11) and human (CaCo-2) cells after incubation with chromium propionate.

Fluorescence (570 nm) was measured from 8E11 (A) and CaCo-2 (B) cell lines incubated with chromium propionate (n=3 ± SEM). An ordinary one-way ANOVA with multiple comparisons was used to determine significance of results. * $p < 0.05$, ** $p < 0.01$.

5.3.2 *Campylobacter* invasion into cell lines pre-treated with feed additives

5.3.2.1 *Campylobacter* invasion into cell lines pre-treated with Butyrate

Pre-treatment of the avian 8E11 cell line with butyrate did not significantly affect the invasion of any of the *Campylobacter* strains (Figure 5.4(A-D)). This was also seen when data from all strains was combined and the results averaged to give an overall '*genera*' effect of butyrate on *Campylobacter* invasion into 8E11 cells (Figure 5.4(E)). A concentration dependent response for strain G28 (Figure 5.4(C)) was observed, however, this was not significant at the 0.05 level.

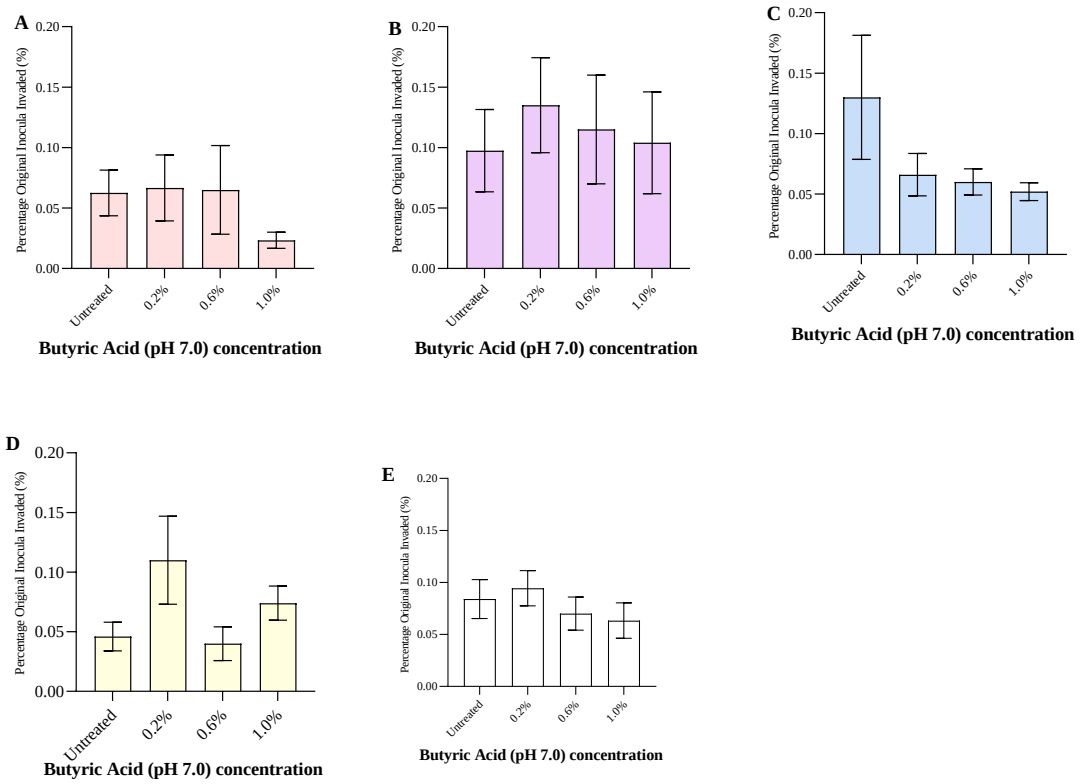

Figure 5.4 Invasion of *Campylobacter* into avian epithelial cell lines pre-treated with Butyrate.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded an 8E11 cell monolayer from an original inoculum *in vitro* (n=5 ± SEM). The 8E11 cells were exposed to varying concentration of butyric acid (pH 7.0±0.2) for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter* C13, C – *Campylobacter* G28, D – *Campylobacter* L29, E – *Campylobacter* mean invasion from four strains. A two-way ANOVA with multiple comparisons was used to determine significance of results.

Similarly, there was no effect of butyrate pre-treatment on invasion of *Campylobacter* into human CaCo-2 cells (Figure 5.5). Again, a concentration dependent response in strain G28 was observed (Figure 5.5(C)), however, this was not significant at the 0.05 level. When averaging the invasion of all strains, to assess the 'genera' effect (Figure 5.5(E)) there was a slight concentration dependent response, however, this was not significant at the 0.05 level.

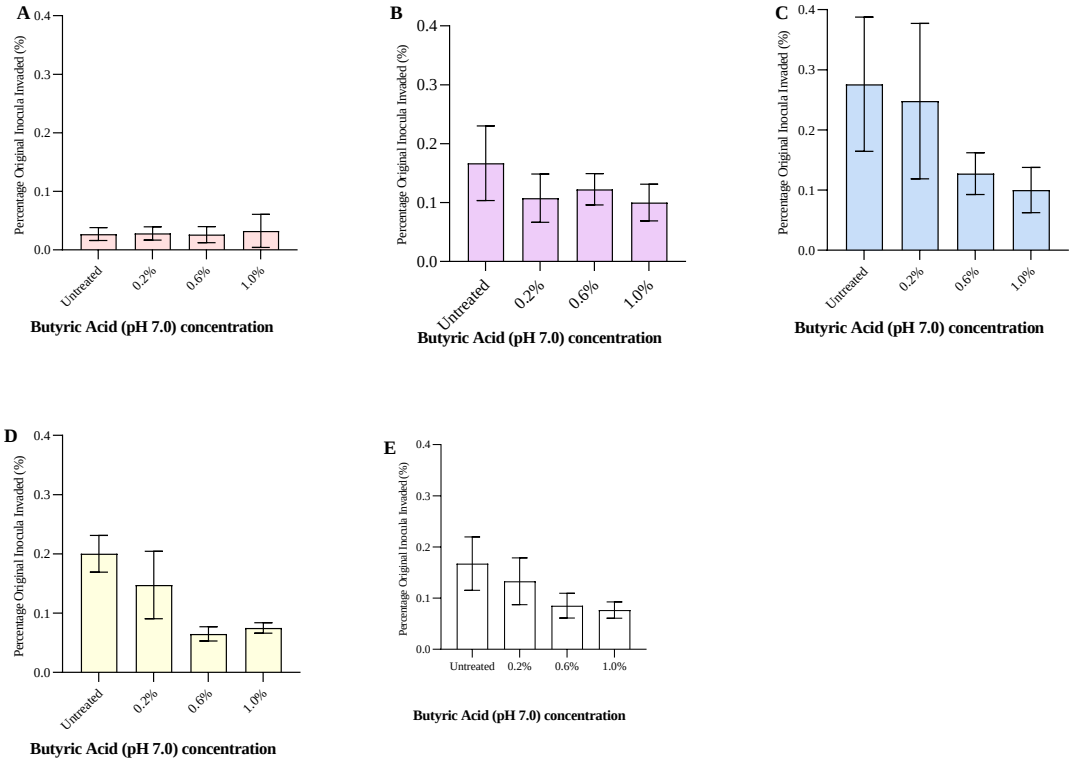

Figure 5.5 Invasion of *Campylobacter* into human epithelial cell lines pre-treated with Butyrate.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded a CaCo-2 cell monolayer from an original inoculum *in vitro* (n=5 ± SEM). The CaCo-2 cells were exposed to varying concentration of butyric acid (pH 7.0±0.2) for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter* C13, C – *Campylobacter* G28, D – *Campylobacter* L29, E – *Campylobacter* mean invasion from four strains. A two-way ANOVA with multiple comparisons was used to determine significance of results.

5.3.2.2 *Campylobacter* invasion into cell lines pre-treated with Caprylate

A large degree of variation in *C. jejuni* NCTC 11168 invasion at strain level into 8E11 and CaCo-2 cells was observed and confirmed by the SEM (Figure 5.6(B); Figure 5.7(B)). Despite a large degree of variation between strains, there was no effect on invasion by *Campylobacter* when 8E11 cells were pre-treated with caprylate at strain or *genera* level (Figure 5.6(A-F)).

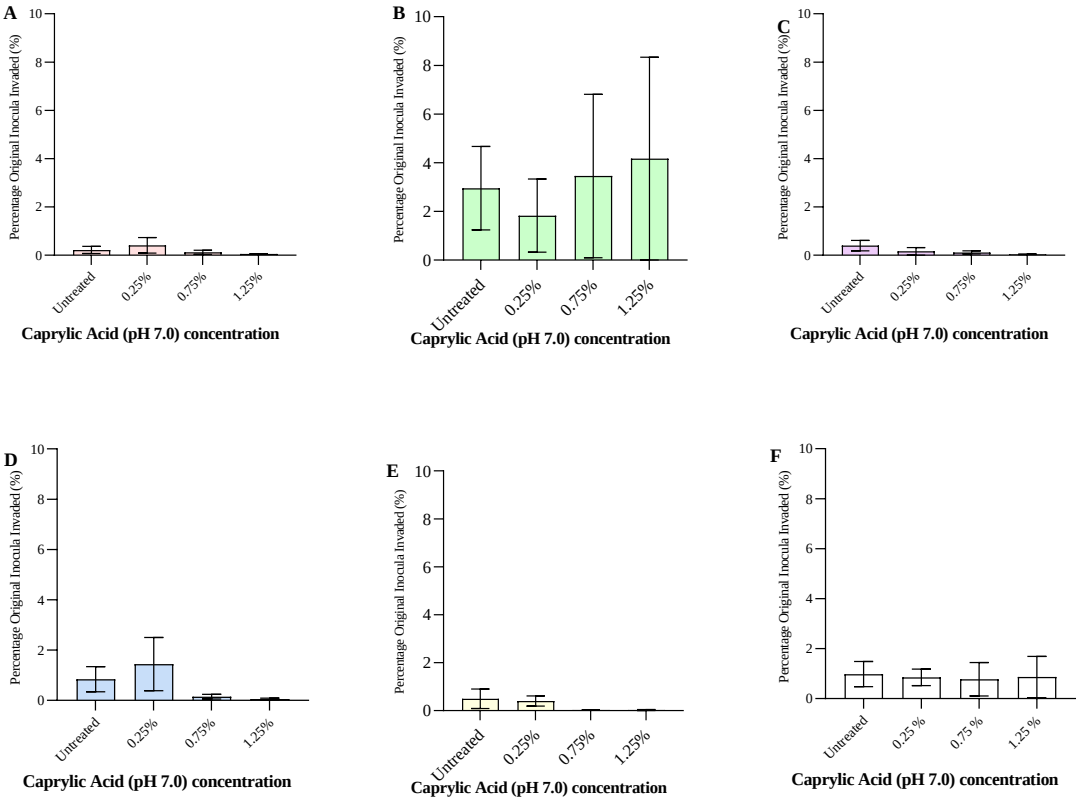

Figure 5.6 Invasion of *Campylobacter* into 8E11 epithelial cell lines pre-treated with Caprylate in various concentrations.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded an 8E11 cell monolayer from an original inoculum *in vitro* (n=5 ± SEM). The 8E11 cells were exposed to varying concentration of caprylic acid (pH 7.0±0.2) for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter jejuni* NCTC 11168, C – *Campylobacter* C13, D – *Campylobacter* G28, E – *Campylobacter* L29, F – *Campylobacter* mean invasion from five strains. A two-way ANOVA with multiple comparisons was used to determine significance of results.

At the strain level (Figure 5.7(A-E)) no significant effect of caprylate on invasion was observed. A minimal response was observed for *C. jejuni* NCTC 11168 as concentration increased (Figure 5.7(B)), however this was not significant at the 0.05 level. No concentration response pattern was seen when invasion of '*genera*' was assessed (Figure 5.7(E)).

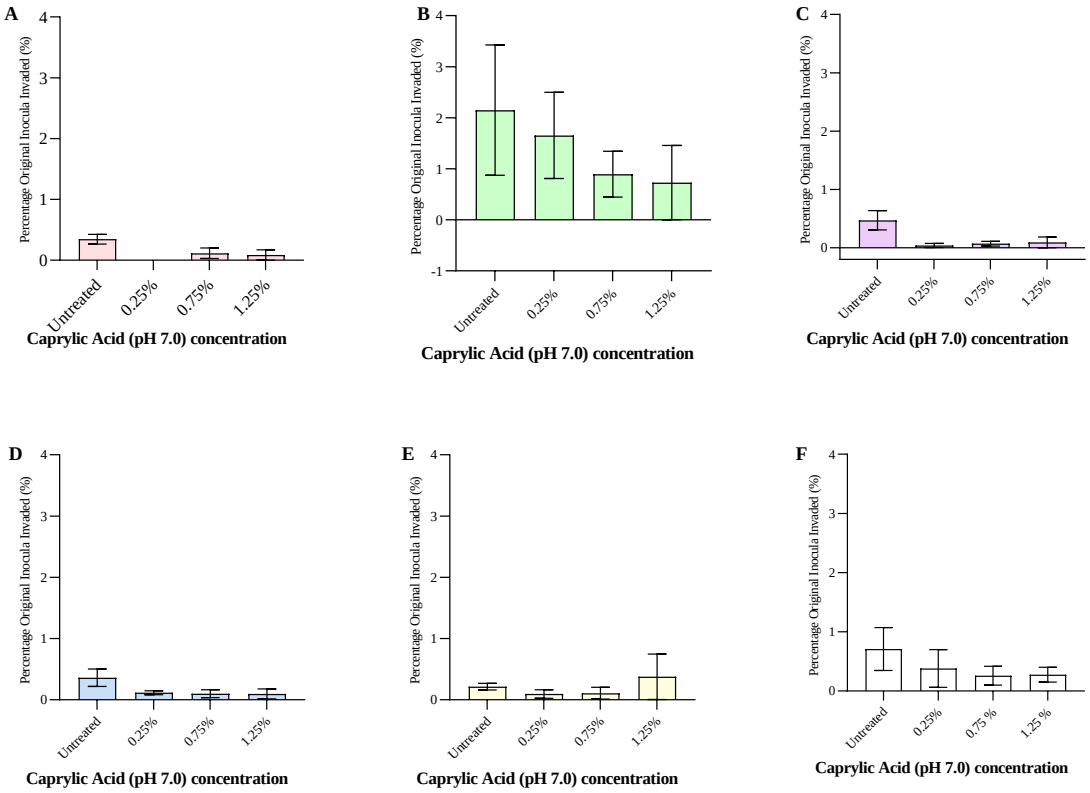

Figure 5.7 Invasion of *Campylobacter* into CaCo-2 epithelial cell lines pre-treated with Caprylate in various concentrations.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded a CaCo-2 cell monolayer from an original inoculum *in vitro* (n=5 ± SEM). The CaCo-2 cells were exposed to varying concentration of caprylic acid (pH 7.0±0.2) for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter jejuni* NCTC 11168, C – *Campylobacter* C13, D – *Campylobacter* G28, E – *Campylobacter* L29, F – *Campylobacter* mean invasion from five strains. A two-way ANOVA with multiple comparisons was used to determine significance of results.

5.3.2.3 *Campylobacter* invasion into cell lines pre-treated with Chromium Propionate

C. jejuni NCTC 11168 invasion into 8E11 and CaCo-2 cells showed a large degree of variation (Figure 5.8(B); Figure 5.9(B)). There was a significant increase of *Campylobacter* L29 invasion into 8E11 cells when pre-treated with 0.00014% chromium propionate compared to the lower three concentrations (Figure 5.8(E)), however this was not significant compared to the untreated control. When averaging results from the five *Campylobacter* strains, to assess '*genera*' chromium pre-treatment of 8E11 cells had no effect on invasion.

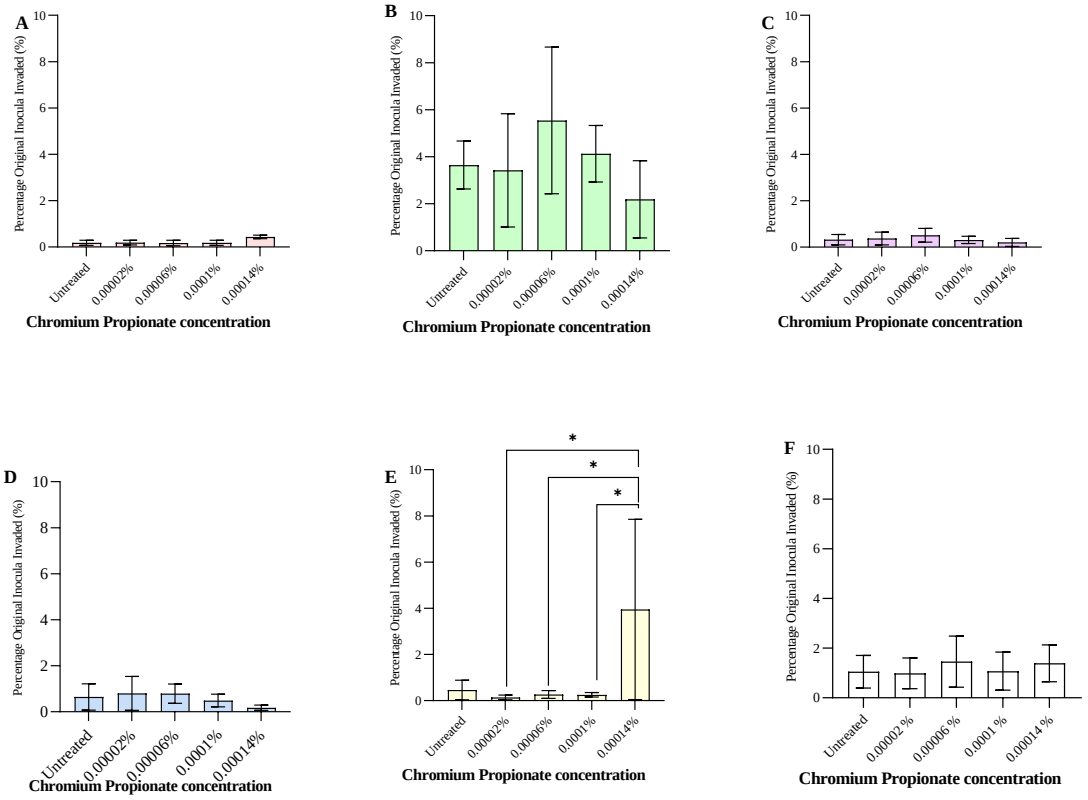

Figure 5.8 Invasion of *Campylobacter* into 8E11 epithelial cell lines pre-treated with Chromium Propionate in various concentrations.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded an 8E11 cell monolayer *in vitro* (n=5 biological replicates ± SEM). The 8E11 cells were exposed to varying concentration of chromium propionate for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter jejuni* NCTC 11168, C – *Campylobacter* C13, D – *Campylobacter* G28, E – *Campylobacter* L29, F – *Campylobacter* mean invasion from five strains. A two-way ANOVA with multiple comparisons was used to determine significance of results. * $p < 0.05$.

Pre-treatment of CaCo-2 cells with chromium propionate had no effect on the average invasion of *Campylobacter* into the epithelial cells (Figure 5.9(F)). At the strain level, *C. jejuni* NCTC 11168 invasion into CaCo-2 cells was significantly lower when pre-treated with 0.00014% chromium compared to 0.00006% chromium (Figure 5.9(B)). None of the treatments significantly affected invasion compared to the untreated control.

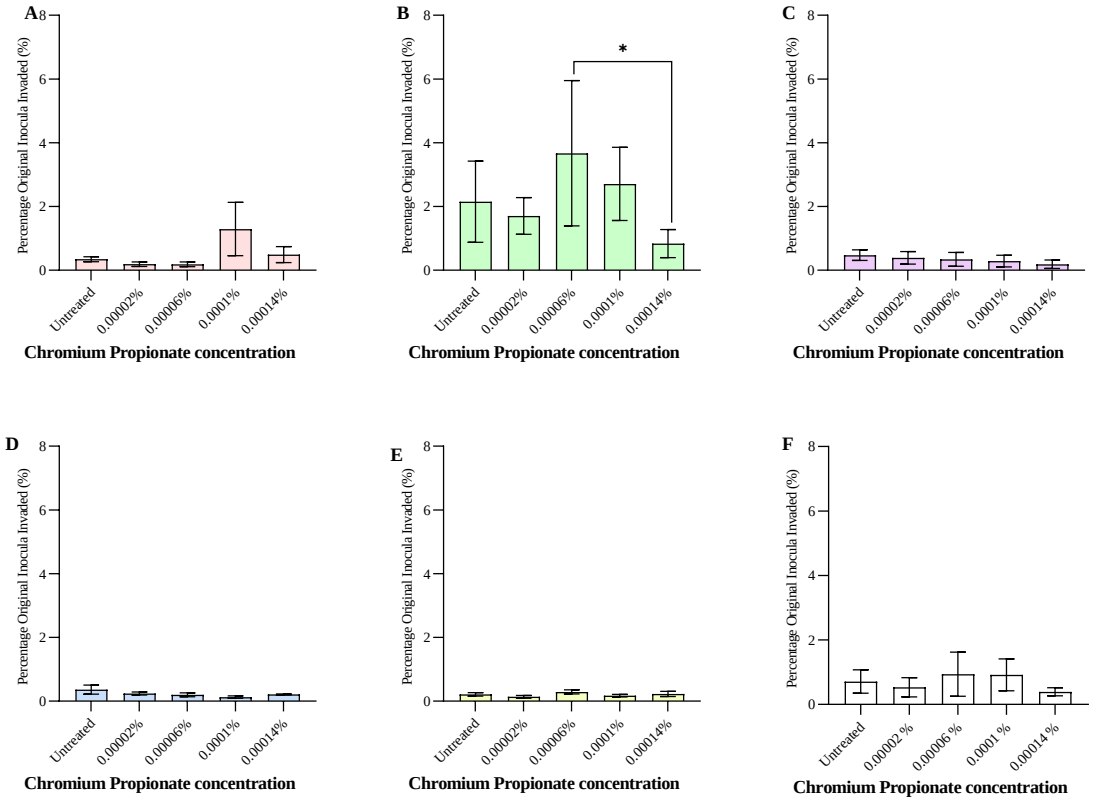

Figure 5.9 Invasion of *Campylobacter* into CaCo-2 epithelial cell lines pre-treated with Chromium Propionate in various concentrations.

A gentamicin protection assay was employed to quantify the percentage of *Campylobacter* cells that invaded a CaCo-2 cell monolayer from an original inoculum *in vitro* (n=5 ± SEM). The CaCo-2 cells were exposed to varying concentration of chromium propionate for 24 h prior to challenge with *Campylobacter* strains. A – *Campylobacter jejuni* M1, B – *Campylobacter jejuni* NCTC 11168, C – *Campylobacter* C13, D – *Campylobacter* G28, E – *Campylobacter* L29, F – *Campylobacter* mean invasion from five strains. A two-way ANOVA with multiple comparisons was used to determine significance of results. * $p < 0.05$.

5.3.3 Cytokine production in avian cells exposed to *Campylobacter* and Butyrate

5.3.3.1 CXCLi1 gene expression is increased in *Campylobacter* infected 8E11 epithelial cells treated with 0.6 % Butyrate

CXCLi1 expression was not significantly different when 8E11 cells were pre-exposed to butyrate in the absence of *Campylobacter*, however at 0.6 % butyrate a spike in CXCli1 production was observed (Figure 5.10A). At the strain level, no significant changes in CXCLi1 levels following addition of sodium butyrate were observed (Figure 5.10 B-F). At the genus level (mean response when results from five strains combined) CXCLi1

gene expression was significantly increased in *Campylobacter* infected cells pre-exposed to 0.6% butyrate (Figure 5.10G). When comparing the effect of *C. jejuni* M1 and *C. jejuni* NCTC 11168 on cells, both isolates stimulated CXCLi1 gene expression in 8E11 cells however there was a large degree of error as seen by the SEM in the untreated observations.

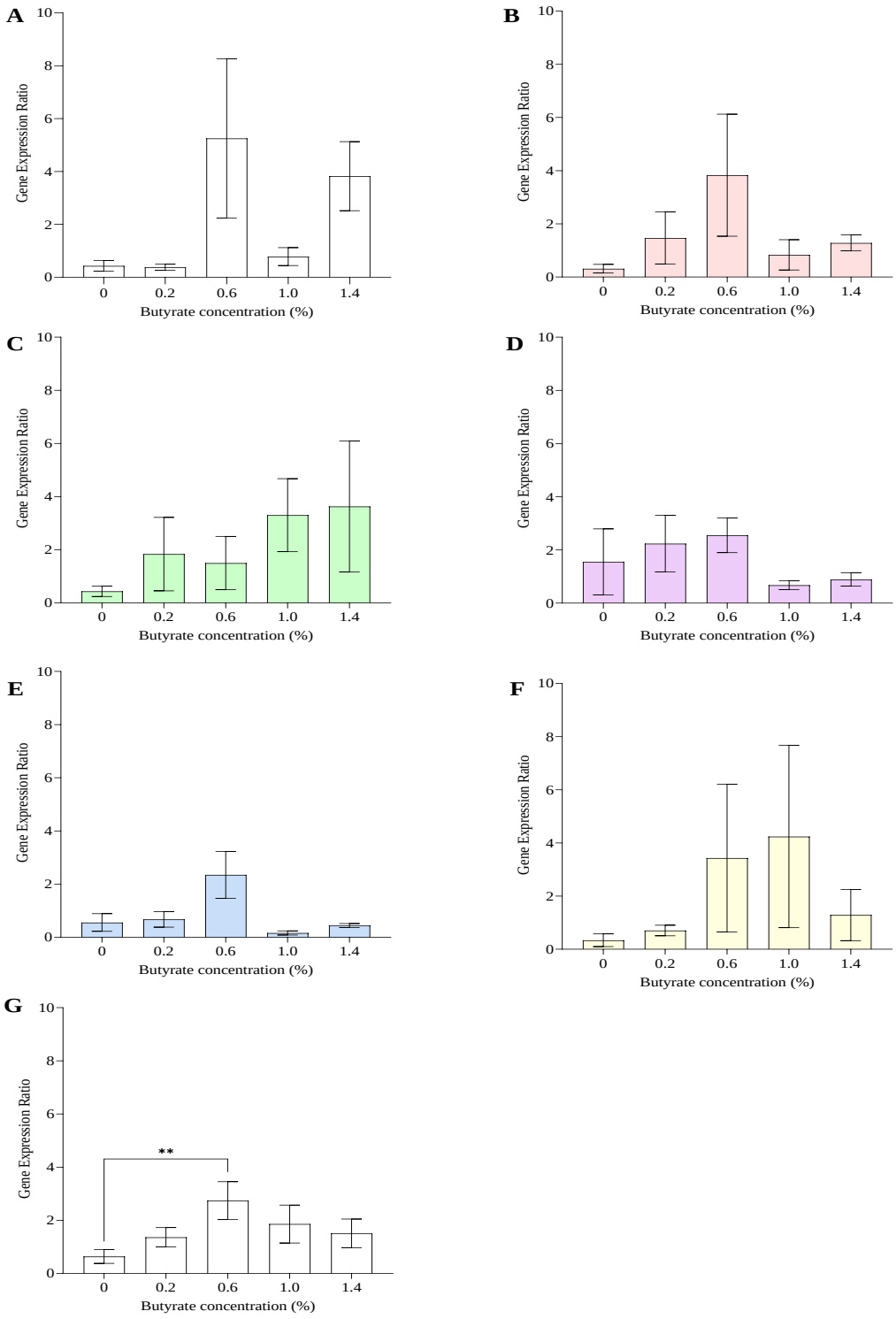

Figure 5.10 CXCLi1 gene expression in 8E11 cells exposed to butyrate.

Quantitative PCR was used to quantify the gene expression of CXCLi1 (IL-8 homologue) in 8E11 cells cultured, infected, and treated *in vitro* (n=3 ± SEM). The 8E11 cells were exposed to varying concentrations of butyric acid (pH 7.0) for 24 h prior to challenge with *Campylobacter* strains. A – Uninfected, B – *Campylobacter jejuni* M1, C – *Campylobacter jejuni* NCTC 11168, D – *Campylobacter* C13, E – *Campylobacter* G28, F – *Campylobacter* L29, G – *Campylobacter* mean invasion from five strains. A Kruskal-Wallis ANOVA with multiple comparisons was used to determine significance of results. * $p < 0.05$, ** $p < 0.01$.

5.3.3.2 CXCLi2 expression is increased in *Campylobacter* infected 8E11 epithelial cells treated with 0.6 % Butyrate

The avian epithelial cells (8E11) showed a similar response to butyrate with regards to CXCLi2 expression in the absence of *Campylobacter* infection, a significant spike at the 0.6 % sodium butyrate treatment compared to all other concentrations (Figure 5.11) suggests that sodium butyrate alone could induce an increase in CXCLi1 and 2 gene expression (Figure 5.11A). At the strain level, no changes in CXCLi2 gene expression were observed with addition of sodium butyrate in varying concentrations (Figure 5.11B-F). At the genus level, a significantly increased expression of CXCLi2 was seen (Figure 5.10G), which were statistically significant (Figure 5.11G). The 0.6% concentration of sodium butyrate increased the gene expression of CXCLi2 significantly compared to the untreated cells and cells treated with 1.4% butyrate. Similar to observations for CXCLi1 it was evident that 0.6 % butyrate spiked CXCLi2 production in both control experiments (Figure 5.11A) and *Campylobacter* infected cells (Figure 5.11G).

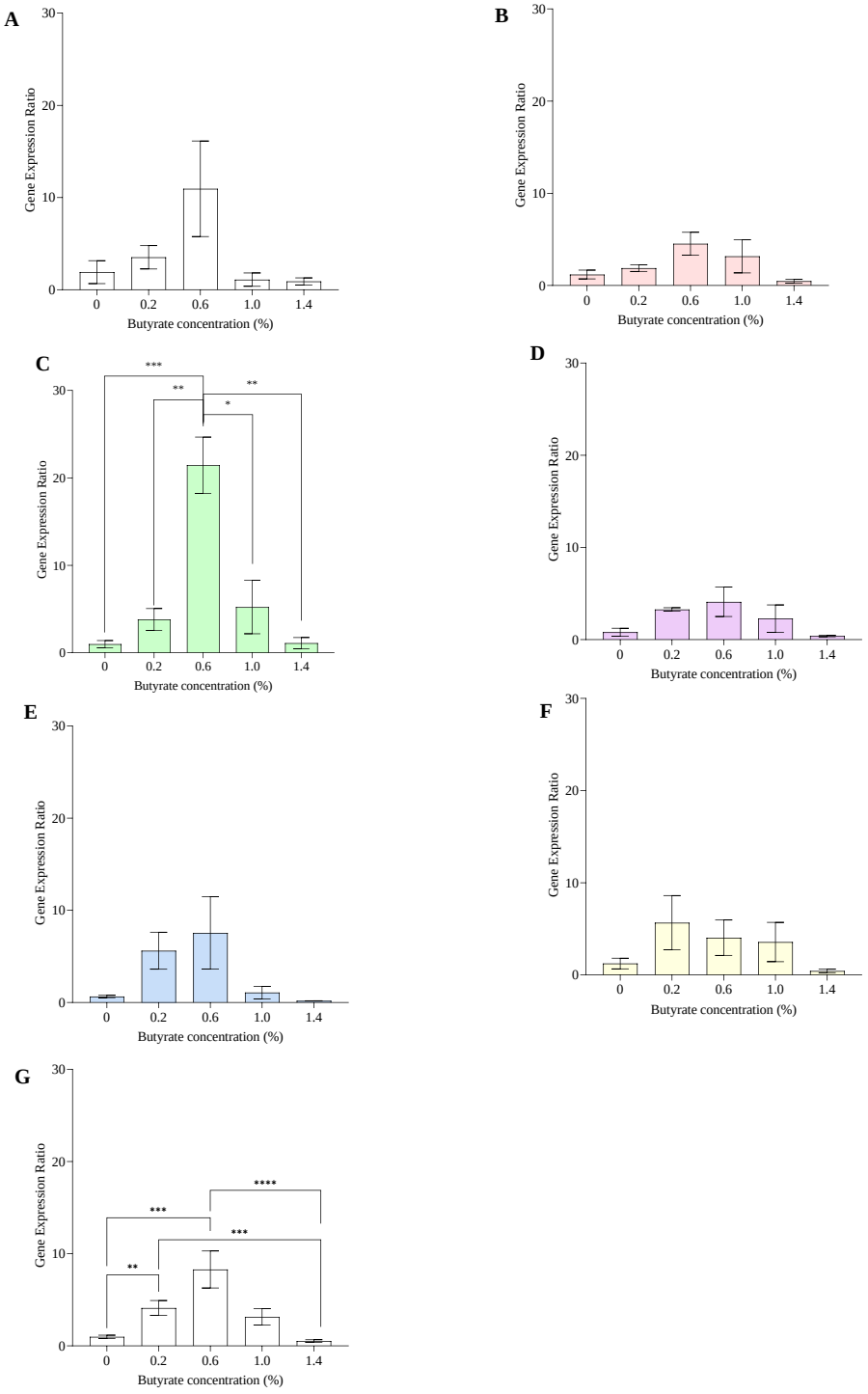

Figure 5.11 CXCLi2 gene expression in 8E11 cells exposed to butyrate

Quantitative PCR was used to quantify the gene expression of CXCLi2 (IL-8 homologue) in 8E11 cells cultured, infected, and treated *in vitro* (n=3 ± SEM). The 8E11 cells were exposed to varying concentrations of butyric acid (pH 7.0) for 24 h prior to challenge with *Campylobacter* strains. A – uninfected, B – *Campylobacter jejuni* M1, C – *Campylobacter jejuni* NCTC 11168, D – *Campylobacter* C13, E – *Campylobacter* G28, F – *Campylobacter* L29, G – *Campylobacter* mean invasion from five strains. A Kruskal-Wallis ANOVA with multiple comparisons and T-tests were used to determine significance of results. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3.3.3 TGFβ expression is not affected by butyrate treatment in infected 8E11 epithelial cells

TGFB expression showed no significant change across any treatments trialled in this study, both at the strain and genus level (Figure 5.12). In uninfected 8E11 cells, butyrate had no effect on TGFβ expression. Despite slight trends observed in the data, the variation in the data recorded resulted in no significance of the results (Figure 5.12).

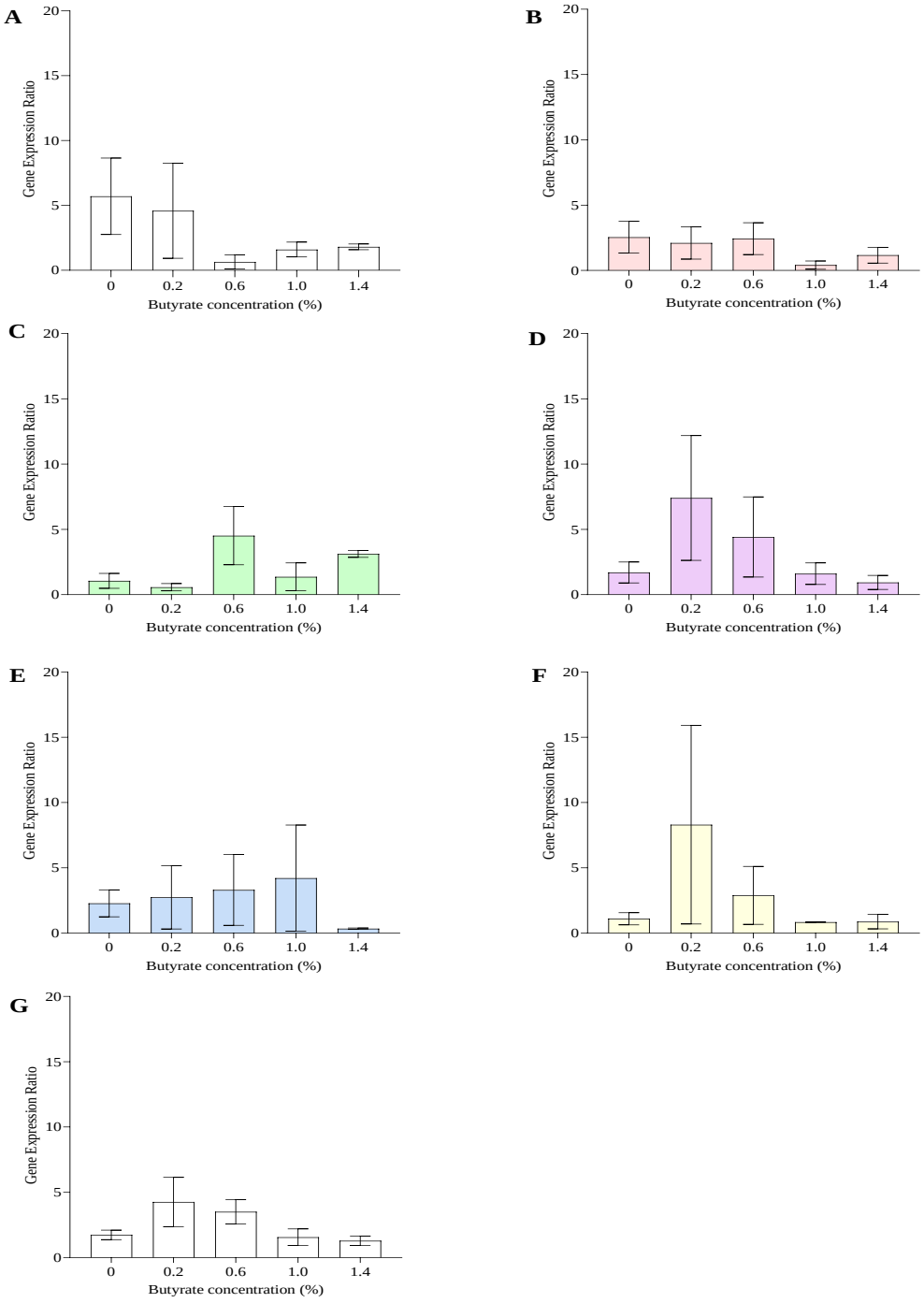

Figure 5.12 TGFβ gene expression in 8E11 cells exposed to butyrate

Quantitative PCR was used to quantify the gene expression of TGFβ in 8E11 cells cultured, infected, and treated *in vitro* (n=3 ± SEM). The 8E11 cells were exposed to varying concentrations of butyric acid (pH 7.0) for 24 h prior to challenge with *Campylobacter* strains. A – uninfected, B – *Campylobacter jejuni* M1, C – *Campylobacter jejuni* NCTC 11168, D – *Campylobacter* C13, E – *Campylobacter* G28, F – *Campylobacter* L29, G – *Campylobacter* mean invasion from five strains. A Kruskal-Wallis ANOVA with multiple comparisons was used to determine significance of results.

5.4 Discussion

The effect of feed additives on the viability human (CaCo-2) and avian (8E11) epithelial cells at defined dilutions was assessed. Furthermore, experiments assessed whether epithelial cells could be protected from *Campylobacter* invasion by feed additive exposure *in vitro*. In addition, as *Campylobacter* infection is associated with a poorly regulated overexuberant inflammatory response, cytokine (CXCLi1, CXCLi2 and TGFβ) induction by the *Campylobacter* isolates chosen for this study were quantified and compared. Finally, epithelial cells were pre-exposed feed additives to determine if this pre-exposure affected cytokine production induced by *Campylobacter*.

Viability was assessed using an AlamarBlue assay which indicates the oxidation and reduction potential of living cells by measuring mitochondrial enzyme activity. A decrease in fluorescence indicates a reduction in cell number assumed to be due to cell death, and an increase in fluorescence indicates an increase in metabolism and cell proliferation, however this could also be a result of increased cell proliferation (Hamid et al., 2004). In the present study, sodium butyrate had no cytotoxic effect on CaCo-2 or 8E11 cell lines. Surprisingly, in human CaCo-2 cells we observed a significantly increased fluorescence, and therefore an indication of increased metabolism, with exposure at various concentrations up to 0.75%. 0.25% butyrate increased cell fluorescence the most compared to untreated epithelial cells. There are potential reasons for this observation. Butyric acid is a product of microbial fermentation, such as *Clostridium* species, which is part of the natural human intestinal flora, and is a known energy source for intestinal and colorectal epithelial cells (Ishikawa et al., 2021; Sakurazawa & Ohkusa, 2005). The cell focussed actions of butyric acid include the modification of nuclear architecture, acetylation, and phosphorylation of nuclear histones, resulting in changes to the cell chromatin structure, and can also alter the differentiation state of cells (e.g., overcoming the resistance of cancerous colonic cells to normal cell death) (J. G. Smith et al., 1998).

It is also relevant to consider the form of the feed additive as acid and salt forms can give different activities. The cytotoxicity of the sodium salts of organic acids (e.g sodium butyrate) have been extensively studied at various pH (Grenier & Mayrand, 1985; A. Hague et al., 1993; Angela Hague et al., 1995; Heerdt et al., 1994; McBain et al., 1997; Soldatenkov et al., 1998). However the *in vitro* cytotoxicity of the acid component has only more recently been investigated (Kurita-Ochiai et al., 2006; Sakurazawa & Ohkusa,

2005). In contrast to the present study, both Sakurazawa and Ohkusa (2005) and Kurita-Ochiai *et al.* (2006) found that butyric acid (0.5 to 50.0 mM and 2.5 to 5.0 mM, respectively) was toxic to various epithelial cell lines (DLD-1, HeLa, Vero, Hep-2, and Jurkat) when an MTT assay was used. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is an alternative method of determining mitochondrial activity without the need for cell quantification and can be used to measure the cytotoxic concentration of solutions where cell death reaches 50% (Sakurazawa & Ohkusa, 2005; van Meerloo et al., 2011). In most cell types, butyric acid has been shown to inhibit growth during the G_1 phase of the cell cycle (Coradini et al., 1997, 2000) and this was replicated by Kurita-Ochiai *et al.* (2006) who observed a negative correlation between increasing butyric acid concentration and a decrease in cell quantity during the G_0/G_1 phases. It is unclear why in the present study results that contradict this previous research was observed (Coradini et al., 1997, 2000; Kurita-Ochiai et al., 2006; Sakurazawa & Ohkusa, 2005). However it could be due to pH alteration that was conducted in the present study, or preparation of solutions. In addition, the MTT assay and AlamarBlue assay do differ, and reagents used during the experiments may have affected the results that were observed.

Caprylic acid at 0.25% significantly increased CaCo-2 cell metabolism. To my knowledge this is the first time the *in vitro* effects of caprylic acid on epithelial cell lines has been reported. Previous *in vitro* findings have been limited to rat skeletal muscle cell lines, however these did report increases in mitochondrial oxygen consumption induced by caprylic acid (Hirabara et al., 2006). This finding in the present study have confirmed the role of caprylate in modulation of mitochondrial energy specifically within human epithelial cells.

Trivalent chromium (Cr3+) is considered to be highly safe (Amata, 2013). Here it was found that Cr3+ in the form of chromium propionate significantly increased the metabolism of 8E11, avian epithelial cells. Pre-exposure of cells to chromium propionate (0.00000002 to 0.00014% v/v) for 24 h was effective at increasing metabolism compared to cells cultured in untreated media. While Cr is not generally considered an essential trace mineral for poultry and therefore not a required supplement in poultry feed, the supplementation of Cr for birds under environmental stress has been shown to reduce the associated negative effects (Amata, 2013; Hayat et al., 2020). Broiler chickens are under constant environmental and heat stresses during rearing, and supplementing feed with

0.15 to 0.4 mg of Cr per kg feed (0.000015 to 0.00004% w/w) has been recommended for improved broiler performance under stress (Hayat et al., 2020; G. Wang et al., 2022). In addition, the US National Research Council (NRC) has previously recommended supplementation of feed with up to 300 µg/kg of Cr (0.00003% w/w) for animals that are experiencing environmental stress, potentially due to its role in protecting RNA from heat induced denaturation (Amata, 2013).

The primary role of Cr on metabolic activity is through enhancing glucose uptake as it is an integral component of the glucose tolerant factor (GTF) (Amata, 2013; Czarnek & Siwicki, 2021). Research has shown that in addition to improving absorption and utilisation of glucose, Cr has additional roles in activating enzymes required for nucleic acid synthesis, improving the health of the GI tract, and improving the immune response (Hayat et al., 2020; Rajalekshmi et al., 2014; G. Wang et al., 2022). There are no data available on the *in vitro* effects of Cr supplementation on avian cell lines, however *in vivo* studies (Hayat et al., 2020; Rajalekshmi et al., 2014; G. Wang et al., 2022) reported a positive correlation between Cr supplementation and expression of genes encoding glucose and amino acid transporters in the GI tract suggesting improved intestinal health. Furthermore, the immune status of broiler chickens was shown to improve with Cr supplementation by significantly increasing the production of corticosterone and by improving the cell mediated immune response which was indicated by the increased proliferation of lymphocytes (Hayat et al., 2020; Rajalekshmi et al., 2014). However, an *in vitro* cell study investigating Cr^{3+} and human fibroblast cells reported a dose dependent innhibitory effect (Czarnek & Siwicki, 2021) as the efficiency of mitochondria in the human fibroblast decreased in conjunction with cell membrane and lysosome damage. Despite the negative *in vitro* observations in human cells, cell proliferation was observed when cells were exposed to 100 and 200 µM Cr chloride. The variable results here may be due to the form in which Cr was introduced to the cells in the respective studies, propionate is a SCFA similar to butyrate and has been shown to increase cryptal cell production rate in the ceca and colon of rats (Sakata and Yajima, 1984; Hamer *et al.*, 2007), whereas chloride was used as a vessel for Cr exposure in other studies. This highlights the importance of the form in which Cr is applied to cells both *in vitro* and *in vivo*.

Treating human epithelial cells with butyrate or caprylate reduced *Campylobacter* invasion. The results reported here were not statistically significant, however previous

studies (both *in vitro* and *in vivo*) have reported a more pronounced effect of these acids on reducing invasion of bacterial enteropathogens (Van Immerseel *et al.*, 2004; Van Deun, Pasmans, Van Immerseel, *et al.*, 2008; Kollanoor-Johny *et al.*, 2012; Gupta *et al.*, 2021). *Salmonella enteritidis* has a similar invasion mechanism to *Campylobacter* as it utilises a T3SS system to alter the actin cytoskeleton and also downregulates the expression of macrophage cellular proteins that regulate these cytoskeletal rearrangements (Gupta et al., 2021). Both butyric and caprylic acids (butyric acid in the form of sodium butyrate) reduce invasion of *S. enteritidis* and *Campylobacter* into CaCo-2 cell lines. Furthermore, caprylic acid was reported to downregulate the expression of invasion genes *hilA* and *hilD* in *S. enteritidis* after 24 h pre-exposure (Gupta et al., 2021; Kollanoor-Johny et al., 2012; Upadhyaya et al., 2015). An alternative explanation of butyrate activity may be that rather than conferring protection to CaCo-2 cells, bacterial invasion decrease may be due to a reduction in bacterial cell number; although cellular differentiation induced by butyrate may also reduce invasion due to a decrease in tyrosine phosphorylation (Gupta et al., 2021; Van Deun, Pasmans, Van Immerseel, et al., 2008). Similarly, the reduction in invasion seen due to caprylate is likely due to the bactericidal properties reducing bacterial populations. When caprylic acid permeates the bacterial plasma membrane it dissociates leading to intracellular acidification resulting in reduced downregulation of critical invasion genes and reduction of virulence properties (Kollanoor-Johny et al., 2012).

One of the primary aims of the current study was to investigate feed additives that may lead to reductions in the intestinal inflammation induced by *Campylobacter* during broiler infection. Both CXCLi1 and CXCLi2 are homologous to the mammalian chemokine IL-8 (Hoshimoto et al., 2002). We found that pre-incubation for 24 h with 0.6% sodium butyrate significantly increased the gene expression of CXCLi1 and CXCLi2 in *Campylobacter* infected avian cells. It is important to note that elevated levels of CXCLi1 and CXCLi2 have also been observed in cells infected with *Campylobacter* compared to uninfected cells (D. John, 2018). In addition, an upregulation of CXCLi1 and 2 in cells treated with 0.6 % butyric acid, independently of *Campylobacter* infection was observed.

Most studies have investigated *Campylobacter* induced cytokine production in human derived cell lines (Andoh et al., 2001; R D Fusunyan et al., 1998; Hoshimoto et al., 2002; Huang et al., 1997; Weng et al., 2007). The use of CaCo-2 cells is often employed due to well documented immunological responses to bacterial pathogens and pattern associated

molecular patterns (PAMPs). PAMPs such as Pam3CSK4 and LPS have been used to mimic the interaction of pathogenic microbes with cells pre-incubated with both butyrate and caprylic acid (Andoh et al., 2001; R D Fusunyan et al., 1998; Weng et al., 2007). IL-8 expression can be both up and down-regulated by butyric acid, this phenomenon is dependent on numerous factors (Andoh et al., 2001; R D Fusunyan et al., 1998; Robert D. Fusunyan et al., 1999; Huang et al., 1997). Down-regulation of IL-8 was observed when CaCo-2 cells were treated with butyric acid in conjunction with PAMP stimulation, however pre-treatment of cells prior to PAMP exposure enhanced the production of IL-8. Furthermore, the A20 negative regulator of the NF-KB pathway is also believed to influence the action of butyric acid (Weng et al., 2007). IL-1β and LPS are additional stimulators of IL-8 production in CaCo-2 cells, interestingly it was reported that LPS induced IL-8 secretion only occurred after CaCo-2 cells were cultured in the presence of sodium butyrate. The upregulatory effects of sodium butyrate in the present study and downregulatory effects of sodium butyrate in previous reports indicate the complex nature of butyric acid action with regards to pro-inflammatory cytokine induction (R D Fusunyan et al., 1998). Rather than classed as a stimulator or inhibitor of IL-8 (CXCLi1 and CXCLi2) production, it could be regarded as a modulator of epithelial cells and their response to inflammatory mediators (Chuntharapai & Kim, 1995; R D Fusunyan et al., 1998; Tran et al., 2019). The differentiation status and type of cells used in studies can have a great impact on the experimental outcome; differentiated cells are believed to reflect the *in vivo* system more accurately than undifferentiated cells (Hoshimoto et al., 2002). In differentiated CaCo-2 cells stimulated with IL-1β, IL-8 secretion can be successfully suppressed by caprylic acid treatment, however the same was not observed in intestine-407 cells challenged with IL-1β where caprylic acid dose dependently enhanced IL-8 secretion (Andoh et al., 2001; Hoshimoto et al., 2002). This dichotomy between results indicates, that the acid should be regarded as a modulator of pro-inflammatory cytokine production dependent on application (Hoshimoto et al., 2002).

5.5 Conclusion

This work in this chapter demonstrated the complex nature of the direct effects of feed additives on human and avian cells (Table 5.3). The non-toxic nature of these compounds, over relevant concentrations, is promising for *in vivo* use as feed additives. The reduction of *Campylobacter* invasion into human cells pre-exposed to caprylate and butyrate is supported by published work. However, this work identified that the mechanism may not

be a result of epithelial cell and additive interaction alone but also the interactions between *Campylobacter* cells and additives. Further work should focus on identifying this mechanism of reduced invasion.

Table 5.3 Summary of main results from Chapter 5

Experiment	**Result**
Epithelial cell viability	Caprylate significantly increased fluorescence from CaCo-2 cells Chromium propionate significantly increased fluorescence from 8E11 cells
Invasion of *Campylobacter* into epithelial cells pretreated with potential feed additives	Chromium propionate at 0.00014% significantly increased the invasion of *Campylobacter* L29 into 8E11 cells
Gene expression	In 8E11 cells exposed to 0.6% butyrate, CXCLi1 expression was increased when challenged with *Campylobacter* In 8E11 cells exposed to 0.6% butyrate CXCLi2 expression was increased when exposed to *Campylobacter*

Chapter 6: General Discussion

The overarching aim of this thesis was to determine the potential of feed additives for reducing *Campylobacter* growth and invasion in the poultry GI tract and extraintestinal spread. *Campylobacter* strains were identified that represent the invasive spectrum in the collection studied. The direct bactericidal properties of feed additives against *Campylobacter* strains, and their indirect effect on reducing invasion of *Campylobacter* into epithelial cells was investigated. Lastly, the potential for feed additives to regulate the inflammatory response induced by *Campylobacter* infection was determined. The rationale behind this work was to improve the reduction of extraintestinal spread of *Campylobacter* and determine the mechanism of action of the feed additives. The results from these *in vitro* experiments will be used to determine the mechanism of action of these compounds and report these findings to the funder of this study, Kemin Animal Nutrition and Health for future *in vivo* investigation.

The current study confirmed the high variability in genes between a small collection of *Campylobacter* isolates, and subsequently the diverse phenotypic behaviour exhibited by this genus – highlighting that a 'one solution fits all' may not apply to this genera of pathogen. Sodium butyrate at pH 7.0 limited the growth of *Campylobacter* isolates *in vitro*. Further investigation is required to determine its exact mechanism of action however it is postulated that this arises from internal acidification of bacterial cells. It is plausible that other mechanisms could be at play, such as disruption of the protonmotive force *of Campylobacter* (van der Stel et al., 2017). This may in fact be a general mechanism of action that weak acids (such as butyrate and caprylate) use to inhibit bacterial growth as similar effects of been has been identified in *Clostridium thermocellum* and *Clostridium acetobutylium and Saccharomyces cerevisiae*. In fact, monitoring the growth inhibitory effects of weak acids has been demonstrated in *B. subtilis* strains using fluorescent sensor proteins (Herrero et al., 1985; Stratford & Anslow, 1996). The interaction between *Campylobacter* and the host is complex and there are various virulence mechanisms which are employed to aid extraintestinal spread and induce a dysregulated immune response. In addition to a growth limiting effect on *Campylobacter*, sodium butyrate may also modulate the epithelial response to inflammatory mediators and confer a protective effect to the gut epithelium (van Beilen & Brul, 2013).

6.1 Reducing *Campylobacter* in the Poultry Industry

Campylobacter is the leading cause of foodborne gastroenteritis and may lead to more serious chronic manifestations such as Guillain-Barre and Miller Fisher syndromes (Soro et al., 2020). In England and Wales, a peak of reported human campylobacteriosis cases occurred in 2000 with over 57,000 cases reported to the Health Protection Agency (HPA) (Newell et al., 2011). The infection of commercial poultry with *Campylobacter* is widespread and the main vehicle for human infection (Chlebicz & Śliżewska, 2018; Myintzaw et al., 2021; Sheppard & Maiden, 2015). 80% of human infections have been traced back to poultry host origins reducing the public health risk from *Campylobacter* infection requires interventions to control or prevent poultry flock colonisation at the farm level and cross-contamination throughout meat processing (Newell et al., 2011; Umaraw et al., 2017).

In chapter 3 the variation in strain types isolated from naturally infected broiler chickens was determined. It is unlikely that a broiler chicken is infected with only one ST of *Campylobacter* at any one time, therefore, to reduce *Campylobacter* levels within the poultry production chain an intervention must be employed that acts consistently upon *Campylobacter* isolates despite variation in genomic and phenotypic behaviours.

There are high levels of poultry products contaminated with *Campylobacter* at the point of sale. Despite statistics varying between studies in the EU, swabs recovered from poultry products were *Campylobacter* positive in 75.8% of cases (Shane, 2000; Soro et al., 2020). Of the *Campylobacter* strains recognised, *C. jejuni* predominated but *C. coli* can be recovered from the GI tract of poultry and may cause human disease (Bull et al., 2006; Epping et al., 2021; Gilbreath et al., 2011; Sheppard et al., 2009).

Reducing the prevalence of *Campylobacter* throughout the poultry production chain will significantly reduce the economic burden related to public health costs (e.g. NHS), industry costs, and costs to the affected individuals. In the UK the economic burden associated with *Campylobacter* associated disease has been estimated at £45.4 million (Roberts *et al.*, 2003; Hansson *et al.*, 2016; Devleesschauwer *et al.*, 2017; Soro *et al.*, 2020; Myintzaw, Jaiswal and Jaiswal, 2021). Furthermore, economic consequences may occur due to recall of products considered unsafe for sale or indeed loss of consumer trust following an outbreak (Devleesschauwer et al., 2017). By reducing the levels of this

pathogen (ideally eradicating it) throughout the poultry processing chain, both the public health risk and economic burden could be alleviated.

6.1.1 Reducing cross contamination through biosecurity

A well-maintained modern poultry house with limited access points should have high biosecurity for holding commercial flocks, as house age is often a factor considered to affect structural integrity (Berndtson et al., 1996; Messens et al., 2009; Newell et al., 2011; Shane, 2000). However, risk factor studies have shown no difference between flock colonisation and house age (Berndtson et al., 1996; Messens et al., 2009). There are also passive and active transgressions of the biosecurity perimeter that are capable of compromising flock security regardless of mitigation strategies and these are still not fully understood (Figure 6.1) (Berndtson et al., 1996; Messens et al., 2009; Newell et al., 2011; Shane, 2000).

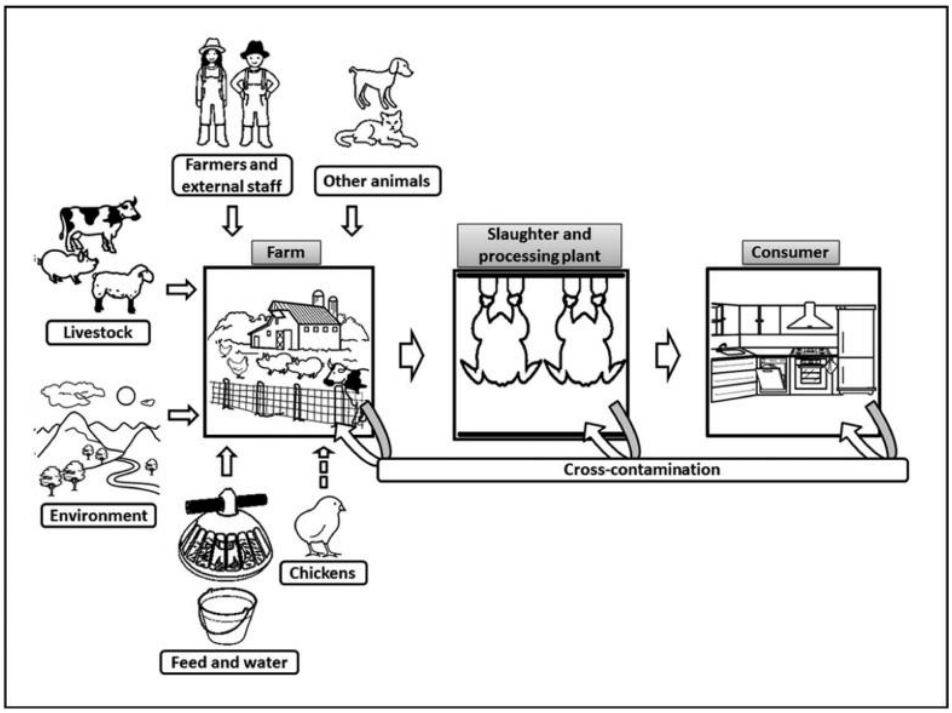

Figure 6.1 Passive and active routes for *Campylobacter* introduction into a poultry house, and cross-contamination potential at stages of poultry processing (Taken from: Soro *et al.*, 2020).

Increased efforts have been made in recent years to reduce the levels of *Campylobacter* contamination in the poultry production chain but despite these efforts there is still no effective strategy that reduces *Campylobacter* to levels that do not pose a significant public health risk (Guyard-Nicodème et al., 2017; Soro et al., 2020). At farm level the EFSA have named several biosecurity practices that theoretically should limit the entry

of *Campylobacter* into broiler houses if strictly adhered to. This includes boot washing (boot dip), overall changes (into designated overalls for that specific house), hand washing and limited personnel entry (Facciolà, Riso, et al., 2017; I Hansson et al., 2010; Koutsoumanis et al., 2020; Soro et al., 2020). Over the last 30 years, it has become evident that this is not always easy in practice (Newell et al., 2011; Soro et al., 2020).

6.1.2 Reducing colonisation through feed additive approaches

Broiler feed formulation and nutritional content is of vital importance. Gut health, immune function, and growth performance are all highly influenced by feed composition and nutritional additives can be utilised to improve bird welfare and productivity (Alagawany et al., 2021; Ali et al., 2021; Choct, 2009; Shakeri et al., 2020). Supplementation of feed with SCFA, MCFA and probiotics has been suggested as an economically and practically viable preventative measure to reduce *Campylobacter* colonisation through mechanisms aimed at reducing faecal shedding of the bacterium, bactericidal properties within the GI tract, competitive exclusion of pathogens within the GI tract, and improvement of gut defences (e.g., reducing inflammation and reducing permeability) (Callaway et al., 2008; Guyard-Nicodème et al., 2017; Pourabedin et al., 2014; Pourabedin & Zhao, 2015; Soro et al., 2020). Research into the efficacy of *Campylobacter* targeted feed additives that are either commercially available, in progress of commercialisation or in the primary stages of testing have shown inconsistent findings, often with a large degree of variation in results, which has been attributed to microbiota composition of broilers during *in vivo* experiments and *Campylobacter* strain variation (Guyard-Nicodème et al., 2017; Orhan Sahin et al., 2015; Soro et al., 2020). It has been shown that feed additives could be successful in reducing the prevalence and intensity of *Campylobacter* colonisation in broilers, however this is speculation as data is primarily based on *in vitro* studies (Bailey, 1993; Shane, 2000).

6.2 *Campylobacter* strain variation

Across the 32 species of *Campylobacter* there is extensive variation in both genetic and phenotypic diversity (Costa & Iraola, 2019; D. John, 2018; Vidal et al., 2016). Infection rate, *in vivo* behaviour and colonisation ability is strain specific and no two strains can be assumed to behave in the same way (D. A. John et al., 2017). In the current study we studied the properties of 29 *Campylobacter* isolates (*C. jejuni* and *C. coli*) all isolated from naturally infected broiler chickens and assessed their *in vitro* behaviour (growth and invasion) and genetic diversity (virulence and antibiotic resistance). Despite conducting

all experiments under controlled conditions in a sterile environment notable phenotypic variation between isolates from the same source was observed. The results also deviated from a previous study using the same isolates under similar conditions. Based on this current study it was demonstrated that not only the variation between isolates but also the variability in results that the same isolate can produce (D. John, 2018).

Genomic analysis confirmed presence of the *cadF* gene in all isolates regardless of species or source (human/poultry: liver, ileum, caeca). However, the presence of other important virulence genes (e.g., *flaA/flaB* and Cdt cluster) was highly variable and the differences between genomic databases affected the detection of some genes.

The caecal and liver *Campylobacter* isolates in the present study showed preference toward avian 8E11 cells with regards to successful invasion as shown in Chapter 3. However, the overall invasion at strain level was diverse with no trend, and this has been previously shown by John (2018) using the same subset of isolates. *In vitro* studies used five isolates (including two reference strains) that showed the most consistent results in screening experiments and could represent the invasive spectrum of the *Campylobacter* isolates within the collection. The species that comprise the *Campylobacter* genus exhibit distinct differences in biological processes (e.g., flagellar biosynthesis, epithelial cell interaction) that are not fully understood (Gilbreath et al., 2011). It is theorised that by experimenting with a variety of strains, the additives tested would be targeted towards *Campylobacter* as a genus, not a specific strain or isolate.

6.3 Mechanisms of action of feed additives: Caprylic Acid, Butyric Acid, Chromium Propionate:

In chapters 4 and 5 the potential for butyric acid, caprylic acid and chromium propionate as *Campylobacter* targeted feed additives was investigated. Organic SCFA (C_1- C_7) and MCFA (C_8- C_{12}) , have been shown to alter micro-environments by reducing pH which leads to pathogen inactivation and are capable of diffusing across the bacterial membrane in an undissociated form causing intracellular acidification (Guyard-Nicodème et al., 2017; Soro et al., 2020). Butyrate is a SCFA known for enhancing production of anti-inflammatory cytokines and increasing transepithelial electrical resistance (TEER) across cell monolayers *in vitro*, which results in protection from pathogen invasion and translocation from the intestinal lumen into the blood stream (Tralongo et al., 2014). Butyrate is additionally involved in immune modulation and is a known energy providing

substrate for enterocytes that line the intestinal tract (Antongiovanni et al., 2007; Fernández-Rubio et al., 2009; Józefiak et al., 2004). Butyric acid is currently a commercially available poultry feed additive in various forms, but most commonly it is administered as a microbead coating (Guyard-Nicodème et al., 2017). Caprylic acid, on the other hand, is not currently a marketed feed additive, however it has been shown to alter the intestinal microbiota *in vivo* and may have a direct effect on *Campylobacter* virulence (F. Solis de los Santos et al., 2009). Chromium is an essential trace element for animals and contributes to metabolic activities. Currently, chromium chloride is the most common form of chromium feed additive, however organic forms such as chromium propionate are more efficiently absorbed and able to interact with the intestinal epithelium (Arif, Alagawany, et al., 2019; Dębski et al., 2004; Safwat et al., 2020).

These compounds were selected due to their known properties, however the studies contained within this thesis support mechanisms of action that involve both direct interaction with *Campylobacter* cells and gut epithelial cells. These mechanisms could reduce *Campylobacter* numbers and protect epithelial cells from invasion and *Campylobacter* induced damage (Figure 6.2).

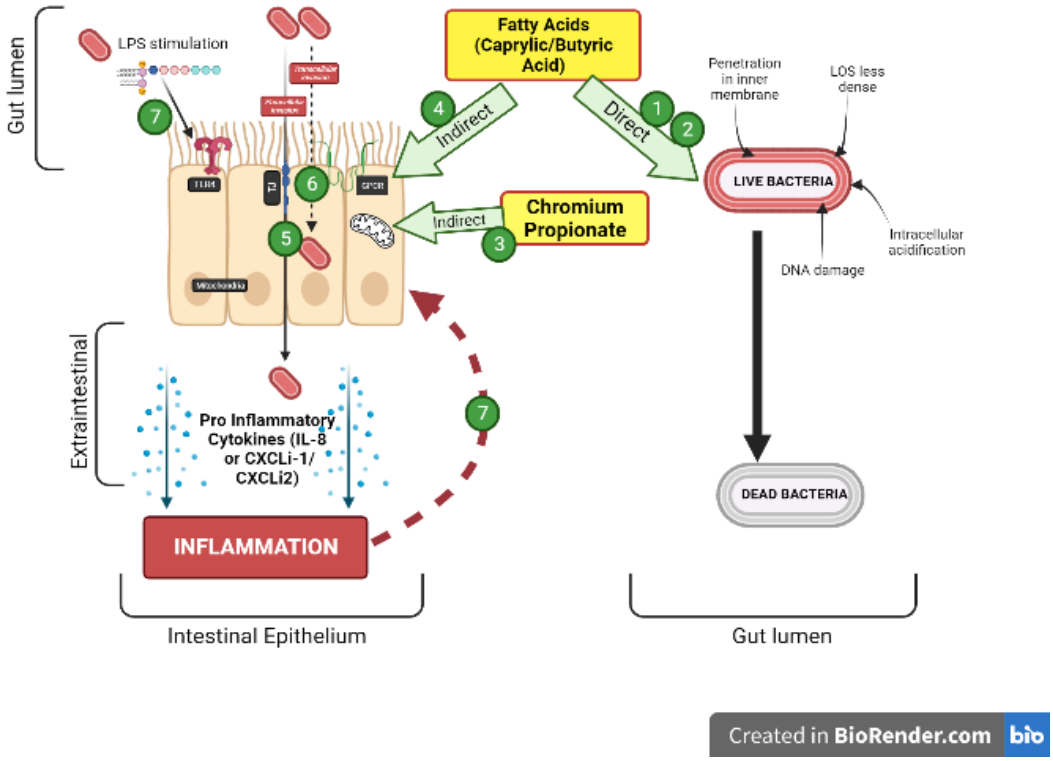

Figure 6.2 Schematic representation of the proposed mechanism of action of feed additives Caprylic acid, Butyric acid, and Chromium propionate (Created in Biorender, 2023)

Caprylic and butyric acid are proposed to be directly bactericidal and can diffuse across the bacterial membrane in the undissociated form where they dissociate and lower the intracellular pH leading to DNA damage, reduction in LOS (Lipooligosaccharide) density and ultimately cell death (1). The acids can also reduce the pH of the GI tract microenvironment, making it unfavourable for *Campylobacter* proliferation and survival; reduction in LOS density may reduce resistance to bactericidal effects of the gut microbiota and complement mediated activity (2). Chromium propionate directly effects the health of the intestinal epithelium by interacting with the mitochondria of enterocytes, thus restoring epithelial health that has been damaged by interaction with *Campylobacter* (3). It was further hypothesised that feed additives may reduce *Campylobacter* invasion (paracellular (5) and transcellular (6)) across the intestinal epithelium by improving integrity of tight junctions (TJ) and providing a protective effect to epithelial cells such as preventing pathogen interaction with G protein coupled receptors (GPCR) which results in pathogen internalisation (4). *Campylobacter* stimulates chicken toll like receptor 4 (TLR4) by lipopolysaccharide (LPS) binding and induces pro-inflammatory cytokine release (CXCLi1/2 in chickens) Release of these inflammatory cytokines results in further damage to intestinal cells and reduction in TEER leading to increased movement of *Campylobacter* from the GI tract to the bloodstream (7).

6.3.1 Bactericidal Properties of Feed Additives

In the present study, sodium butyrate (pH 7.0 ±2.0) was the source of butyric acid, which has known bactericidal properties in the dissociated form (Leeson et al., 2005; Panda et al., 2009). A significant decrease in *Campylobacter* growth at 1.0% and 1.4% (v/v) sodium butyrate was observed. It was suggest that the sodium butyrate may be

internalised in its associated form, leading to intracellular dissociation and bacterial cell death by cytoplasmic acidification (Figure 6.2) (D. Hermans et al., 2010).

The other feed additives trialled, i.e. chromium propionate and caprylate, had no significant effect on *Campylobacter* growth. This observation for chromium propionate (Figure 6.2) was unexpected, as chromium is known to have modulatory effects on glucose metabolism and immune modulation (Hayat et al., 2020; R. U. Khan et al., 2014; Rajalekshmi et al., 2014; Spears et al., 2019). The pH of the caprylic acid used was altered with HEPES buffer (to pH 7.0 ±2.0). Here it was suspected that the lack of effect on *Campylobacter* growth seen in caprylic acid treatments was associated with the buffering effect of the HEPES. Unlike butyric acid (sodium butyrate), a previous *in vitro* study has shown a pH dependent bactericidal effect of caprylic acid (D. Hermans et al., 2010). Adverse effects of HEPES on cell lines *in vitro* have been reported (upregulation of inflammatory signalling and cytotoxicity) (Liu et al., 2023). Zwitterionic betaine-based pH buffers are an organic buffer that do not induce the adverse effects on cells seen in HEPES treated media and should be considered as a safer alternative for altering caprylic acid pH (Liu et al., 2023).

6.3.2 Improving integrity of the intestinal epithelium using an *in vitro* model

Campylobacter can spread from the GI tract of poultry using a number of mechanisms that result in compromising the intestinal epithelium therefore it is important to design practical interventions to prevent gut wall damage, therefore preventing the bacterium from reaching edible tissues. *Campylobacter* can migrate transcellularly and paracellularly between individual intestinal epithelial cells where they can proliferate, damage the host cell and spread into the blood stream (Figure 6.2) (Ó Cróinín & Backert, 2012). Paracellular passage of *Campylobacter* occurs by redistributing the central tight junction proteins (occludin and ZO-1) allowing movement between cells and agitating cell to cell contact (Figure 6.2) (Ó Cróinín & Backert, 2012).

Pre-treatment of human epithelial cells with sodium butyrate and caprylate led to a decrease in transcellular invasion, measured by bacterial internalisation, although this was not significant. Similar results have been reported with a more pronounced effect but with different enteropathogens (Gupta et al., 2021; Kollanoor-Johny et al., 2012; Van Deun, Pasmans, Ducatelle, et al., 2008; F. Van Immerseel et al., 2004). It is suggested that a

protective mechanism of these feed additives on human cell lines occurs by decreasing tyrosine phosphorylation during CaCo-2 differentiation (Gupta et al., 2021; Van Deun, Pasmans, Ducatelle, et al., 2008). However there could be other effects and it is the sum of these effects that is important. For instance, the limitation of bacterial growth by residual feed additive on the cell monolayer; additionally, permeation of the bacterial membrane by acids could cause reduction in virulence properties by downregulating invasion genes (Kollanoor-Johny et al., 2012).

6.3.3 Regulating inflammatory response using an *in vitro* model

Campylobacter is known to induce the pro-inflammatory cytokines IL-8 and CXCLi1/2 in human and avian cells respectively (D. John, 2018). In the present study it was found that 0.6 % sodium butyrate significantly increased the expression of CXCLi1 and CXCLi2 genes in *Campylobacter* infected cells compared to cells infected with *Campylobacter* only. TGFβ expression in *Campylobacter* infected 8E11 cells was not significantly affected by butyrate treatment. *In vitro* models of *Campylobacter* induced cytokine production have been used in previous research, however the cell lines used in these have been primarily human, such as CaCo-2, HT-29, T84, and SW480 (Andoh et al., 2001; R D Fusunyan et al., 1998; Hoshimoto et al., 2002; Huang et al., 1997; D. John, 2018; Weng et al., 2007). An increase in CXCLi1 and CXCLi2 expression in some sodium butyrate treatments was expected. A previous study has reported small induction of IL-8 (homologous to CXCLi1/2) in butyrate treated CaCo-2 (R D Fusunyan et al., 1998).

Despite an indication that sodium butyrate results in upregulation of CXCLi1/2 in *Campylobacter* infected cells in this study, there is evidence may act as a modulator of IL-8 expression, and under the correct conditions could also be a downregulator CXCLi1/2 expression in 8E11 cells.

6.4 Implications for the poultry industry

The production of poultry products needs to create profit for all stakeholders involved in the process To meet customer demand there must be a constant flow of broiler flocks, and to achieve this the crop times are short, farming is intensified, and profit margins are low (Newell et al., 2011). Any *Campylobacter* targeted feed additives or new biosecurity practices must be supported by strong research evidence that would justify potential extra

costs, or ideally low-cost interventions that would likely be more appealing and welcomed by the poultry industry (Newell et al., 2011; Soro et al., 2020).

Despite previous studies reporting commensal carriage of *Campylobacter* within poultry (Humphrey *et al.*, 2014; Awad *et al.*, 2015; Pielsticker *et al.*, 2016) it has been more recently acknowledged by the scientific community that some strains of *Campylobacter* are harmful to chickens, causing intestinal inflammation and diarrhoea (Hermans *et al.*, 2011b; Williams *et al.*, 2013; Humphrey *et al.*, 2014; Awad *et al.*, 2015; Awad, Hess and Hess, 2018). At the farm level a reduction in *Campylobacter* infection will reduce the frequency of diarrhoea and this will lead to an improvement to animal welfare as litter in the house will be drier and thus reduce the instances of hock marks and pododermatitis.

6.5 Implications for Public Health

Campylobacter is the leading bacterial cause of foodborne human gastroenteritis worldwide, and the true incidence of the disease is unknown due to underreporting, especially within developing countries (Ingrid Hansson et al., 2016; Heimesaat et al., 2021; Myintzaw et al., 2021; Sheppard & Maiden, 2015). Despite this, educated estimates are that a 2-3 $\log_{10}$ reduction in *Campylobacter* poultry colonisation would result in a 76 to90% reduction in human campylobacteriosis (Gracia *et al.*, 2016).

As the global population grows year-on-year, the need for cheap sources of safely produced food increases. By reducing *Campylobacter* prevalence in poultry, the industry will be able to continue to meet demand and provide safe and affordable food to feed the planet. However, the reduction of *Campylobacter* would only result in a medium-term solution for the poultry industry and public health risk. A long-term solution to completely reduce the public health risk with the assurance that intensive poultry farming does not pose a significant risk to public health would be to develop an intervention strategy that is effective at preventing poultry colonisation at the primary level rather than controlling the extra-intestinal spread once infection has occurred.

6.6 Limitations

In Chapter 3 three isolates were selected to carry forward for testing *in vitro* with feed additives alongside two control isolates. Due to consistency in results and ability to culture, the three strains were all *C. coli* and assessed in comparison to two *C. jejuni* control strains. As *C. jejuni* is the most common species that causes human campylobacteriosis it would have been preferable to have used a *Campylobacter* isolate

from each sequence type. However, we could not identify a consistent isolate that represented each sequence type and therefore could not carry these forwards without incurring high degrees of variation in results.

The pH of the chicken caeca, small and large intestine, range from 6.4 to 6.6 in the most common white hybrid chicken for fast growing meat, Ross 308 birds (Mabelebele et al., 2013), therefore the caprylic acid feed additive may be more efficient *in vivo* at limiting *Campylobacter* growth if tested *in vitro* with a lower pH. Here, studies further tested the feed additives using epithelial cells optimally cultured at pH 7.0 for *in vitro* testing, therefore using additives with a lower pH could have compromised epithelial cell viability.

The only feed additive trialled with subsequent cytokine analysis was butyric acid in the form of sodium butyrate. When culturing the epithelial cells with caprylate, the epithelial RNA was unrecoverable after lysing. It is speculated that intracellular acidification of the epithelial cells might have occurred, however previous cytotoxicity assays suggested that the concentrations of caprylic acid trialled were non-toxic to both cell lines. To confirm the toxicity of the caprylic acid, a different cytotoxicity assay could be employed such as the LDH release assay (measure loss of membrane integrity) or the MTT assay (measures metabolic activity of viable cells) (Weyermann et al., 2005). The outcome of the cytotoxicity assay is dependent on the cell death mechanism hypothesised and therefore not all assays may report that a toxic compound is in fact toxic. Had an alternative assay been used concentrations could have been adjusted accordingly and it would have been possible to trial caprylate as an inflammatory regulator of CaCo-2 cells.

6.7 Future Work

This research has provided a basis for continued exploration of feed additives as safe and viable to combat *Campylobacter* extraintestinal spread at primary production (Soro et al., 2020). Repeating the experiments presented here should be done with a wider range of isolates, including *C. jejuni* isolates of poultry origin and clinical importance (e.g., ST-21 and ST-437).

The current study focusses highly on transcellular movement of *Campylobacter*; future research should explore the paracellular movement of *Campylobacter* between intestinal epithelial cells and identify to what degree feed additives can improve intestinal integrity by acting upon tight junctions and TEER (transepithelial electrical resistance). The pH of

caprylic and butyric acid used here was adjusted to pH 7.0 with buffers (HEPES or sodium hydroxide). It would be recommended to repeat the *in vitro* experiments here with additives at a pH that represents the average pH of the chicken GI tract.

Any future *in vitro* research should be followed up with an *in vivo* trial designed with critical parameters in mind such as broiler age, broiler lineage, form of additive administration, and additive constitution (one active ingredient or a combination). It is of the upmost importance that *in vivo* trials are conducted to ensure that observations in *in vitro* experiments are not a product of the controlled and sterile environments.

The industrial partner sponsoring this research, Kemin Animal Nutrition and Health Ltd., should consider the possibility of a *Campylobacter* targeted feed additive composed of more than one active ingredient. There are possibilities for additive and synergistic effects and these could be further tested in vitro in the systems used here. We have highlighted the variable nature of *Campylobacter* and its response to the additives. Additive development should consider reduction of multiple virulence mechanisms that are utilised by *Campylobacter* in its pathogenesis, in addition to improving the gut health of the poultry host. It is clear from the results in this thesis that feed additives have both direct effects (on *Campylobacter*) and indirect effects on the host (chicken epithelial cells) that ultimately may affect *Campylobacter* levels.

Chapter 7: References

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Appendix and Supplementary Data

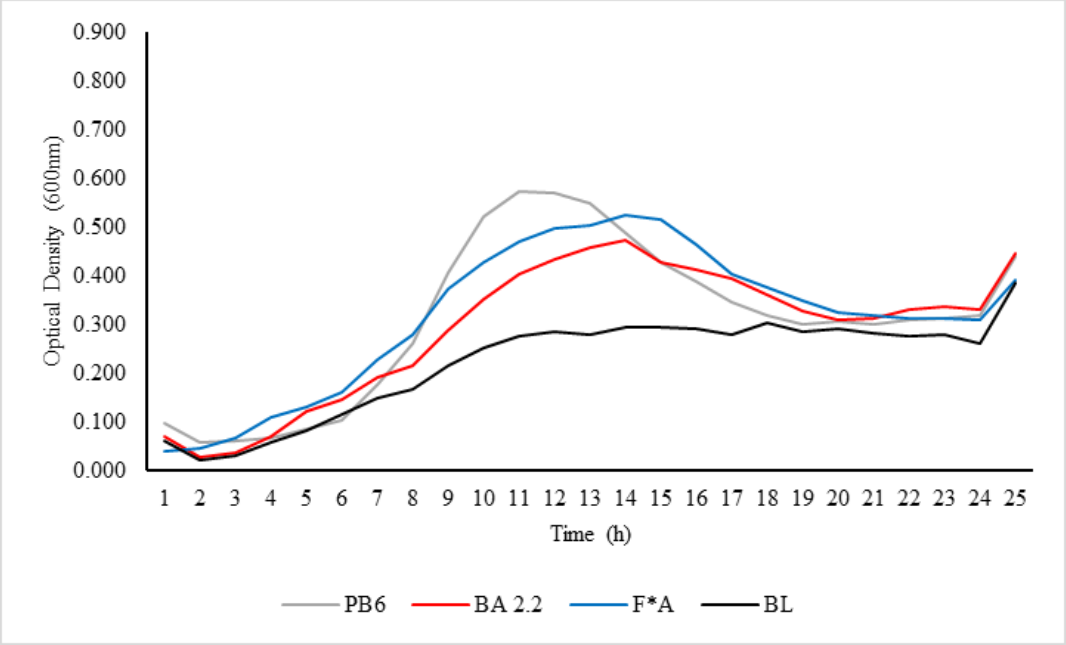
Appendix 1 Atmospheric conditions for the culture of *Bacillus* spp. conditioned media

Atmospheric conditions	Temperature (˚C)	Incubation time (h)
Aerobic	42	4
Aerobic	42	24
Microaerobic	42	4
Microaerobic	42	24

Appendix 2 Optical density (600 nm) readings after culture of *Bacillus* spp. for conditioned media

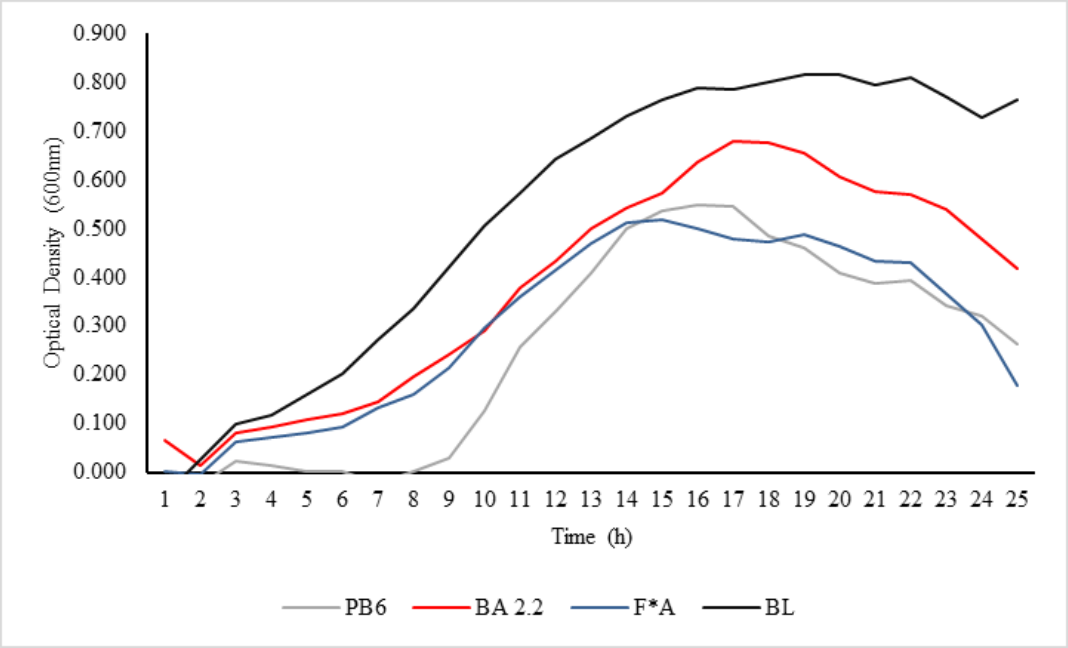
Solution	OD (600 nm)	Dilution
0.2 g *Bacillus subtilis* PB6, 20 mL 8E11 antibiotic free media incubated aerobically for 4 h at 42˚C	0.878	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL 8E11 antibiotic free media incubated aerobically for 24 h at 42˚C	2.432	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL 8E11 antibiotic free media incubated microaerobically for 4 h at 42˚C	0.708	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL 8E11 antibiotic free media incubated microaerobically for 24 h at 42˚C	2.409	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL CaCo-2 antibiotic free media incubated aerobically for 4 h at 42˚C	0.738	n/a

0.2 g *Bacillus subtilis* PB6, 20 mL CaCo-2 antibiotic free media incubated aerobically for 24 h at 42˚C	2.456	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL CaCo-2 antibiotic free media incubated microaerobically for 4 h at 42˚C	0.801	n/a
0.2 g *Bacillus subtilis* PB6, 20 mL CaCo-2 antibiotic free media incubated microaerobically for 24 h at 42˚C	2.482	n/a
0.2 g *Bacillus licheniformis*, 20 mL 8E11 antibiotic free media incubated aerobically for 4 h at 42˚C	0.79	n/a
0.2 g *Bacillus licheniformis*, 20 mL 8E11 antibiotic free media incubated aerobically for 24 h at 42˚C	2.495	n/a
0.2 g *Bacillus licheniformis*, 20 mL 8E11 antibiotic free media incubated microaerobically for 4 h at 42˚C	1.873	n/a
0.2 g *Bacillus licheniformis*, 20 mL 8E11 antibiotic free media incubated microaerobically for 24 h at 42˚C	2.076	n/a
0.2 g *Bacillus licheniformis*, 20 mL CaCo-2 antibiotic free media incubated aerobically for 4 h at 42˚C	1.864	n/a
0.2 g *Bacillus licheniformis*, 20 mL CaCo-2 antibiotic free media incubated aerobically for 24 h at 42˚C	2.237	n/a
0.2 g *Bacillus licheniformis*, 20 mL CaCo-2 antibiotic free media incubated microaerobically for 4 h at 42˚C	1.622	n/a
0.2 g *Bacillus licheniformis*, 20 mL CaCo-2 antibiotic free media incubated microaerobically for 24 h at 42˚C	1.493	1:1 (culture:fresh media)



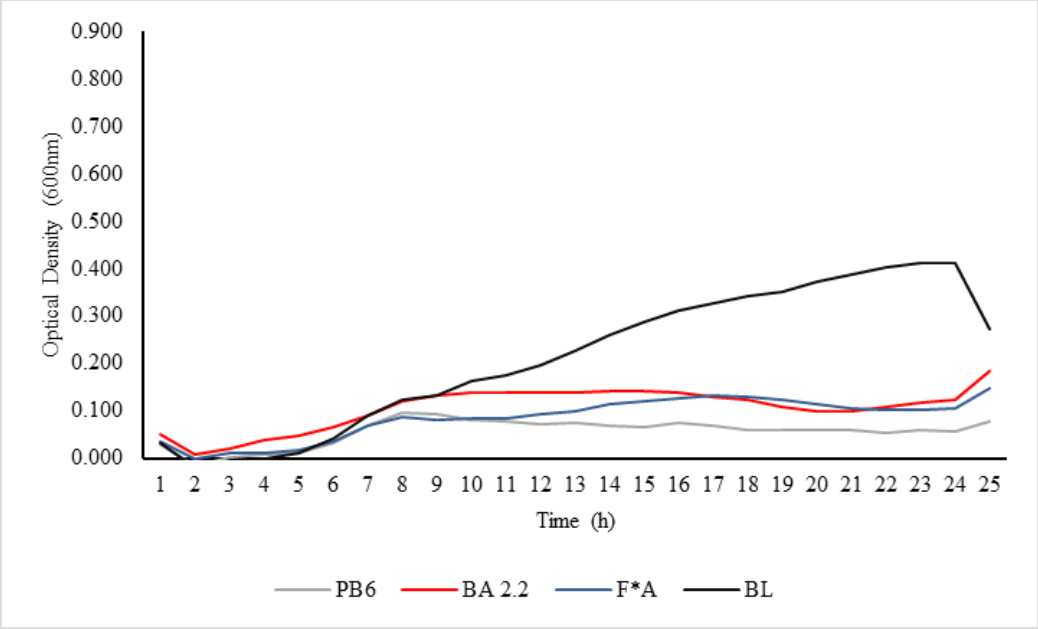
Appendix 3 Growth of *Bacillus* species in Brucella broth over 24 h

The optical density (600 nm) of four species of *Bacillus* were grown for 24h under aerobic conditions at 37˚C using a BMG omega plate reader (experimental repeats n = 3). The four species used were *Bacillus subtilis* PB6 (PB6), *Bacillus subtilis* BA2.2 (BA2.2), *Bacillus subtilis* F*A (F*A) and *Bacillus licheniformis* (BL).

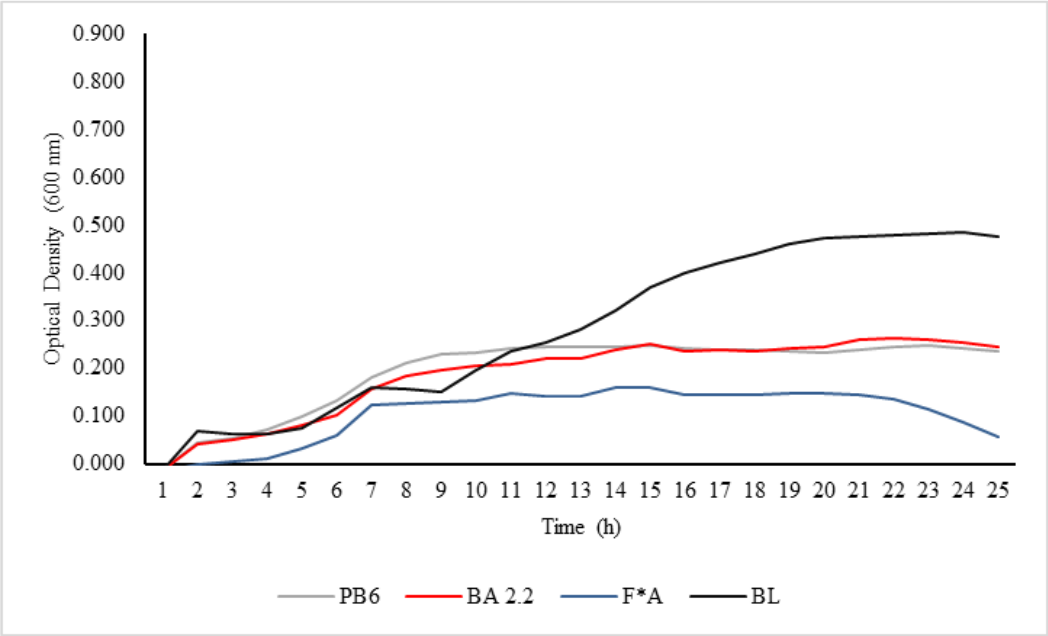


Appendix 4 Growth of Bacillus species in TSB broth over 24 h

The optical density (600 nm) of four species of *Bacillus* were grown for 24h under aerobic conditions at 37˚C using a BMG omega plate reader (experimental repeats n = 3). The four species used were *Bacillus subtilis* PB6 (PB6), *Bacillus subtilis* BA2.2 (BA2.2), *Bacillus subtilis* F*A (F*A) and *Bacillus licheniformis* (BL).



Appendix 5 Growth of Bacillus species in CaCo-2 media over 24 h The optical density (600 nm) of four species of *Bacillus* were grown for 24h under aerobic conditions at 37˚C using a BMG omega plate reader (experimental repeats n = 2). The four species used were *Bacillus subtilis* PB6 (PB6), *Bacillus subtilis* BA2.2 (BA2.2), *Bacillus subtilis* F*A (F*A) and *Bacillus licheniformis* (BL).



Appendix 6 Growth of *Bacillus* species in 8E11 media over 24 h

The optical density (600 nm) of four species of *Bacillus* were grown for 24h under aerobic conditions at 37˚C using a BMG omega plate reader (experimental repeats n = 2). The four species used were *Bacillus subtilis* PB6 (PB6), *Bacillus subtilis* BA2.2 (BA2.2), *Bacillus subtilis* F*A (F*A) and *Bacillus licheniformis* (BL).